Severe Dengue

2016

8th ACPID

SEAMEO

TROPICAL MEDICINE AND PUBLIC HEALTH NETWORK · TROPMED ·

Severe Dengue

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PREFACE

The Division of Infectious Diseases, Department of Internal Medicine, Faculty of Medicine, Chulalongkorn University and the Dengue Project Banpong-Photharam, Mahidol Univeristy, Bangkok, Thailand are very pleased to introduce the proceedings of "**Severe Dengue**" with the main purpose of sharing knowledge on all aspects of severe dengue.

Dengue is the most common mosquito-borne viral disease in the world. WHO has announced a global strategy of prevention and control of dengue during 2012-2020 aiming at the reduction of dengue mortality by 50% and morbidity by 25%. Five technical elements to be applied are: diagnosis and case management, integrated surveillance and outbreak preparation, sustainable vector control, future vaccine implementation, and basic operational and implementation research.

These proceedings contain written versions of most topics presented under an educational session on "**Severe Dengue**" during the 8th Asian Congress of Pediatric Infectious Diseases during 7-8 November 2016 at the Queen Sirikit National Convention Center, Bangkok, Thailand. We truly hope that you will find the proceedings as an enriching guide to help implement effective strategies in severe dengue.

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DENGUE: AN OVERVIEW

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Abstract. Dengue affects millions of people annually, and it is a re-emerging disease in the tropical world. The increasing number of dengue cases over the last decades has been explained by association with unplanned urbanization and lack of efficient health facilities, demographic transition, travel/commercial development and limit efficacy of the vector control efforts. The clinical presentations of dengue range from mild illness to the life-threatening severe forms of the disease associated with plasma leakage, shock, severe bleeding, or multi-organ failure, which may be fatal. Although shock and plasma leakage seem to be more prevalent as age decreases, the frequency of severe bleeding or internal hemorrhage augments as age increases. Increases in liver enzymes, unlike conventional viral hepatitis, indicate liver involvement during dengue infections. Fatal cases were found to have significant frequencies of shock, altered consciousness, massive gastrointestinal bleeding, renal/hepatic failure, and concurrent bacteremia. The early recognition of dengue infection, bleeding tendency, and signs of circulatory collapse would reduce mortality rate in patients with dengue infection. The implementations of effectively sustainable vector control and effective dengue vaccines are keys to success for prevention and control of this disease.

Keywords: dengue, clinical manifestation, diagnosis, pathogen, pathogenesis, prevention, treatment

INTRODUCTION

Dengue is one of the most common mosquito-borne viral infections. The dengue virus is a single-stranded RNA virus and is transmitted to humans by the *Aedes aegypti* and *Aedes albopictus* mosquito species. This mosquito-borne arboviral infection is endemic in more than 140 countries with a geographic distribution in Asia, the Americas, the Eastern Mediterranean, and Africa. The World Health Organization (WHO) has reported that more than 2.5 billion people are at risk of dengue infection by one or more dengue viruses, and this risk is mainly in children living in tropical and subtropical countries. Estimates of the disease burden suggest nearly 100 million symptomatic dengue infections worldwide every year with the majority (75%) occurring in Asia and the Western Pacific region (Bhatt *et al*, 2013). In recent decades, outbreaks of increasing severity of dengue infection have been reported worldwide.

Changing factors in ecology and demographics are thought to contribute to the emergence of dengue infection in the last half-century. Contributory factors associated with the geographical expansion of dengue into new countries and urban settings include the increasing geographical range of *Ae. aegypti*, population growth, urbanization, slum growth, human population migration, movement of dengue virus by infected travelers, trade development, and improved diagnostic capabilities in medical practice (Kyle and Harris, 2008; Cummings *et al*, 2009).

Urban areas in the Tropics have experienced increased transmission of dengue virus. This trend has been caused by unplanned urbanization that leads to poor quality housing and high urban

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population density, and crowding along with poor quality water, sewer, and waste management systems (Barbazan *et al,* 2002; Guzman and Kouri, 2003; Nakhapakorn and Tripathi, 2005; Anyamba *et al,* 2006). Thus, an expansion of its geographical distribution and an increasing burden of health care resources caused by dengue are predicted in future decades.

Effective vector control management is the only method to reduce dengue infection in endemic areas. Nevertheless, historic vector control management efforts have had limited success in reducing transmission rates. Therefore, an effective dengue vaccine used in the target population and historic preventative measures such as raising public awareness may effectively control dengue in endemic areas (Pang *et al,* 2015).

Hyperendemic dengue is a major public health problem in many countries in South and Southeast Asia. In these regions, *Ae. aegypti* and *Ae. albopictus* are commonly found in urban and rural areas, and multiple dengue serotypes are circulating. A trend of general increase in the number of dengue cases has been seen in these regions. In particular, the disease is a leading cause of hospitalization and death in children. Within a population, the extent of hyperendemicity and timing of the introductions of differing serotypes mainly determine serotype-specific immunity. This serotype-specific immunity then determines the age distribution of clinically detectable dengue infections.

Past age distributions of indigenous dengue cases in South and Southeast Asia and the Americas have differed. Dengue syndromes in South and Southeast Asia occurred mainly in children, whereas they occurred in all age groups, including the elderly in the Americas. Recently, several Asian countries have reported an epidemic shift in age groups of dengue from mainly children to adolescents and young adults with increased disease severity (Charoensook *et al,* 1999; Pancharoen *et al,* 2002; Pongsumpun *et al,* 2002; Kulanatne *et al,* 2005). Of dengue virus infection cases in Thailand, adults aged over 15 year are estimated to be 30-40% of cases (Patumanond *et al,* 2003).

Children and adults with dengue show some differences in clinical presentations, laboratory findings, and severe complications (Kittigul *et al,* 2007; Hanafusa *et al,* 2008; Namvongsa *et al,* 2013). The incidence of dengue amongst travelers has been reported to be increasing (1.0-6.7%), which has been suggested to be a potential hazard for international travelers returning from endemic areas (Jelinek, 2000; Brien *et al,* 2001; Stephen *et al,* 2002; Pongsumpun *et al,* 2004). Recent reports have indicated that adult travelers returning from Asia to Western countries are more likely to have dengue than malaria, resulting in a greater probability that healthcare providers in Western countries will be confronted with travel-acquired dengue infections (Schwartz *et al,* 2008; Burdino *et al,* 2011; Wilder-Smith, 2012; Leder *et al,* 2013).

In travelers returning with fever, clinical manifestations of dengue infection are comparable with observations in the endemic area where dengue may go unnoticed. This situation highlights that surveillance should be maintained in non-endemic countries, and that febrile travelers returning from countries that are dengue endemic areas should be properly evaluated and followed up (Freedman *et al,* 2006; Massed and Wilder-Smith, 2009). Travelers should be encouraged to protect themselves from mosquito bites to avoid infections and onward transmission of dengue in new areas where *Ae. aegypti* is established.

PATHOGEN AND PATHOGENESIS

Dengue virus is a single-stranded RNA virus of the genus *Flavivirus* in the family Flaviviridae. It is the etiological agent of dengue infection. The four serotypes of dengue virus are DEN-1, DEN-2, DEN-3, and DEN-4, all of which are transmitted by the *Aedes aegypti* and *Ae. albopictus* species of mosquito.

The rainy season is the time of peak transmission of dengue virus in hyperendemic and endemic areas, and high temperatures also contribute to transmission. Rare cases of dengue transmission by needlestick, receipt of infected blood component, tissue or organ transplantation, and transplacental infection have been documented, although

the vast majority of cases are transmitted by mosquitoes (Chen and Wilson, 2004; Wagner *et al*, 2004; Tan *et al*, 2005; Mohammed *et al*, 2008; Tambyah *et al*, 2008; Wilder-Smith *et al*, 2009; Tangnararatchakit *et al*, 2012; Costa *et al*, 2015).

Any dengue virus has an incubation period of 4-8 days, after which there is an dengue virus infection that may manifest as a wide spectrum of illness ranging from asymptomatic or subclinical infection, undifferentiated fever, dengue fever (DF), and severe forms of the disease associated with plasma leakage, including dengue hemorrhagic fever (DHF), dengue shock syndrome (DSS), severe bleeding, encephalopathy, and multi-organ failure (WHO, 2009). DHF is characterized by rapid onset of capillary leakage accompanied by thrombocytopenia, hemoconcentration, vascular collapse, abdominal pain, and hemorrhagic manifestations (WHO, 1997). DF and DHF have been classified as distinct clinical entities, yet they are probably a continuum of the same pathophysiology with divergent outcomes in vascular integrity dysfunction.

Asymptomatic cases of dengue occur more frequently than symptomatic cases at a variable ratio (0.9:1, respectively to 18:1, respectively), and the ratio depends on geographical area, epidemiological context and the immunologic characteristics of an individual (Hadinegoro *et al*, 2016; Olivera-Botello *et al*, 2016). However, patients with asymptomatic infection may be the reservoir for dengue virus transmission to mosquitoes and subsequently to humans and should be considered in estimating disease burden.

Although recovery from dengue infection with one serotype confers lifelong homologous immunity, protection against other serotypes is short-term. Thus, a secondary infection can occur with other dengue serotypes. Previous epidemiologic data reveals that secondary heterotypic dengue virus infection is a risk factor to develop severe DHF/DSS, mediated most likely by antibody-dependent enhancement (ADE) of infection. Pre-existing homotypic antibodies bind to heterotypic dengue virions (virus-antibody complexes) and enable Fcγ receptor-mediated uptake by target Fcγ receptor-

bearing cells (*eg*, monocyte/macrophage) resulting in increased viral replication and viremia (Hadinegoro *et al*, 2016). Changing inflammatory cytokine production (such as TNF∝, interleukin-1, interleukin-2 interleukin-6, interleukin 12, macrophage migration inhibitory factor, and HMGB1, MCP-1) produced by T-lymphocytes, monocytes/macrophages, and endothelial cell is observed in dengue patients who have increased vascular permeability, thrombocytopenia, and activation of coagulation and fibrinolysis (Green and Rothman, 2006; Guzman and Harris, 2015). In addition, secreted NS1 protein, anti-NS1 antibodies and increased complement activation (C3a, C5a) might be involved in increased production of inflammatory cytokines, causing intravascular coagulopathy and virus-induced vascular leakage by implicated local and systemic effects. Thrombocytopenia caused by bone marrow suppression, shortened platelet survival and increased platelet consumption due to platelet adhesion occurs during the dengue infection and reaches nadir during the day of defeverescence (toxic stage) (Chuansumrit and Chaiyaratana, 2014; Guzman and Haris, 2015).

Although secondary dengue infection remains the strongest known risk factor for DHF/DSS, viral genetics, serotype sequence, host factors, and time interval between primary and secondary infections can modulate severity of illness (Gamble *et al*, 2000; Guzman *et al*, 2002; Hammond *et al*, 2005; Anderson *et al*, 2014; Guzman and Harris, 2015).

CLINICAL MANIFESTATION

Dengue infection should be suspected if the patient in a dengue epidemic or endemic area has a fever of 10 days or less with myalgia, headache, flushing, anorexia, nausea or vomiting, arthralgia, bone pain, and peri-orbital pain with no apparent signs or symptoms of respiratory tract infection or organ-specific signs of other infectious diseases.

The clinical spectrum of dengue infection ranges from mild illness (undifferentiated fever, non-severe DF) to the life-threatening severe forms of the disease associated with plasma leakage (DHF/DSS), severe bleeding or multi-organ failure, which may be fatal.

Classical DF is a non-fatal febrile illness with a duration of around 5-7 days that is associated with sudden onset, anorexia, myalgia, headache, and occasional rash.

DHF is characterized by a high continuous fever of 2-7 days and a rapid onset of capillary leakage with thrombocytopenia, hemoconcentration, vascular collapse, abdominal pain, and hemorrhagic manifestations.

Shock (DSS) results from severe loss of plasma volume into serous spaces (*eg*, the pleural space or peritoneal cavity) or severe internal hemorrhage. Clinical symptoms of DSS in the acute febrile phase, which usually lasts 3-8 days, are mostly similar to those of DF and severe dengue (DHF), including fever, nausea/vomiting, headache, rash, and myalgia, but abdominal pain and severe or widespread bleeding are more frequent in DSS and less frequent in DF.

Dengue patients sometimes experience minor hemorrhagic manifestations including petechiae, epistaxis, gingival bleeding, and menorrhagia, but DF is rarely associated with severe hemorrhage leading to shock.

The reasons for age-related differences in dengue severity are not well understood. The clinical course has been observed to differ between children and adults. Children experience plasma leakage (DHF) and DSS more frequently compared with adults. This may reflect age-dependent differences in intrinsic vascular permeability. However, bleeding manifestations, in particular severe internal hemorrhage and hepatic dysfunction, have been reported to be more common in adults and older age groups than in children (Guzman *et al,* 2002; Hammond *et al,* 2005; Wichmann *et al,* 2005; Kittigul *et al,* 2007; Guilarde *et al,* 2008; Tantawichien, 2012; Namvongsa *et al,* 2013; Souza *et al,* 2013; Chuansumrit and Chaiyaratana, 2014; Tantawichien, 2015).

The symptoms of dengue generally last for 3-7 days before the fever subsides and symptoms remit. During the convalescence stage, the patients with dengue infection, even in DSS, may have rapidly increasing appetite, convalescence rash on lower extremities (a confluent rash with characteristic, scattered, round areas on pale skin), and sinus bradycardia. Most dengue infections are self-limiting with normalization of all abnormal hemostasis occurring in the convalescent stage or within 1-2 weeks after defervescence.

The apparent increased prevalence in the complications of dengue in the adolescent, adult, and elderly have resulted from the emergence of severe bleeding, fulminant hepatic failure, and encephalopathy in DF and DHF cases (Tsai *et al,* 1991; Anuradha *et al,* 1998; Agarwal *et al,* 1999; Wichmann *et al,* 2005; Pungjitprapai and Tantawichien, 2008; Tantawichien, 2012; Tantawichien, 2015). High mortality rates have previously been reported in elderly patients with dengue infection because of medical co-morbidity and waning of host immunity (Rigau-Perez and Laufer, 2006; Kuo *et al,* 2007; Lee *et al,* 2008; Gautret *et al,* 2012; Pang *et al,* 2012).

The prognosis of dengue infection largely depends on early diagnosis, recognition of plasma leakage, and treatment with immediate replacement of fluid along with intensive supportive care. Severity classification is important because the practice of the physician in patient observation, place of management, intensity of management (intravenous fluids, blood or plasma transfusion, and medicines) are likely to depend on the classification system used. WHO released a new classification in 2009, which is dengue with or without warning signs and severe dengue because the previous WHO 1997 classification (DF, DHF, and DSS) has had some issues. Its classification system has poor classification of disease severity and difficulties in usage in the clinical setting as well as in triage during outbreaks, and it could have led to inaccurate reporting of severities of dengue worldwide because of the difficulty for reporting clinicians in using it (WHO, 1997; WHO, 2009; Srikiatkhachorn *et al,* 2010; Hadinegoro, 2012).

Clinicians should monitor the progression of a dengue infection if the following warning signs manifest: persistent or severe vomiting, abdominal

pain or tenderness, liver enlargement, drowsiness or alteration of consciousness, fluid accumulation with respiratory distress, epitaxis, gum bleeding, gastrointestinal bleeding, retinal hemorrhage, oliguria, and hemoconcentration with severe thrombocytopenia. Physicians should note any of these warning signs, as they may lead to severe dengue (Leo *et al,* 2011; Horstick *et al,* 2012; Prasad *et al,* 2013). Severe dengue is defined by one or more of the following: plasma leakage (DHF) possibly resulting in shock (DSS), severe bleeding, and severe organ impairment such as hepatic failure, acute renal failure, and encephalopathy (Gamble *et al,* 2000; Guzman *et al,* 2002; Hammond *et al,* 2005; Wichmann *et al,* 2005; Green and Rothman, 2006; Kittigul *et al,* 2007; Guilarde *et al,* 2008; WHO, 2009; Anderson *et al,* 2014; Chuansumrit and Chaiyaratana, 2014; Guzman and Harris, 2015). Without treatment, mortality may be as high as 20%, but proper management, including intravenous rehydration, can dramatically reduce mortality to less than 1%. Viral factors such as serotypes, structural and nonstructural proteins of dengue virus, and viral load as well as host factors such as age, gender differences, genetic, nutritional status, immune reaction, and co-existing medical conditions may contribute towards the severity of dengue infection.

Dengue hemorrhagic fever and dengue shock syndrome

Typically, DHF resembles DF in many clinical respects, but it is characterized by high continuous fever 2-7 days, hemorrhagic diathesis, hepato-megaly, and circulatory disturbance (DSS).

The critical stage associated with plasma leakage (20% increase in hematocrit over baseline) and marked thrombocytopenia ($<100 \times 10^9$/l) associated with bleeding frequently occurs at the end of febrile phase of illness (WHO, 1997).

Right-sided pleural fluid detected by chest roentgenogram or free fluid in the peritoneal cavity and thickening of gall bladder wall detected by ultrasonography has been interpreted as evidence of plasma leakage, and this is usually only clinically detectable after intravenous fluid therapy unless plasma leakage is significant (Setcawan *et al,* 1995; Srikiatkhachorn *et al,* 2007; Wang *et al,* 2007). The right-sided or bilateral pleural effusion is generally not prominent, but becomes increasingly more so after excessive intravenous fluid administration.

In mild DHF cases, the changes in blood pressure and pulse may be minimal and transient with patients recovering shortly after treatment. In more severe DHF cases, the classification of DSS is established by a rapid and weak pulse, narrowing of the pulse pressure to less than 20 mmHg, or an unobtainable blood pressure (WHO, 1997). Clinical indicators of impending DSS, include severe abdominal pain, change from fever to hypothermia, restlessness, sweating, prostration, and tender hepatomegaly. When continuous loss of plasma that becomes excessive occurs, the patient may progress rapidly to profound shock.

Prolonged shock is often complicated metabolic acidosis, severe gastrointestinal bleeding, and disseminated intravascular coagulopathy (DIC). DSS was an independent risk factor (odds ratio 220) for development of acute renal failure in adult patients with DHF (Lee *et al,* 2009). In a few patients, cardiac involvement ranging from abnormality of electrocardiogram, mild elevation of cardiac biomarkers to myocarditis and/or pericarditis was observed, and some of these patients died (Miranda *et al,* 2013). Acute respiratory failure is a rare complication but has a high mortality rate (Wang *et al,* 2007a).

Although children have a greater likelihood of developing hypovolemic shock in DHF characterized by increased microvascular permeability compared to adults, adults and elderly with dengue infection still have a high mortality rate (Agarwal *et al,* 1999; Gamble *et al,* 2000; Rigau-Perez and Laufer, 2006; Kuo *et al,* 2007; Lee *et al,* 2008; Gautret *et al,* 2012; Pang *et al,* 2012; Tantawichien, 2012). High fatality rates of dengue in adults were significantly associated with pre-existing co-morbid medical illnesses such as cardiac diseases and renal diseases (Kuo *et al,* 2007; Lee *et al,* 2008; Leo *et al,* 2011; Gautret *et al,* 2012; Pang *et al,* 2012; Tantawichien, 2015).

Because the altered vascular permeability is short-lived and spontaneously converts to normal level, the period of clinically significant plasma leakage usually lasts 24-48 hours after deferverscence. Diuresis ensues as plasma leakage terminates, convalescent rash, transient hypertension and sinus bradycardia have been described during convalescence in patients with DHF/DSS.

Hemorrhage associated with dengue infection

Hemorrhage often occurs between 5-to-8 days after the onset of illness and is a contributory factor to morbidity and mortality, particularly during the severe thrombocytopenia (Chuansumrit and Chaiyaratana, 2014).

The pathogenesis of abnormal bleeding in dengue is multifactorial and encompasses severe thrombocytopenia, platelet dysfunction, blood coagulation defects, and vasculopathy.

Typically seen coagulopathies are increased aPTT and low fibrinogen levels, but the likely major causes of clinical hemorrhage are severe thrombocytopenia and platelet dysfunction.

Variable degree of hemorrhage may occur at any sites, most commonly petechiae, epistaxis, and gingiva or vaginal bleeding, and usually on days 5-to-8 of the illness. Bleeding from the nose, gums, and upper gastrointestinal tract are not uncommon in patients with dengue infection.

Vaginal bleeding (menorrhagia) was a common site of bleeding (24.6% in adults with dengue infection) and hormonal therapies, such as premarin and primulute N, are suggested for patients exhibiting excessive vaginal bleeding (Tantawichien, 2012).

Of the dengue patients with plasma leakage (DHF), severity of bleeding varied markedly with spontaneous petechiae, hematemesis, melena, menorrhagia, and epistaxis. Risk factors of severe bleeding are platelets ≤20,000/mm³ (20x10⁹/l), high aspartate aminotransferase (AST), or alanine aminotransferase (ALT), prolonged prothrombin time (PT), severe plasma leakage (DSS), patients with DIC, or fulminant hepatic failure (Chamnanchanunt et al, 2012).

Pre-existing peptic ulcer or hemorrhagic gastritis in adults with DF or DHF may result in massive hematemesis, and this kind of hemorrhaging may be not associated with profound shock in adults dissimilar to previous reports in children. The literature contains few reports of endoscopic findings in dengue-infected adults with upper gastrointestinal bleeding, and those findings reported hemorrhagic gastritis most commonly (40.9-58.5%), followed by gastric ulcer, and duodenal ulcer (Tsai et al, 1991; Chiu et al, 2005). However, the utility of endoscopic therapy in upper gastrointestinal bleeding in dengue patients has not been established (Wung et al, 1990).

Subcapsular splenic hemorrhaging and ruptures are rare and life-threatening internal hemorrhaging that may occur spontaneously or due to trauma and may not be noticed. Splenectomy is still the favored treatment for splenic rupture, but favorable outcomes with conservative treatment have been recently and numerously reported (Imbert et al, 1993; Pungjitprapai and Tantawichien, 2008).

Averting a fatal outcome in the dengue patient with severe hemorrhaging requires early diagnosis, intensive supportive care and replacement therapy.

Reports about pregnant women with DF or severe dengue in Asia have emphasized the concept that young women in hyperendemic and endemic area are susceptible to dengue infection (Thaithumyanon et al, 1994; Bunyavejchevin et al, 1997; Corles et al, 1999).

Obstetricians must be alert to the risk of dengue infection in pregnant women and should take history and order laboratory test relevant to dengue infection. Dengue during pregnancy is of particular importance in pregnant women who are traveling from non-endemic countries to endemic ones (Carroll et al, 2007).

Spontaneous abortion and severe postpartum bleeding were reported to be caused by uterine hemorrhage in dengue-infected pregnant women (Thaithumyanon et al, 1994). Unexpected hemorrhage that is challenging to control in post-operative period may be caused by surgical

procedures, including cesarean sections that are performed on patients with dengue infection, may reveal previously undetected dengue-induced hemostatic defects (Adam *et al,* 2010).

Vertical transmission of dengue from mother to fetus has been reported that caused a full-blown illness in the neonate similar in manifestations to those seen in children and adults (Bunyavejchevin *et al,* 1997). The effects of dengue infection on pregnant women and their fetuses or newborns are unclear. Nevertheless, recent studies have shown that dengue infection may have been the culprit for fetal deaths and morbidity in pregnant women but did not appear to cause any infant abnormalities (Basurko *et al,* 2009; Pouliot *et al,* 2010; Chitra and Panicker, 2011).

Severe organ impairment and unusual manifestations

Hepatomegaly and increased levels of AST and ALT were more commonly found in patients with dengue infection, especially DHF (Kuo *et al,* 1992; Kalayanarooj *et al,* 1997; Souza *et al,* 2004; Trung *et al,* 2010; Kittitrakul *et al,* 2015; Treeprasertsuk and Kittitrakul, 2015). Due to these reports that certain liver enzymes may be elevated in dengue infections, the clinician should include dengue infection in the differential diagnosis of acute viral hepatitis in Asia. Dissimilar to conventional viral hepatitis, the dengue patient has levels of AST that are higher than that of ALT possibly owing to excessive release of AST from damaged myocytes during dengue infections, and these liver enzymes increase to maximum levels at 7-9 days after onset of illness, after which they decrease to normal levels within 2 weeks (Kuo *et al,* 1992; Kalayanarooj *et al,* 1997; Trung *et al,* 2010; Tantawichien, 2012; Tantawichien, 2015). Potential mechanisms of liver injury could involve a range of potential insults, including direct effects of infected virus serotypes, an adverse consequence of abnormal host immune responses to liver cells, compromised circulation and/or hypoxia due to hypotension or localized vascular leakage with the liver capsule, drug-related hepatotoxicity such as hepatotoxicity to acetaminophen or traditional herbal remedies, co-infection with other hepatitis-causing viruses such as hepatitis A, B, and C, as well as pre-existing underlying diseases (*eg*, hemoglobinopathies and alcoholic liver diseases) (Parkash *et al,* 2010).

Clinicians should consider carefully using hepatotoxic drugs such as acetaminophen, antibiotics, and antiemetic drugs, all of which have the potential to aggravate liver damage in patients with dengue. Acetaminophen overdose may play an important role in causing acute liver failure in dengue patients (Ling *et al,* 2007; Kye Mon *et al,* 2016), and adult dengue patients probably have relatively more liver impairment than child dengue patients. Pre-existing liver diseases such as chronic infection with virus hepatitis B or C, alcoholic liver disease, and cirrhosis may aggravate the severity of liver impairment during a dengue infection.

Dengue patients with vascular leakage and abnormal bleeding who have abnormal liver enzyme levels have been associated with disease severity and poor outcomes (Kittitrakul *et al,* 2015; Treeprasertsuk and Kittitrakul, 2015). Increased levels of bilirubin and alkaline phosphatase have been reported in a few patients. Severe liver impairment such as acute hepatic failure contributing directly to severe hemorrhaging as well as potentiating the severity of DIC may occur in the late stage of dengue disease, complicating the outcome of the patient (Ling *et al,* 2007; Kye Mon *et al,* 2016). Severe jaundice and high mortality are observed in dengue patients with fulminant hepatic failure. The management of fulminant hepatic failure in dengue is primarily intensive supportive care; however, therapies with N-acetylcysteine or artificial liver support have been described in the literature (Treeprasertsuk and Kittitrakul, 2015).

The unusual manifestations of dengue infection that have been recognized, include severe internal hemorrhage, fulminant hepatic failure, encephalopathy, cardiomyopathy, cardiac arrhythmia, adult respiratory distress syndrome, rhabdomyolysis, pancreatitis, appendicitis, co-infection with other viruses or tropical infectious diseases, and neurological complications (*eg*, altered consciousness, seizures, paresis, and coma resulting from encephalitis and encephalopathy)

(Thakane *et al,* 1996; Solomon *et al,* 2000; Garcia-Rivera and Rigue-Perez, 2002; Davis and Bourke, 2004; Promphan *et al,* 2004; Misra *et al,* 2006; Premaratna *et al,* 2007; Sam *et al,* 2013).

The neurological manifestations secondary to dengue infection, including encephalopathy, encephalitis, myelitis, neuro-ophthalmic complications, polyradiculopathy, neuropathy, and neuromuscular complications were ascribed in 0.5-21 % of hospitalized patients (Solomon *et al,* 2000; Garcia-Rivera and Rigue-Perez, 2002; Misra *et al,* 2006; Carod-Artal *et al,* 2013). Possible causes of encephalopathy in patients with dengue, include hypotension, cerebral edema, focal hemorrhage, hypernatremia, fulminant hepatic failure, and the direct invasion of dengue virus in the central nervous system (Lum *et al,* 1996; Chokephaibulkit *et al,* 2001).

Acute renal failure is an accompanying presentation in DSS or dengue-associated fulminant hepatic failure. Previous studies have revealed that 5.5% of the patients with DHF/DSS also had dual infection (*eg*, urinary tract infection, diarrhea, or bacteremia) (Pancharoen and Thisyakorn, 1998; Tantawichien, 2012). Dual infection should be suspected in patients with atypical manifestations; for example, fever for more than 10 days, mucus diarrhea, jaundice, persistent abdominal pain, recurrent fever, WBC >10,000/mm^3 (>10x10^9/l) with neutrophilia, or presence of the band form of neutrophil and acute renal failure (Lee *et al,* 2005).

The patient with severe dengue infection may have secondary bacterial sepsis (*eg*, bacteremia or UTI after hospitalization). Failure in making a diagnosis of concurrent infection in patients with dengue may lead to otherwise preventable mortality (Trunfio *et al,* 2016).

DIAGNOSIS

Most efforts to clinically differentiate dengue infection from other acute febrile illnesses are likely to be unsuccessful. The diagnostic attempt may be assisted by laboratory examination, indicating leukopenia, neutropenia, thrombocytopenia, or mildly elevated AST levels.

Early definite diagnosis of dengue infection allows the clinician to initiate supportive care and adequate fluid management early on and also identifies patients with severe dengue for close monitoring for signs of plasma leakage, bleeding, and end-organ damage. A definite diagnosis might also prevent the use of potentially harmful drugs, ensure the adequate use of treatment guidelines, and promote effective control of dengue outbreaks.

A positive tourniquet test has been considered by the WHO to have utility as a clue for probable dengue infection for a long time (WHO, 2009). Unfortunately, recent reports have found that the sensitivity and specificity of tourniquet test is poor (34-56% and 68-94%, respectively), and a negative test does not exclude the disease (Gregory *et al,* 2011; Mayxay *et al,* 2011; Halsey *et al,* 2013).

Laboratory diagnosis of dengue infection is established either directly by isolation or detection of viral components in serum or tissue, or indirectly by detection of virus-specific antibodies in serum (de Oliveira *et al,* 2005). The sensitivity of either molecular or serological testing depends partially on the duration and severity of the illness in a patient.

Only reverse transcriptase polymerase chain reaction (RT-PCR) or dengue virus NS1 antigen assay can reliably confirm the diagnosis of dengue during the 2 to 3 days after the onset of illness. The presence of the dengue virus in serum, tissues, saliva or urine can be definitely detected most satisfactorily by RT-PCR, and this molecular testing modality can detect dengue viruses up to 7 days after the onset of the symptoms, especially in severe cases (Alcon *et al,* 2002; Yamada *et al,* 2002; Lanciotti, 2003).

Various ELISA assays in the plasma and/or sera of dengue patients have shown a high circulating level of dengue virus NS1 in early stage of dengue infection (Vazqueza *et al,* 2010). Detecting antibodies for rapid diagnosis in the early stage of the illness is not practical due to their adequate detection ability starting to occur around 5 days after the onset of the illness. To date, ELISA for

detecting acute phase (IgM) and convalescent phase (IgG) antibodies has been considered the test of greatest utility in diagnosing dengue owing to its high sensitivity and ease of use.

There are several commercial kits of rapid tests for IgM and IgG detection; nevertheless, they vary in sensitivity, specificity, and accuracy (Kittigul and Suankeow, 2002; Blacksell *et al*, 2006). Various combination tests for elevated levels of NS1 and dengue IgM/IgG in serum have reported sensitivities ranging from 75.5% to 92.9% and specificities ranging from 75% to 100%, and these combination tests are a pragmatic diagnostic approach in a patient with a suspected dengue infection.

Even without the availability of laboratory tests that are accurate and facilitate early detection, the clinician must consider dengue infection in the differential diagnosis of an acute undifferentiated febrile illness in every presenting child or adult in a dengue endemic area.

MANAGEMENT

Until now, due to the lack of a specific therapeutic agent for dengue infection, treatment is supportive care, especially careful fluid administration. Reduction in morbidity/mortality rates in patients with dengue infection could be aided by the early recognition of warning signs, plasma leakage, abnormal bleeding, signs of circulatory collapse, and other serious complications. Dengue patient without warning signs may be treated at home with oral hydration and antipyretics with instructions to follow up at outpatient care. The clinician needs to provide appropriate safety netting advice by telling the patient return to the hospital promptly if hemorrhaging or warning signs suggestive of severe dengue develop.

Oral rehydration is indicated to replace fluid lost by vomiting and high fever. Patients and their care givers should be made aware that acetaminophen, salicylates, non-steroidal anti-inflammatory drugs (NSAIDs) and traditional medicines that are commonly prescribed to febrile patients may cause either severe bleeding or hepatic injury if used or

improperly used as appropriate. If warning signs develop, the patient requires close observation and hospitalization with appropriate use of intravenous fluids in patients with inadequate oral intake or a rapidly increasing hematocrit (WHO, 2009).

Monitoring all of the patients with warning signs to identify developing warning signs of severe disease, which is recommended by the WHO, may greatly burden healthcare services in limited-resource countries. Nonetheless, the clinician should hospitalize a patient with dengue infection immediately if any of the followings are observed: severe nausea/vomiting, restlessness or lethargy, severe hemorrhage (*eg*, hematemesis or hematochezia), narrowing of pulse pressure (≤20 mmHg) or hypotension, a sudden rise in hematocrit or continuously elevated hematocrit despite the administration of fluid, a platelet count of ≤20,000/mm^3 (≤20x10^9/l), AST or ALT >500 U/ml, oliguria or acute renal failure, liver failure, heart failure, severe hypoxemia, pregnancy, and no opportunity to be followed up in an out-patient setting (Nimmannitaya, 1987; Ngo *et al*, 2001; Royal College of Physicians of Thailand, 2015).

Close clinical monitoring of patients with severe dengue or suspected DHF/DSS and intensive supportive treatment are lifesaving and have reduced fatality rates.

Critical activities for the clinician taking care of hospitalized dengue patients are monitoring of abnormal bleeding, circulation and vascular leakage by serial clinical assessments of hypovolemia/shock and rising of hematocrit to inform the decision to give intravenous fluid or blood component transfusion.

Assessment of the severity of plasma leakage by close monitoring measurements (*eg*, vital signs, urine output, and serial hematocrit levels) and timely appropriate intravenous fluid resuscitation with crystalloid to counteract massive plasma leakage are necessary to reduce morbidity and mortality in the critical stage of DHF. DHF patients require close monitoring for signs of shock until at least 24-48 hours after defervescence. The main therapy for patients with plasma leakage and shock

(DSS) is early and appropriate replacement of lost plasma. The WHO recommends immediate volume replacement with Ringer's lactate or physiologically normal saline solution, followed by a plasma expander such as fresh frozen plasma or colloid solutions (albumin and dextran) if shock persists.

Therapeutic responses to colloid and crystalloid solutions from two randomized controlled trials have found that Ringer's lactate is inferior to other options and that the more severely ill patients identified by a narrow pulse pressure have a greater benefit from initial resuscitation with colloid compared with crystalloid solution (Dung *et al,* 1999; Wills *et al,* 2005; Akech *et al,* 2011). Adequate volume replacement should assessed by adjusting the rate of intravenous fluid during the period of plasma leakage with frequent assessments of vital signs, hematocrit, and urine output. Volume replacement should be kept to the minimum needed to maintain cardiovascular stability until vascular permeability returns to a normal level. Internal or concealed bleeding should be suspected in the patient with persistent shock in spite of a declining hematocrit after fluid resuscitation with crystalloid or colloid solutions.

To correct the bleeding tendency, anemia, coagulopathy, and hypovolemia in a dengue patient with severe hemorrhaging, blood transfusion therapy with packed red cell, concentrated platelets, and fresh frozen plasma is still the treatment of choice. Blood or platelet transfusions for prophylaxis against severe thrombocytopenia may cause harm and should not be performed in uncomplicated cases (Lye *et al,* 2009). Invasive procedures should be minimized to avoid hemorrhagic complications.

Because metabolic acidosis and hyponatremia occur more commonly in DSS, sodium bicarbonate infusion should be considered along with early adequate fluid replacement.

Co-morbidities in adult and elderly patients such as coronary artery disease, peptic ulcer, hypertension, diabetes mellitus, cirrhosis, or chronic kidney disease should be considered for proper management, and these co-morbidities

may contribute to the severity of dengue infection (Rigau-Perez and Laufer, 2006; Kuo *et al,* 2007; Lee *et al,* 2008; Lye *et al,* 2010; Sam *et al,* 2013).

No evidence supporting the use of chloroquine, corticosteroid, interferon, immune globulin, desmopressin, or carbazochrom sodium sulfonate (AC-17) for severe dengue infection exists (Tassniyom *et al,* 1993; Tassniyom *et al,* 1997; Kularatne *et al,* 2009; Tricou *et al,* 2010).

Vasculopathy and circulatory failure are usually self-limiting, and spontaneous resolution of these can be expected to occur within 2-to-3 days, followed by complete recovery. In the recovery period, the patient usually has improved appetite, bradycardia, and a convalescent rash and may have fatigue or mood disturbance for several weeks.

PREVENTION

To reduce burden of dengue, the WHO has set out specific objectives in global dengue control strategy: estimate the true burden of dengue by 2015 and a reduction of dengue morbidity and mortality by 2020 by at least 25% and 50%, respectively (using 2010 as the baseline reference measurement) (WHO, 2012).

It seems clear that implementations of effectively sustainable vector control and effective dengue vaccines are keys to success for this disease control.

The WHO has recommended the concept of integrated vector management (IVM). This is an evidence-based approach based on evidence specific to a country to promote the optimal use of its resources. Development and deployment of vector-control strategies that effectively minimize dengue replication and transmission are still challenging.

At present, public health and community-based *Ae. aegypti* control programs that use chemical or biological methods to remove and destroy mosquito-breeding sites are the mainstay of dengue prevention (WHO SEARO, 2011). However, the IVM approach has been unsuccessful in most of the Asian countries, especially dengue endemic regions. Thus,

new tools and strategies are needed to prevent and control dengue, including the development of a safe and efficacious dengue vaccine.

The potential dengue vaccine is one consisting of a tetravalent combination of attenuated dengue strains, which simultaneously induce protective and durable immune responses against all four dengue serotypes. Recent studies in Asia and Latin America show that recombinant live-attenuated tetravalent dengue vaccine (CYD-TDV) was safe and moderately efficacious when given three injections at months 0, 6, and 12 to children and adolescents (Capeding *et al,* 2014; Hadinegoro *et al,* 2015). Overall vaccine efficacy of CYD-TDV was estimated to be 60% against virologically confirmed dengue infection (VCD) with high levels of protection offered against hospitalization (80%) in subjects aged 2-16 years. However, variations of vaccine efficacy against VCD were observed in endemic settings, dengue serotype, and the pre-existing dengue antibody type in terms of serotype in the individual (Capeding *et al,* 2014; Hadinegoro *et al,* 2015). Recent pooled analyses of the first 2-3 years of long-term follow-up provided further supportive evidence of efficacy against hospitalized dengue in children 9 years of age or older (Guya *et al,* 2015). This vaccine has recently obtained licensure for use in children 9 years of age or older (9-45 year old) in Mexico, the Philippines, Brazil, Thailand, Singapore, and several other endemic countries. Due to the high disease burden in endemic countries, this vaccine could have a substantial effect on public health despite its moderate overall efficacy (Endo *et al,* 2016; Lopez-Gatell *et al,* 2016; Pang, 2016). The implementation of an efficacious dengue vaccine will shift the burden of disease, the age-related differences in clinical manifestations and prognoses described here, indicating the importance of comparing a wide range of ages in future clinical studies of dengue.

CONCLUSION

The increasing number of dengue cases is a major public health problem in many countries in South and Southeast Asia where *Ae. aegypti* and *Ae. albopictus* are widespread in both urban and rural areas. Several countries in Asia have reported an epidemic shift of dengue from mainly affecting children to affecting adolescents and young adults with increased severity. The clinical spectrum of dengue ranges from undifferentiated fever or DF to the life-threatening infection associated with plasma leakage (DHF/DSS), severe bleeding or multi-organ failure, which may be fatal.

The early recognition of warning signs, plasma leakage, abnormal bleeding, circulatory collapse, and other serious complications would reduce mortality rates in patients with dengue infection. The implementations of effectively integrated vector management and efficacious dengue vaccines are the keys to success for disease control in hyperendemic/endemic areas.

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A SHORT HISTORY OF DENGUE AND MAHIDOL DENGUE VACCINE

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Abstract. In 1980, Mahidol University committed to develop a live-attenuated tetravalent DENV vaccine. The DENV vaccine development project was supported by a grant from the WHO Regional Office for South-East Asia (ICP RPD 002/DHF). DENV-1 and -2 obtained from DHF patients and DENV-4 obtained from DF patients were serially passaged in primary dog kidney (PDK) cells certified to be free from human and canine infectious agents. DENV-3 obtained from DHF patients was first passaged in primary green monkey kidney (PGMK) cells and then in certified Fetal Rhesus Lung (FRhL) cells. The degree of attenuation was empirically based on certain biological markers. Bulk seed productions were eventually prepared in pilot production facilities at Mahidol University's Centre for Vaccine Development at the Institute of Molecular Biosciences. They were subjected to general safety tests and monkey neurovirulence tests in accordance with the US FDA requirements. These pre-clinical tested candidate DENV viruses were approved for proceeding to the clinical evaluation phase by a WHO-appointed Scientific Steering Committee and by the Ethical Review Committee of the Thai Ministry of Public Health. The monovalent live-attenuated viruses – DENV-1 PDK-13, DENV-2 PDK-53, DENV-3 PGMK-30/FRhL- 3 and DENV-4 PDK-48 – were first tested in flavivirus non-immune adult subjects, followed by bivalent, trivalent, and tetravalent vaccine clinical trials. All vaccine recipients developed either a mild or no adverse reaction to the vaccine. The immunogenicity data were discussed. Due to viral interference of each DENV components in the combinations, 12 DENV formulations were evaluated for confirmation of safety and immunoginecity profiles in 155 Thai children aged 3-15 years. Preliminary data were analyzed and processed for further development.

In order to make productive use of this research, Mahidol University entered into a collaborative licensing agreement in DENV vaccine production in 1993 with France based Sanofi Pasteur, the vaccine division of Sanofi-Aventis Group and the largest company in the world devoted entirely to human vaccines. DENV vaccine based on this approach was prepared for production on an industrial scale in France using specific-pathogen-free (SPF) dog colony and FRhL cells. The vaccine is presented in a lyophilized (freeze-dried) form and reconstituted with water for injection in order to deliver a 0.5 ml specified dose. Multiple dose presentations were planned for a target population of children and adults living in or travelling to DEN-endemic areas. The current strategy of creating tetravalent DENV vaccine formulations can lead to an unbalanced immune response. This is attributed to viral interference that apparently comes into play when three monovalent vaccine viruses DENV-1, DENV-2 and DENV-4 are mixed with DENV-3 to create a tetravalent formulation. More research is needed on a priority basis to work out the viral interference factor in order to make the production of a tetravalent vaccine out of our attenuated DENV-3 candidate vaccine strain a success.

Keywords: live attenuated tetravalent dengue vaccine, WHO/SEARO, PDK cells

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INTRODUCTION

Dengue fever and dengue hemorrhagic fever (DF/DHF) are caused by dengue (DENV) viruses. There are four antigenically related, but distinct, DENV serotypes (DENV-1 through DENV-4).

Humans are the amplifying vertebrate hosts, and *Aedes* mosquitoes are the primary mosquito vectors as well as the reservoir of infection. DENV infections cause a spectrum of diseases, ranging from asymptomatic infections to infections that are complicated by hemorrhage, shock, and death. Infection with DENV of one serotype results in apparent life-long monotypic immunity against that serotype but not against any other serotype. Thus, separate infections with all four DENV serotypes are theoretically possible in a single host. It should be noted that in Thailand, all the four DENV serotypes co-circulate, thereby resulting in multiple exposures and the potential for re-infection with different serotypes.

HISTORICAL DEVELOPMENT

The name dengue was accepted for standard usage by the Royal College of Physicians of London in 1869. Bancroft in 1906 published the first evidence of the fact that *Aedes aegypti* mosquitoes are vectors of the disease. In 1931, Simmons proved that *Aedes albopictus* is also an efficient vector. The demonstration that dengue virus can produce inapparent infection in certain species of monkeys led to the accumulation of evidence indicating that certain monkeys may be infected in nature that the infection can be transmitted by mosquitoes from monkey to monkey as well as from monkey to man and that monkeys may constitute one of the links in the chain of events which perpetuate the virus in nature. During World War II, extensive studies resulted in the demonstration of multiple immunologic types of the virus, known later on as dengue 1-4 serotypes.

Dengue Hemorrhagic Fever (DHF) was recognized as a new disease first in Manila in 1954. The disease affected mainly children and was characterized by acute onset of high fever, petichial hemorrhagic and shock. The second large outbreak occurred in Manila again in 1956 affecting more than 1,200 cases with 6% case-fatality rate. In 1958, an outbreak of DHF occurred in Bangkok and nearby areas. Almost 2,500 cases with 10% case-fatality rate were recorded.

Since then, DHF has become a serious public health problem, causing large scale of morbidity and mortality among children in South-East Asia and Western Pacific Regions of WHO. Well-established epidemics have been reported from Myanmar, China, Cambodia, Indonesia, Lao PDR, Malaysia, Philippines, Thailand and Vietnam.

In the WHO South-East Asia Region, DHF is a major public health problem in Indonesia, Myanmar and Thailand.

The first meeting of the South-East Regional Advisory Committee on Medical Research (SEA/ACMR) held in New Delhi, 5-9 January 1976, recommended to the Regional Director that research on DHF be considered to be of high priority. During the second session of the SEA/ACMR held in New Delhi, 23-27 August 1976, a review on the history of the spread of this disease through several countries of the Region, with an evaluation of the current state of knowledge on its epidemiology, virology, pathogenesis and the related problems of clinical management.

A meeting of the Research Study Group on DHF was held in New Delhi on 24-25 February 1977. Several measures with potential for the prevention and control of this disease were considered. After detailed discussions, the group made its recommendations, of which the two important ones were: (i) vaccine research; and (ii) control of *Aedes aegypti*. The first plan of vaccine research was developed, which, inter alia, proposed that virologists from the South-East Asia and the Western Pacific regions be trained in research and development of the vaccine at the School of Tropical Medicine and Medical Microbiology, University of Hawaii. On the completion of the training, the participants on return to their respective countries were impressed upon to get directly involved in the national DENV vaccine development program. The time frame needed for the development of the DENV vaccine program was proposed to be 3-5 years.

It was understood that most countries with DHF problem would like to participate in the field trials of DENV vaccine at a later stage when the vaccines would be ready.

In 1978, a research steering committee recommended to WHO to take positive steps towards DENV vaccine development by designating the then Department of Pathology, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, now known as the Centre for Vaccine Development, Mahidol University at Salaya, Thailand, to undertake the research for the development of the vaccine.

Funding of this project began in April 1980. Three laboratories were equipped for DENV vaccine research and development. A virologist was recruited and sent to the University of Hawaii for the initial phase of research as well as for advanced training while equipping of the laboratory continued. The laboratory was ready for operation in early November 1980. Detailed and comprehensive standard operating procedures (SOPs) for vaccine development were prepared. Protocols were available. Tests were signed by operators and were checked and signed by the supervisor.

In 1987, the site for DENV vaccine development moved to the Centre for Vaccine Development of Mahidol University at Salaya, Nakhon Pathom, Thailand. Equipments for the Government. Another four additional buildings were constructed between 1988-1990, which included an experimental animal house and vaccine pilot plant buildings. The entire vaccine compound was designated for DENV vaccine development. Airlocks and a hepafiltered air supply were generated to control potential cross-contamination.

DENGUE VACCINE DEVELOPMENT

Rationale for dengue vaccine development

The scientific hypothesis behind the development of tetravalent DENV vaccine against DHF could be summarized as follows:

1. Adults develop a higher rate of seroconversion of antibody response against DENV viruses and appeared to be less susceptible to DHF. The naturally acquired immunity appeared to protect the individuals against the infection. The immunization of target populations could result towards development of protective antibody response in individuals and could help in protecting the disease.

2. It has also been shown that a mono- or bitypic antibody response could be a risk factor for DHF if sequential infection by other types of DENV viruses occurred. It was imperative that DENV vaccine should be able to confer the protective immunity against all four types of DENV infection and provide a life-long immunity. This called for the development of a live-attenuated tetravalent DENV vaccine.

3. The target population for immunization against DHF should be toddlers 1-3 years old.

Technical consideration on dengue vaccine development

The objectives of this program were to select strains of DENV-1, -2, -3, and -4, which showed promise of being attenuated for human use and produced in cell substrates. All the four DENV virus serotypes being developed in Thailand were passaged serially in cell culture without specific selection.

Dengue virus strains selected for attenuation attempts

Dengue 1 (16007-TC-10 2/14/74). Isolated from a DHF patient in Thailand in 1964 had been passaged in tissue culture before inoculation into *Toxorhynchitis amboinensis*. The first intrathoracic passage is No. 167164 and the second in 167376 (received from Dr Robert E Tesh on June 17, 1980).

Dengue 2 (16681 LLC-1 1/22/73). Isolated from a DHF case in 1964 from Thailand had been passaged in tissue culture before inoculation into *Toxorhynchitis amboinensis*. The first intrathoracic passage was Nº 167165 and the second passage is 167377 and was received on 17 June 1980. The parent culture strain was virulent for man, having produced typical DF in a laboratory worker who was exposed accidentally (unpublished observations, Dr SB Halstead).

Dengue 3 (16562 TC-7 1/31/72). Virus was isolated in 1964 from a DHF case in the Philippines. It had been passaged in cell culture before inoculation of *Toxorhynchites amboinensis*. The

first intrathoracic passage was N° 167166; the second passage was N° 167378 (received for PDK cell passage on 17 June 1980). The parent culture passaged virus was demonstrated to be virulent for man having produced DF in an accidentally infected laboratory worker (unpublished observations, Dr SB Halstead).

Dengue 4 (1036). Virus was isolated from a DF cases in Indonesia in 1976 using *Aedes aegypti* and kindly furnished by Dr Duane G Gubler. The fourth passage was used to initiate the vaccine studies.

Mosquito inoculation

At the University of Hawaii (Pacific Research Unit), five adult laboratory-reared *Toxorhynchitis amboinensis* were inoculated intrathoracically with strains of DENV-1 to -4. The inoculum was approximately 0.0003 ml. Mosquitoes were maintained on 10% sucrose solution at 28°C for 12 days. At the end of the incubation period, each group of insects was killed by freezing, their heads removed and triturated in 5.0 ml phosphate buffer saline, containing 0.5% gelatin, 30% heat-inactivated calf serum and penicillin and streptomycin. After centrifugation at 5°C for 30 minutes, each supernatant fluid was inoculated into another group of five *Toxorhynchitis amboinensis*. These insects were also held at 28°C for 13 days. At the end of the incubation period, freezing killed them.

Preparation of mosquito suspensions

The head was removed from the infected mosquitoes with a sterile surgical blade and placed in a mortar. Body parts were kept in a sterile vial and frozen at -70°C. The virus diluent, with 30% heat inactivated calf serum in phosphate-buffered saline, pH 7.5, penicillin/streptomycin, was added to ground mosquito heads, 2.5 ml/5 mosquitoes. After centrifugation at 10,000 rpm for 30 minutes at 5°C, the supernatant fluids were filtered through a 0.45 micron Millipore filter. Filtrates were used to inoculate primary dog kidney and Green Monkey Kidney (GMK) cells.

Preparation of primary dog kidney cells

The work was done in the laboratories of the Department of Tropical Medicine and Medical Microbiology, University of Hawaii School of Medicine, and was supported, in part, by a Grant from the Rockefeller Foundation to Dr SB Halstead.

Each lot and sub-lot of dog kidney cells were subjected to safety tests to assure that the cells and supernatant fluid were free of infectious agents. Tests included for exclusion of bacterial, fungi, mycoplasma and cytopathic and hemadsorbing agents.

Development of attenuated strains of DENV 1-4 viruses

Mosquito suspension of the DENV-1 (16007), DENV-2 (16681), and DENV-4 (1036) were attenuated by serial passages in PDK cell culture ate 32°C without cloning or deliberate selection. The procedure relied on the spontaneous appearance of variants and selection for attenuated variants by the biological pressure of the abnormal host cell. This general method had been successful with several other live virus vaccines, *eg* rubella and mumps.

The DENV-3 (16562) virus was attenuated by serial passages in GMK cell; however, attempts to adapt it to PDK cells had failed.

DENV viruses were serially passaged in PDK cells (Fig 1).

At every fifth passage level, a moderate-sized virus seed was prepared. This virus was studied for plaque size morphology in LLC-MK2 cells, temperature sensitivity to of replication shut-off, suckling mouse neurovirulence and growth in human monocytes, viremia and antibody response in primates.

When the passaged virus presented a reduction in plaque size, temperature sensitivity for replication and absence of viremia and reduced antibody response in monkeys, a "Master seed", "Production seed" and "Candidate vaccine" were prepared. Safety tests on the Production seed and Candidate vaccine included the inoculation of neutralized virus into adult and suckling mice, guinea pigs, rabbits and several tissue culture systems. Furthermore it was also the assured that the candidate vaccine produced no neurovirulence

DENV VIRUS

↓

Mosquito (2 passages)

GMK
(parental virus)

PDK 1

↓

PDK 5

↓

PDK 10

↓

etc
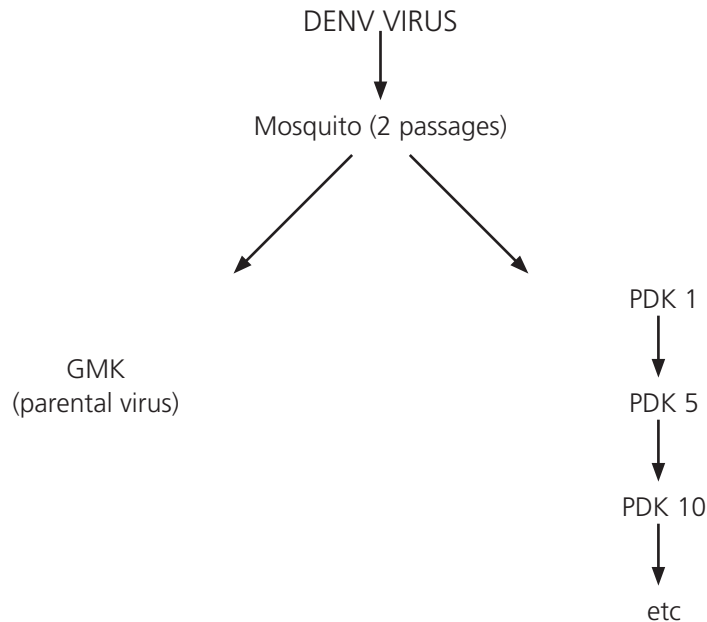
Fig 1–Schema for attempted attenuation of dengue viruses.

following intracerebral inoculation in monkeys. The attenuated strains this developed could help towards worldwide stock of candidate dengue vaccines.

What constitutes a satisfactory level of attenuation remains uncertain. Hypothetically, we would like to have a vaccine which is avirulent, that is, viruses which do not have the capability to cause direct cell injury, but the protective antigenic epitopes of these avirulent viruses should still be preserved and effectively presented to both the B and T lymphocytes of the vaccines to confer both humoral and cellular immunity. It is very difficult to define the attenuation of the dengue viruses by a specific series of the biological markers. These observations remained unsubstantiated due to the fact that there was, no known animal model for DHF. Man represented the only alternative testing model of vaccine efficacy.

Markers of attenuation

To define the level of attenuation of the viruses at the present, it could at best be empirical. The assessment was based on the findings of a combination of markers.

Evidence for attenuation was based on a comparison of the high passage viruses with the parent virus in several *in vivo* and *in vitro* tests: plaque size, temperature sensitivity, replication in human monocytes, and monkey viremia. These characteristics had been shown to be related to human virulence with other experimental dengue vaccines.

The DENV-1 PDK 43, DENV-2 PDK 53, DENV-3 GMK 33 and DENV-4 PDK 48 candidate viruses produced a uniform plaque size when assayed in LLC-MK2 cells. They revealed temperature sensitivity by the plaquing efficiency test. High PDK or GMK passages had significantly reduced virulence for suckling mice by the intracerebral route. All the DENV candidate viruses produced low or no ability to replicate in human monocytes *in vitro*. All of them showed low or no viremia after inoculation with 104-105 plaque forming unit (pfu) of each candidate viruses with moderate specific neutralizing antibody responses. Reduced neurovirulence for mice was observed with DENV-1, DENV-2 and DENV-3 candidate viruses. However, the DENV-4 PDK48 candidate viruses still revealed

modulate neurovirulence in suckling mice.

Safety test

Safety tests of the cell substrate, the candidate viruses and the candidate vaccines were designed according to the United States FDA regulations as applied to live attenuated vaccines produced in the United States. Tests included microbial sterility; and search for adventitious agents in PGMK cells, adult and infant mice, guinea pigs, and rabbits. Hemadsorption agents were sought in cell-culture experiments. A second tier of tests required for additional safety were performed at the virology laboratory of the Department of Tropical Medicine and Medical Microbiology, University of Hawaii.

The team could establish the capability to perform monkey neurovirulence test in Thailand and slides of monkey tissue were independently reviewed by an experienced neuropathologist.

Peer review of the vaccine development project

Candidate DENV vaccines considered to be sufficiently attenuated were submitted to an international panel of experts in DENV for review. This panel had met annually once a year in Bangkok for twelve times from 1983 to 1994 (Table 1). The function of the panel of experts was to review the scientific work, including visit to the site of vaccine development, in order to pursue and examine the facilities, and to audit the raw data. The record books were reviewed by two of the peer reviewers in detail for completeness and for the accuracy of summary data presented. The peer group gave recommendations to the Ministry of Public Health of Thailand and to WHO-SEARO based on their assessment whether the candidate vaccines were suitable for vaccine trials in human beings or not. This process was unique for WHO programs.

The DENV-1 (16007) PDK 43, DENV-2 (16681) PDK 53, DENV-3 (16562) GMK 30 FRhL 3 and DENV-4 (1036) PDK 48 met the US FDA requirements for microbial safety and monkey neurovirulence test for live attenuated viral vaccine conducted by laboratory at Mahidol University as well as by an independent laboratory at the Walter Reed Army Institute of Research, USA. They were

approved by an international peer review group based on the examination of the result of safety test and by an on-site examination of the facilities, laboratory record and log books. Confirmatory histopathological examination was done at the ethical review conducted by a committee for human experimentation of the Mahidol University and by a similar committee of the Ministry of Public Health, which was satisfactory and these candidate vaccines were approved for clinical trials.

CLINICAL TRIALS OF DENGUE VACCINES

Clinical trials of monovalent dengue vaccines

The site for the small-scale experimental clinical trial was Lamphun and Loei Provinces, an area where there was low prevalence of *Ae. aegypti* mosquitoes. The trials were conducted during the cold season so as to minimize the risk of other arbovirus infections and possible transmission of vaccine viruses. The population chosen to conduct the trials consisted of flavivirus non-immune young male adults. The initial trial was conducted in two phases using first two and then eight volunteers to increase the safety factor. The protocol called

Table 1. Dengue Virus Development Program, WHO Peer Review Meeting, Bangkok, Thailand [a].

Serial No.	Date		
1st	1-5	August	1983
2nd	23-25	August	1984
3rd	31	July-2 Agust	1985
4th	20-22	August	1986
5th	27-30	July	1987
6th	1-5	August	1988
7th	7-11	August	1989
8th	29-30	September	1990
9th	26-28	August	1991
10th	24-26	August	1992
11th	23-25	August	1993
12th	29-31	August	1994

[a] Organized by WHO/SEARO, WHO Project: ICP RPD/002.

for close observation during the first 21 days.

DENV-1 (16007) candidate vaccines

The candidate DENV-1 (16007) PDK 43 vaccine was passed 43 times in PDK cell. The evolution of the biological markers tested was as follows: plaques in LLC-MK2 cells became of small size (=1 mm) after passages 10-15. Temperature sensitivity at 39°C was achieved at passage 30. Ability to grow in human peripheral blood lymphocytes (PBL) was lost at passage 20. Suckling mouse neurovirulence was reduced to minimal level at passage 15. After 43 passages, all monkeys that received the DENV-3 PDK 43 showed none or low viremia. Based on these results, passage 43 was selected for phase 1 trial.

Six flavivirus non-immune subjects were inoculated with a dose of 2.1 to 3.5 × 10⁴ pfu. Clinical symptoms were mild in all volunteers and only one of them showed very minimal nose bleeding, without other hemorrhagic manifestations. In no case was absolute eukopenia observed. Clinical chemistry was normal in all volunteers. Immune response, as measured by plaque reduction neutralization test (PRNT), was detected in only 1 out of 6 flavivirus non-immune volunteers. Two of the seronegative subjects were challenged again with the same dose of DENV-1 PDK 43 at 3 months. Again, they failed to develop any neutralizing antibody. The conclusion was that DENV-1 PDK 43 had been over-attenuated and that it was necessary to try lower passage levels.

A candidate vaccine was then prepared from DENV-1 PDK 30, and after the usual safety tests, was infected into five adult male volunteers who were seronegative for both JE and DENV viruses. Two of the five seroconverted, but the responses were low. The exercise was thus repeated using DENV-1 PDK 20 but again the antibody responses were low and only three of the five seroconverted. The conclusion was thus reached that DENV-1, PDK 20, PDK 30 and PDK 43 were over-attenuated to be useful as candidate vaccines for DENV-1 in people that were seronegative for previous exposure to flaviviruses. The DENV-1 PDK 13 virus that was used in the next trial showed evidence of lesser attenuation than PDK 20, PDK 30 or PDK 43 in that it still replicated in human monocytes. When seven DENV and JE antibody negative male volunteers were injected with DENV-1 PDK13, five seroconverted to DENV-1 within 30 days. There was some evidence of rhinitis but this may have been coincidental and the significance of the observation could not be assessed. There was also a slight fall in leukocyte counts on day 10, but the virus was not isolated from blood at any stage.

DENV-2 (16681) candidate vaccine

The trial of the DENV-2 candidate vaccine, DENV-2 (16681) PDK 53, was carried out as phase 1a and 1b trial.

The initial phase 1a of DENV-2 PDK 53 candidate vaccine in ten 18-30-years-old male human subjects showed encouraging results. None of the 10 persons vaccinated were febrile or incapacitated; side-reactions possibly attributable to the candidate vaccine were limited to slight leukopenia, occasionally abnormally large platelets and a few transient complaints such as mild aches and pains.

All vaccinated persons developed DENV-2 neutralizing antibody. Those subjects with preexisting antibody to JE virus responded serologically more rapidly than those subjects without preexisting flavivirus antibody before vaccination.

Serological tests carried out one year after the vaccination showed that neutralizing antibodies were present in 100% of the volunteers.

The phase 1b trial of DENV-2 PDK 53 candidate vaccine involved sixteen 15-30-year-old male volunteers, 15 of whom were flavivirus non-immune. Four doses of varying virus dilutions were given to groups of four +volunteers each, and every person developed DENV-2 neutralizing antibodies, regardless of vaccine virus dilutions. Abnormal lymphocytes and a slight decrease in lymphocyte numbers were consistently observed between days 6 and 10. As in the phase 1a trial, no adverse reactions to the vaccine were observed.

Viremia was detected in one volunteer and virus isolated in C6/36 cells from day 6 serum.

The virus had growth characteristics similar to those of the candidate DENV vaccine virus. On the basis of 1a and 1b studies it was suggested that viremia occurred between days 6 and 10. It is unlikely to occur after day 14 because of the onset of neutralizing antibodies. It is possible that viremia may precede the time of the lowest white cell counts, which frequently occurred on day 6.

A dose response study in adults, based on 5-fold dilutions of the vaccine, showed an estimated 50% infectious dose of 5-7 pfu.

DENV-3 (16562) candidate vaccines

The DENV-3 (16562) parent virus did not grow in PDK cells and was passaged in GMK cells. At passage GMK 30, the virus was still able to replicate in PBL and produced plaques of varying sizes. After passage 34, plaques were uniformly small. Two GMK passages were selected for adaptation to FRhL cells: 30 and 35. In FRhL cells, DENV-3 attained titers one log higher than in GMK cells. With both passages (PGMK 30/F2) and GMK 35/F2), biological markers were considered to be satisfactory: plaques were of small size, no CPE in LLC-MK2 cells, temperature sensitive at 38.3°C, no growth in human PBL, and reduced neurovirulence for suckling mice.

No adventitious agents were found in GMK cells analysed by electron microscopy. Safety tests of GMK cells were being completed at the National Institute for Biological Standards and Control (London) and the National Biological Standards Laboratory (Canberra). The cells had been found to be free of mycoplasma, mycobacteria and other adventitious agents. Tests to detect simian retroviruses, SV5 and SV40, were negative.

Biological characteristics of DENV-3 candidate vaccine viruses

The DENV-3 (16562) GMK 30 passage virus had mixed plaque morphology (medium and small), a restrictive temperature of 40°C caused CPE in LLC-MK2 cells and grew in human PBL. DENV-3 GMK 30, FRhL-3 virus had small and pinpoint plaque morphology, restricted growth at 38°C, and did not cause CPE in LLC-MK2 cells. Considerable change, presumably selection, had

occurred with FRhL passage. The virus recovered from a volunteer who received PGMK 30, FRhL-3 vaccine had biological markers (medium) plaque size, CPE in LLC-MK2 similar to earlier passage vaccine was either genetically prone to reversion or contained an undetected subpopulation of more virulent virus.

Three passage levels of DENV-3 (16562) were given to volunteers; GMK 33, PGMK 30-FRhL-2, and GMK 30 FRhL-3. The FRhL passaged viruses differed from the GMK 33 in being more temperature-sensitive, less able to produce CPE in LLC-MK2 cells, and having uniform small plaque morphology.

Four volunteers received the GMK 30 FRhL-3 virus at doses of 1×10^4 to 6.5×10^4 pfu. One of two volunteers seroconverted at the lower dose. The volunteer who failed to convert at the lower dose was revaccinated at the higher dose and seroconverted. Two volunteers seroconverted at a higher dose. Only minor symptoms and no fever were observed. Satisfactory primary immune responses were observed in three volunteers; the fourth, who was JE immune, had a secondary-type serological response. The virus isolated from the serum of one volunteer exhibited medium-sized plaque morphology and its characteristics of earlier passage virus.

In other trials, two volunteers received GMK 33 vaccine and four volunteers received GMK 30 FRhL-2 vaccine. Both of those vaccines contained both medium and small plaque sizes and were less temperature sensitive than the GMK 30 FRhL-3 vaccine. Both vaccines immunized satisfactorily at doses of 10^4; however, brief febrile responses and mild symptoms were observed.

The GMK-30, FRhL-3 vaccine appeared to be less reactogenic than the other two DENV-3 candidate vaccines and was immunogenic at a dose of 5×10^4.

DENV-4 (1036) PDK 48 candidate vaccine

DENV-4 (1036) virus was passaged in PDK cells to passage 48. Biological markers of PDK 48 included small plaque and no cytopathic effect in

LLC-MK2 cells, temperature replicative shutoff at 39°C, and average survival of 12 days in suckling mouse. PDK 48 virus replicated in peripheral blood mononuclear cells. Rhesus monkeys inoculated with PDK 48 virus did not develop viremia but seroconverted. On evaluation of the monkey neurovirulence tests, the panel of experts concluded that there was no significant difference between the parental DENV-4 virus and DENV-4 PDK 48 candidate vaccine, and it was thus acceptable to proceed with phase 1a clinical trial. An additional four rhesus monkeys had been tested with PDK 48 virus; enhanced neurovirluence was not found. The monkey neurovirulence test result was considered satisfactory, and it appeared feasible to proceed with PDK 48 as a candidate vaccine.

The phase 1a clinical trial was the inoculation of five flavivirus seronegative volunteers with $1-2 \times 10^4$ pfu of DENV-4 PDK 48. All volunteers developed specific neutralizing antibodies, which first appeared from days 13-16, and peaked in titer at day 30 post-inoculation. Clinical signs were unremarkable in all volunteers, and no volunteer developed fever. Clinical symptoms were generally absent, although two volunteers reported eye pain and headache. In one of these volunteers the headache re-occurred for a period of about 2 weeks. All volunteers showed normal blood chemistry profiles. Hematological studies revealed a transient increase in atypical lymphocytes in three volunteers. All volunteers showed a temporary depression in total white blood cell counts; however, there was no absolute leukopenia. Virus was recovered only from plasma, and the viremia appeared to be low in titer, since no virus could be detected by direct plaque assay. The recovered virus strains shared all biological markers with the vaccine candidate, except that one strain showed an extended mean day to death in suckling mice of 20 days.

The phase 1b trial was designed to determine the minimum infective dose of the DENV-4 vaccine candidate. The 1b trial was temporarily divided into two phases with groups of seven and five volunteers. The vaccine was diluted from 1:5 (3,700 pfu) to 1:1,000 (12-15 pfu) and each dilution was inoculated into 1-3 volunteers. None of the volunteers showed fever or rash, and clinical signs and symptoms were mild, although eight of the twelve volunteers reported transient headache and eye pain. Blood chemistries were normal, and hematological findings were similar to those seen in the phase 1a trial.

All groups of volunteers inoculated with a dilution of 7×10^2 pfu or greater developed specific neutralizing antibody. Two of the two volunteers at 7×10^2 pfu seroconverted, one of the two at 1.5×10^2 pfu seroconverted, and none of the three volunteers at 63-77 pfu seroconverted. In total, combining the phase 1a and phase 1b results, 10 of the 10 volunteers inoculated with vaccine doses of 7×10^2 pfu or greater seroconverted.

Polyvalent vaccine clinical trials
Bivalent vaccine DENV-2 (16681) PDK 53 and DENV-4 (1036) PDK 48 clinical trial. The aim of the DENV vaccine development program was to develop and administer a vaccine containing a mixture of multiple DENV serotypes. The rationale was based on the provision of providing protection to all serotypes that would minimize any chances of future DENV infection enhancement and adverse host reaction. This trial was designed to conduct in humans in support of the hypothetical concept that multiple simultaneous infections with candidate vaccines were possible, safe, and effective. Eleven male flavivirus non-immune subjects aged 16 to 31 years received bivalent DENV-2 and DENV-4 candidate vaccines.

The neutralizing antibody responses to DENV-2 at 6 months ranged from 1:52 to 1:440 and that of DENV-4 ranged from 1:44 to 1:310. A low titer of neutralizing antibodies to DENV-1, DENV-3 and JE viruses were detected early, but this disappeared by 6 months.

The bivalent DENV-2–DENV-4 candidate vaccine was both immunogenic and without unacceptable reactions. Moreover, the dose of DENV-2 and DENV-4 viruses was acceptable and formed to be the basis for future trials.

Bivalent vaccine DENV-1 (16007) PDK 13 and DENV-4 (1036) PDK 48 clinical trial. The bivalent DENV-1 and DENV-4 vaccine's human clinical trial was conducted in Loei Province, Thailand. Seven male flavivirus nonimmune subjects, aged 16 to 30 years, received bivalent DENV-1 and DENV-4 candidate vaccine.

All seven subjects seroconverted to both DENV-1 and DENV-4 since the presence of neutralization antibodies were detected by PRNT to both DENV-1 and DENV-4. There was no difference in response between those receiving candidate vaccines in separate arms and among those receiving mixed vaccine in another arm.

The bivalent DENV-1 and DENV-4 candidate vaccine induced specific response to both DENV-1 and DENV-4 but low titers of heterologous neutralizing antibody were found and the vaccine, was without any adverse reactions, among the recipients.

Bivalent vaccine DENV-1 (16007) PDK 13 and DENV-2 (16681) PDK 53 clinical trial. Seven male subjects aged between 17 and 32 years received bivalent DENV-1 and DENV-2 candidate vaccine.

All seven subjects seroconverted to both DENV-1 and DENV-4 since the presence of neutralization antibodies were detected by PRNT to both DENV-1 and DENV-4. There was no difference in response between those receiving candidate vaccines in separate arms and among those receiving mixed vaccine in another arm.

The bivalent DENV-1 and DENV-4 candidate vaccine induced specific response to both DENV-1 and DENV-4 but low titers of heterologous neutralizing antibody were found and the vaccine was without any adverse reactions among the recipients.

Bivalent vaccine DENV-1 (16007) PDK 13 and DENV-2 (16681) PDK 53 clinical trial. Seven male subjects aged between 17 and 32 years received bivalent DENV-1 and DENV-2 candidate vaccine.

All subjects seroconverted to DENV-1 and DENV-2 by 30 days. Titers of DENV-1 neutralizing antibody ranged between 1:27 and 1:70 in the six subjects who were non-immune before vaccination. Titers of DENV-2 ranged from 1:26 to 1:120 and at 30 days no cross-reaction with other flaviviruses was observed.

The bivalent DENV-1 and DENV-2 candidate vaccine was both immunogenic and without any adverse reactions.

Trivalent vaccine DENV-1 (16007) PDK 13, DENV-2 (16681) PDK 53 and DENV-4 (1036) PDK 48 clinical trial. The human clinical trial comprised of a mixture of three monovalent DENV vaccines (DENV-1, -2 and -4), which was inoculated into each of 12 male adults. The study was performed in a subdistrict of Loei Province in Northeast Thailand, where the prevalence of *Ae. aegypti* or *Ae. albopictus* mosquitoes was low. The objective of this study was to determine the safety and feasibility.

Of the 12 recipients, nine were flavivirus non-immune; they all developed serum neutralizing antibodies to all the three DENV viruses.

The results of this study showed that it was possible to infect humans safely with three attenuated DENV viruses. The median dose of DENV-1 virus was close to optimum whereas the dose of DENV-4 was too low. The successful administration of a trivalent DENV vaccine indicated that Mahidol University had achieved another important milestone on the road to the development of a tetravalent dengue vaccine.

Trials of tetravalent vaccine in children aged 5-12 years. The tetravalent DENV vaccine candidate appeared to be safe when administered to children aged 5-12 years. Children became just febrile, and this usually did not last for more than a day. One volunteer had a rash that persisted for three days.

Two trends of serological response to the tetravalent vaccine were observed among the volunteers. First, the infectious dose that was calculated for adults was not equivalent for the children in the age groups studied. A trend of an

increasing rate of seroconversion among children was noted with a decreasing vaccine dosage; however, an optimum dose for children 5-12-years-old still had to be determined.

Second, children with preexisting antibodies to either DENV or JEV appeared to respond better to the tetravalent vaccine than did children who were completely non-immune. Even so, not all volunteers with preexisting flavivirus antibodies responded to all four DENV serotypes.

Collaboration with manufacturer

The Mahidol vaccine was licensed to Pasteur Mérieux (PMsv, now Sanofi Pasteur) in France for large-scale production under Good Manufacturing Practice (GMP) conditions.

The master seed, the production seed and the candidate DENV vaccines were sent to Pasteur Merieux in February 1993, shortly after the agreement was signed in January 1993. Three technical meetings at Pasteur Merieux were held in Lyon, France, between 1994-1996. Industrial production of the four monovalent vaccines was achieved by 1995. All biological studies, including monkey neurovirulence studies were repeated.

The first clinical trial carried out using the Mahidol/PMsv tetravalent vaccine in US volunteers suggested that the combination of four attenuated strains appeared to result in increased reactogenicity and diminished tolerability. Antibody responses were predominantly directed against DENV-3 with low or undetectable titers against the remaining three serotypes. This outcome appeared to be the result of preferential replication of DENV-3 in the tetravalent vaccine. The mechanism of such viral interference was not known. But it had been suggested that the ratio of the four attenuated viruses in the tetravalent formulation may be an important factor. A subsequent clinical study in Thailand showed that varying and reducing the concentrations of the DENV-3 strain resulted in an improved clinical safety profile of the tetravalent vaccine. About 71% seroconversion (against all 4 serotypes) was observed after a two-dose vaccination schedule in this study. Several different reformulations of the tetravalent vaccine were being evaluated in order to provide a more balanced immune response to each serotype.

Second generation recombinant vaccines

A Cooperative Research and Development Agreement (CRADA) was entered into with the Division of Vector-Borne Infectious Diseases, National Center for Infectious Disease, Centers for Disease Control and Prevention (CDC), Fort Collins, Colorado, USA, to develop the second-generation recombinant vaccines using complementary DNA (cDNA) technology. As per the memorandum of understanding (MoU), the first shipment of candidate DENV vaccines (DENV-1 and DENV-2) was sent to CDC in August 1994. The DENV-3 and DENV-4 vaccines were sent shortly thereafter. The agreement called for Mahidol University to provide support for one locally trained technician and for CDC to provide support to one Thai investigator engaged in research and capacity-building activities at Fort Collins. Vaccine development studies were realized at CDC while biological marker testing was partially done in Thailand.

Conclusion on present status of PDK-based live-attenuated dengue vaccine

The importance of DENV vaccine development was imperative in order to improve public health throughout the world and was highly desirable for WHO to provide financial support for this program. The peer group summarized the progress as follows:

(1) Monovalent candidate vaccines

    (a) DENV-1: A usable candidate vaccine.
    (b) DENV-2: A near-perfect candidate vaccine.
    (c) DENV-3: The most recently developed candidate vaccine, somewhat more reactogenic than the other candidate vaccines. A search for a better vaccine should proceed.
    (d) DENV-4: A very good product.

(2) Bivalent and trivalent combinations using DENV-1 PDK 13, DENV-2 PDK 53 and DENV-4 PDK 48 had undergone phase 1 trials in adults with satisfactory results.

(3) Tetravalent vaccine was acceptably safe. Interference was noticed after mixing of the DENV-3 GMK-30/F3 in the combination.

LESSONS LEARNED

There was a general consensus that vaccination can be one of the most cost-effective ways to prevent DF and DHF. The aim of this project was to develop a safe and immunogenic vaccine against the four DENV serotypes. Each of the four monovalent vaccines as well as the bivalent and the trivalent vaccines were developed and tested step by step in the laboratory and in human volunteers. By 1992, the attenuated, tetravalent vaccine was being tested for immunogenicity and safety in human volunteers. Formal phase 1 and phase 2 clinical trials had proven the vaccines to be both safe and immunogenic in humans. Human trials of the tetravalent vaccine were successfully concluded.

In November 1992, WHO headquarters and WHO/SEARO announced the attainment of the objective of the dengue vaccine development project at Mahidol as follows: "Vaccine for Dengue Hemorrhagic Fever". From this study, it was proved that PDK cells could be used successfully for attenuation attempts. The DENV-2 PDK 53, which was one of the important outcomes of this study, has been further used as a backbone to construct live molecular DENV vaccines in the USA.

Research as well as relevant capability building activities at Mahidol University were established with the advice of the international peer group which met annually. However, the initial expectation in 1985 that DENV vaccine development would be completed within three years proved too optimistic.

Considerable research capacity building took place as part of the research project support during that decade. The various technologies required for vaccine development and laboratory-scale production were transferred. They included continuous tissue culture, development of PDK and other cell lines, monkey tests for neurovirulene, etc. The annual meetings of the peer group itself provided valuable scientific advice to the project.

In addition, Mahidol University scientists were supported for visits and contacts with various scientists and institutions in other countries.

Meanwhile, Mahidol University expanded the physical and other infrastructure required for vaccine development and pilot scale up. A vaccine development center building, and a laboratory animal center were completed at the new Salaya campus. Equipment for up scaling was received as donation from the Italian Government.

The DENV vaccine development project was acknowledged to be a worthy scientific achievement in the area of health. Such achievements could occur due to the long-term commitment of scientists in Thailand, the continuous support of the Government of Thailand, and the initial impetus and sustained commitment and support provided by WHO/SEARO. The Government of Thailand and Mahidol University provided the major resources. WHO provided about USD 2.5 million during a period of 15 years. Other donors contributed substantial amounts at various stages of the project for specific components of the program. Success was due to scientific correctness of the research, the outstanding leadership of the late Prof N Bhamarapravati of Mahidol University, sound research management by several parties, and the sustained commitment and technical support through the years of the WHO Regional Office for South-East Asia.

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PROSPECTIVE STUDY OF DENGUE IN BANGKOK DURING 2015-2016

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Abstract. Dengue patients admitted to 5 hospitals of the Department of Medical Service, Bangkok Metropolitan, Bangkok, Thailand during 2015-2016 were prospectively studied. The diagnoses of all dengue patients were adhered to clinical and laboratory criteria for the diagnosis of dengue patients as established by the WHO 1997. Blood for dengue serotype detection was collected from all patients. The disease was seen all year round with higher incidence during the rainy season. Dengue patients were seen in all age groups with increase in the number of cases in adolescents and adults. All four dengue serotypes were detected during the period of study with the following descending order: 45% DEN-4, 29% DEN-3, 17% DEN-2, 8% DEN-1 and 1% combined serotypes. Infections with any of all 4 dengue serotypes were seen in all age groups. All severity of dengue diseases can be seen in all age groups who were infected with any of dengue serotypes. The study showed that all four dengue serotypes circulated in Bangkok from 2015-2016 with DEN-4 as the predominant serotype.

Keywords: dengue, epidemiology, serotype, severity

INTRODUCTION

Dengue, a global health threat is the most common mosquito-borne viral infection transmitted by *Aedes* mosquitoes. There are four antigenically distinct, closely related serotypes of dengue virus (DEN1-4) which belong to genus *Flavivirus* in the family Flaviviridae. A continuum of dengue disease includes undifferentiated febrile illness (UFI), dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (Thisyakorn and Thisyakorn, 2015).

The World Health Organization's (WHO's) Global Strategy for Dengue Prevention and Control (2012-2020) aims to reduce the burden of dengue through the following objectives: reduce dengue mortality by 50% and reduce dengue morbidity by 25% by 2020 and estimate the true burden of disease by 2015. The global strategy is based on five technical elements of: diagnosis and case management; integrated surveillance and outbreak preparedness; sustainable vector control; vaccine implementation; and basic operational and implementation research. Five enabling factors support the technical elements: advocacy and resource mobilization; partnership; coordination and collaboration; communication to achieve behavioral outcomes; capacity building; and monitoring and evaluation (WHO, 2012).

Integrated surveillance is important for risk assessment and situation awareness, and can support outbreak preparedness and development of appropriate public communication. However,

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the focus must be on feasibility as resources are often limited at the national level. The combination of surveillance techniques employed at a national level must be prioritized while ensuring that surveillance can be sustained and dengue disease identified early for a locally appropriate response.

The objective of this study is to identify infecting dengue serotype in all dengue patients admitted to 5 hospitals of the Department of Medical Service, Bangkok Metropolitan, Bangkok, Thailand during 2015-2016.

MATERIALS AND METHODS

A prospective study was conducted with all dengue patients admitted to five hospitals of the Department of Medical Service, Bangkok Metropolitan, Bangkok, Thailand; namely, Charoenkrung Pracharak Hospital, Wetchakarunrasm Hospital, Lat Krabang Memorial Hospital, Sirindhorn Hospital, and Klang Hospital. All patients were seen by one of the listed authors. The diagnosis of dengue patients adhered to clinical and laboratory criteria for the diagnosis of dengue patients as established by the WHO (WHO, 1997).

Blood collection for dengue serotype detection was done in all patients according to the following: Viral RNA was extracted from serum/plasma using the QIAamp, according to the manufacturer's instructions. Viral genomic RNA is converted to cDNA by Reverse Transcriptase (RT). The cDNA is used as template to amplify the DNA fragment [511 base pairs (bp) from the region between capsid and PreM genes by AmpliTaq DNA polymerase with a primer pair D1/D2 in the first round polymerase chain reaction (PCR)]. The type-specific DNA fragments for DENV-1 (482bp), DENV-2 (119bp), DENV-3 (290bp) and DENV-4 (392bp) were then amplified by AmpliTaq DNA polymerase using the 511 bp DNA fragment (the product of the first round PCR) as a template and mixed primer pairs (one forward primer D1 and four reverse primers: TS1, TS2, TS3, and TS4) in the nested PCR (the second round PCR). The DNA fragments amplified by the nested PCR are loaded onto a 1.5% agarose gel and electrophoresised, followed by staining of the gel with ethidium bromide. The specific DNA bands for each DENV serotype are visualized under ultraviolet light (Fig 1) (Lanciotti *et al*, 1992).
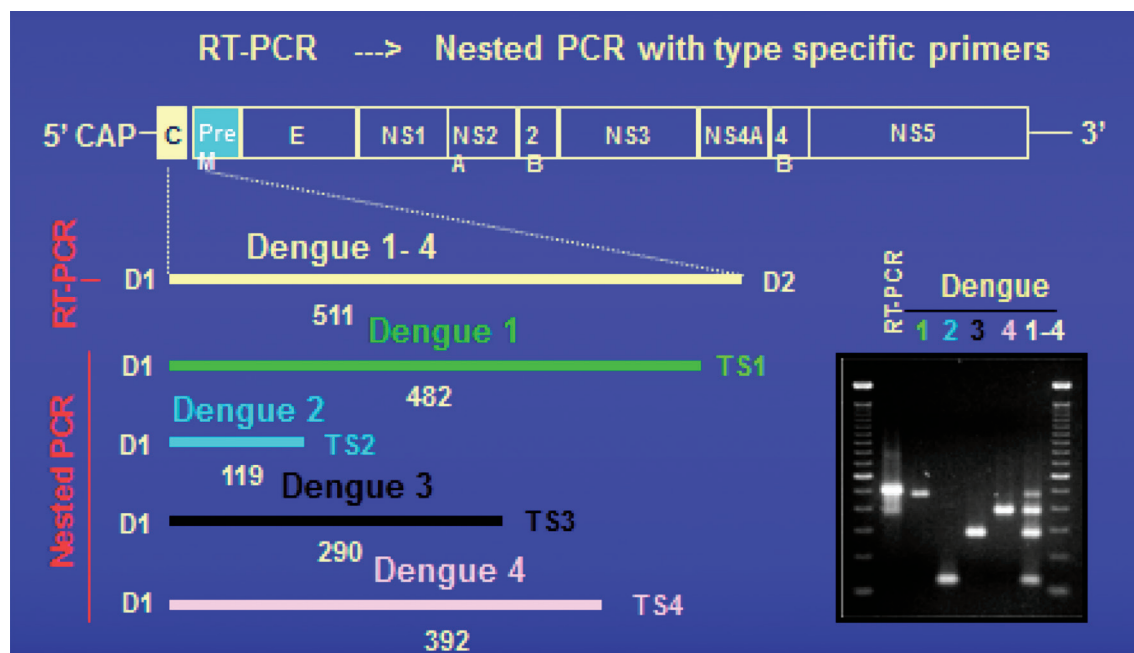
Fig 1– Dengue serotype detection (Lanciotti *et al*, 1992).

Only dengue patients with positive dengue serotype-specific were included for analysis.

RESULTS

From all 660 dengue suspected patients, 472 were positive for dengue serotype-specific which included 254 males and 218 females. Five cases of which were in the 0-1 year age group (1.06%), 19 cases in the 2-5 years age group (4.03%), 60 cases in the 6-8 years age group (12.71%), 214 cases in the 9-15 years age group (45.34%), 103 cases in the 16-30 years age group (21.82%), 60 cases in the 31-60 years age group (12.71%), and 6 cases in the >60 years age group (1.27%) (Fig 2). Detection of dengue serotype in all dengue patients showed 8% DEN-1, 17% DEN-2, 29% DEN-3, 45% DEN-4 and 1% combined serotypes (Fig 3). All dengue serotypes were detected in all age groups and in all severity of dengue diseases (Fig 4, Fig 5).

DISCUSSION

There were two deaths, one was a 22 years old man with DHF, another was an 8 years old girl with DF. Both were infected with DEN-4. Outbreak of dengue virus infection in Bangkok started in 1958. In the early stages, the outbreak usually occurred every other 1-2 years. Later, the outbreak was unpredictable, and patients were found throughout the year, mostly during the rainy season. During the past decades, the rate of dengue patients in Bangkok varied from 27.99 per 100,000 population in 1992 to 292.24 per 100,000 population in 2001. The case fatality rate was between 0.0-0.21%. A trend towards higher ages of dengue patients was seen (Liulak, 2013).

Our study has indicated that all four dengue serotypes circulate continuously in Bangkok during the period of study with one predominant dengue serotype. Our study confirms the uniqueness of predominant dengue serotype in emerging as the cause of each periodic epidemic as has been seen in the past (Burke *et al*, 1981; Burke *et al*, 1988; Nisalak *et al*, 2003; Nisalak *et al*, 2016). In this study, all severity of dengue diseases can be seen in all age groups no matter they acquired any serotype-specific dengue virus. According to the
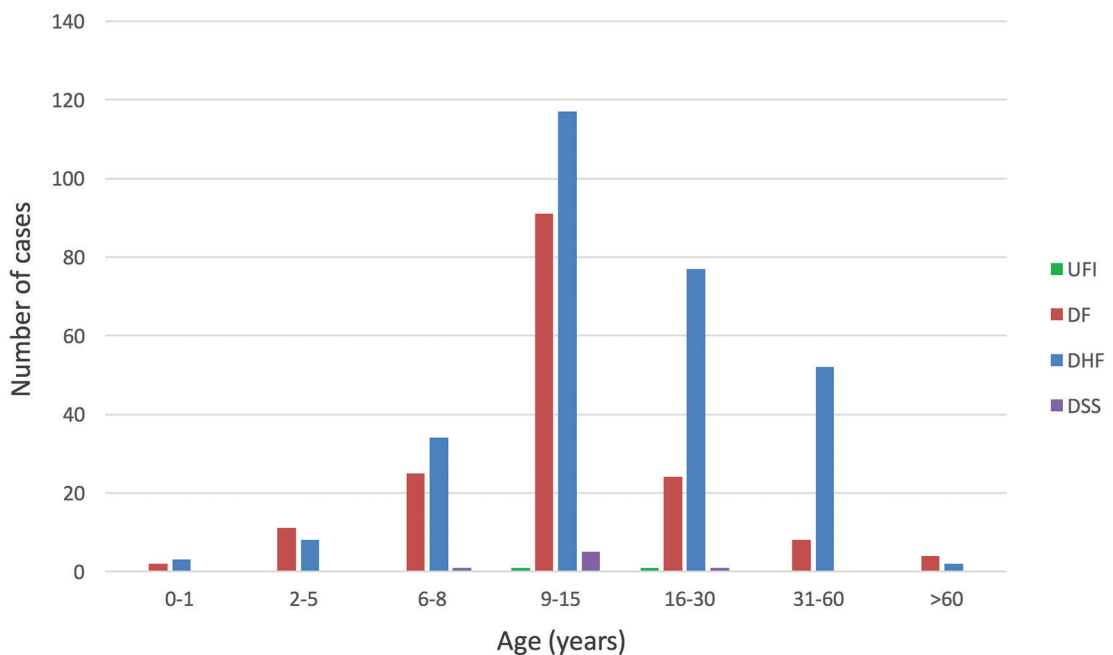
Fig 2– Age distribution *vs* dengue severity in Bangkok 2015-2016.
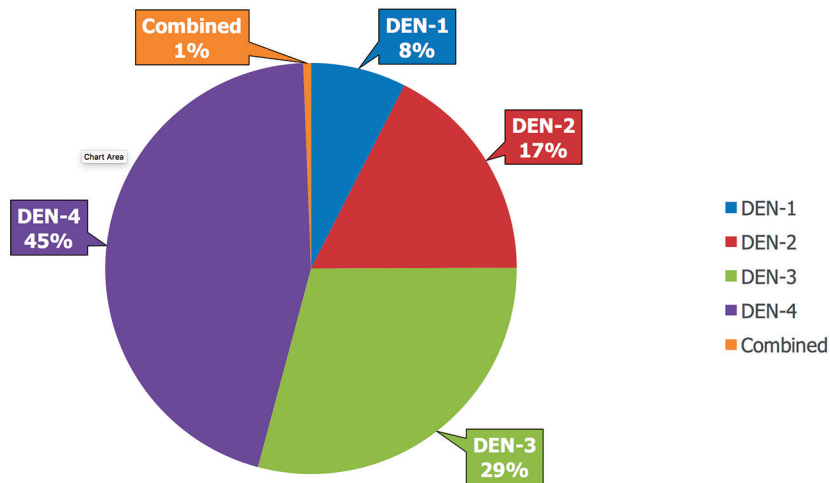
Fig 3– Dengue serotype in Bangkok 2015-2016.
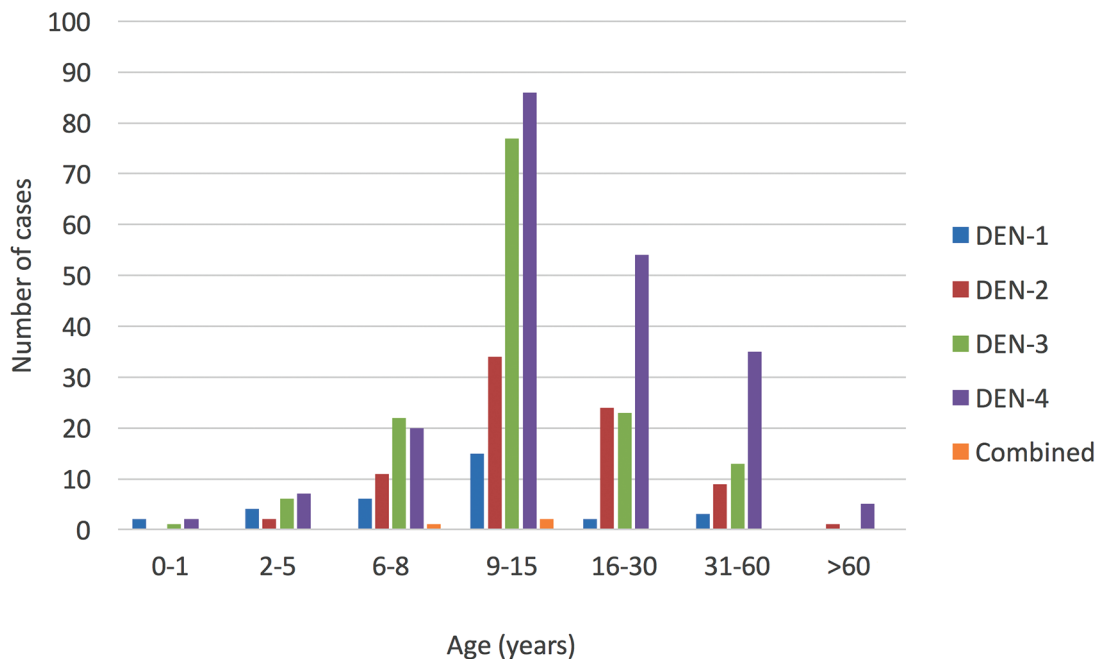
Fig 4– Age distribution *vs* dengue serotype in Bangkok 2015-2016.

high numbers of cases that were seen in adolescents and adults in this study, co-morbidities in adult may also contribute to the severity of dengue.

Clinical profiles show a difference in some aspects across all age groups and need to

be considered since recognition of clinical characteristics in different age groups are essential in early diagnosis and treatment. Successful treatment, which is mainly symptomatic and supportive, depends on early diagnosis of the
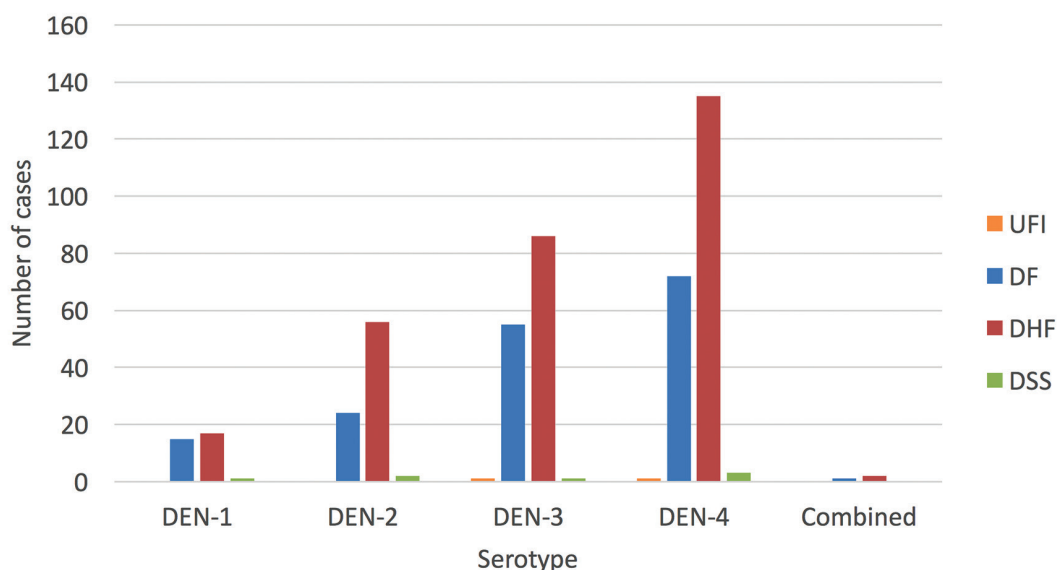
Fig 5– Dengue serotype *vs* dengue severity in Bangkok 2015-2016.

disease and careful monitoring for the disease severity. (Tantawichien, 2015; Thisyakorn and Thisyakorn, 2017).

Dengue is one disease entity with different clinical manifestations, often with unpredictable clinical evolutions and outcomes. Variations of clinical characteristics in different age group of dengue patients need to be considered for proper management (Royal College Physician Thailand of Thailand, 2015).

We conclude that a long-term surveillance of dengue disease is one of the key strategies for proper prevention and control of dengue epidemic. Our findings show changing dengue epidemiology in Bangkok in terms of age group distribution of dengue patients and dengue serotype-specific distribution in comparison to the past. These results will be an evidence for developing an effective guideline for prevention and control of dengue.

ACKNOWLEDGEMENTS

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EPIDEMIOLOGY OF DENGUE AT THAMMASAT UNIVERSITY HOSPITAL DURING 2006-2015

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Abstract. Dengue is the most common mosquito-borne viral disease in humans. Over the past several years, the epidemiological profile of dengue has been changing progressively and is currently characterized by an increase in the number of cases in adults. Between 2006 and 2015, there were 5,633 dengue patients at Thammasat University Hospital (TUH) including 4,132 (73.4%) with dengue fever (DF), 1,501 (26.6%) with dengue hemorrhagic fever (DHF). Of all dengue patients, 3,542 (62.9%) were treated in the outpatient department (OPD), and 2,091 (37.1%) were treated in the inpatient department (IPD). During the years 2006 to 2015, 1,540 cases (27.3%) were children (aged <15 years), and 4,093 cases (72.7%) were adults. The highest numbers of dengue cases were reported in individuals aged 15-19 years (17.5%), followed by 20-24 years (17.1%), and 10-14 years (13.3%). Rates have constantly been high amongst adolescents and young adults (aged 10-24 years). The overall case fatality rate from dengue was 0.1%. The case fatality rate was higher for children (0.19%) than for adults (0.07%). The epidemiology has certainly changed and appears to be shifting from child to adult aged population. However, children still remain at risk for infection and death. This changing epidemiology is important for our public health control programs.

Keywords: age distribution, dengue, dengue hemorrhagic fever, epidemiology

INTRODUCTION

Dengue is the most common mosquito-borne viral disease in the world. The number of dengue cases worldwide reported annually to the World Health Organization (WHO) has increased from 0.4 to 1.3 million in the decade 1996-2005, reaching 2.2 million in 2010 and 3.2 million in 2015 (WHO, 2012; 2016). The incidence of dengue fever (DF)/,dengue hemorrhagic fever (DHF) has continuously increased since the first recognized outbreak in 1958 in Thailand (Halstead *et al*, 1963; Halstead, 1990). By the late 1970s, the disease was widespread among countries in Southeast Asia and

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DHF had become a leading cause of hospitalization and death among children in Thailand (WHO, 1986).

Four closely related dengue serotypes (DEN-1, DEN-2, DEN-3, and DEN-4) cause the disease, which ranges from asymptomatic infection to undifferentiated fever, DF, and DHF. DHF is characterized by fever, bleeding diathesis, and a tendency to develop a potentially fatal shock syndrome (Thisyakorn and Thisyakorn, 2015). Currently, there are no specific medications to treat a dengue infection. This makes prevention the most crucial weapon in the fight against this disease. The availability of a safe, efficacious, and cost-effective vaccine would significantly alter the paradigm of dengue prevention.

The first dengue vaccine (CYD-TDV) was registered in several countries in 2015. It was registered for use by individuals, 9-45 years-of-age living in endemic areas. Pooled vaccine efficacy amongst all participants aged 9 years or over was 65.6%, and it was 44% in participants aged <9

Table 1. Epidemiology of dengue at Thammasat University Hospital (2006-2015).

Characteristic	2006 No. (%)	2007 No. (%)	2008 No. (%)	2009 No. (%)	2010 No. (%)	2011 No. (%)	2012 No. (%)	2013 No. (%)	2014 No. (%)	2015 No. (%)	Total No. (%)
Sex											
Male	228 (53.6)	263 (53.7)	385 (52.2)	298 (52.7)	291 (51.6)	280 (53.8)	261 (51.0)	332 (51.6)	217 (49.1)	392 (53.5)	2,947 (52.3)
Age, mean, years	26.3	29.1	24.9	24.6	24.4	24.7	22.4	22.5	24.2	24.5	24.8
Age group											
Children	99 (23.3)	107 (21.9)	154 (20.9)	156 (27.6)	162 (28.7)	164 (31.5)	179 (34.9)	215 (33.5)	124 (28.0)	180 (24.6)	1,540 (27.3)
Adult	326 (76.7)	383 (78.1)	583 (79.1)	410 (72.4)	402 (71.3)	356 (68.5)	333 (65.1)	429 (66.5)	318 (72.0)	553 (75.4)	4,093 (72.7)
Hospitalization	246 (57.9)	287 (58.6)	411 (55.8)	216 (38.2)	165 (29.3)	143 (27.5)	156 (30.5)	179 (27.8)	56 (12.7)	232 (31.7)	2,091 (37.1)
Type of infection											
DF	258 (60.7)	297 (60.6)	419 (56.9)	365 (64.5)	384 (68.1)	351 (67.5)	372 (72.7)	511 (79.3)	375 (84.8)	584 (79.7)	4,132 (73.4)
DHF	167 (39.3)	193 (39.4)	318 (43.1)	201 (35.5)	180 (31.9)	169 (32.5)	140 (27.3)	133 (20.7)	67 (15.2)	149 (20.3)	1,501 (26.6)

DHF, dengue hemorrhagic fever; DF, dengue fever.

years (Capeding *et al*, 2014; Villar *et al*, 2015). Age and seropositivity were highly correlated in the trials.

Generally, dengue used to predominantly affect children. Evidence-based decision-making on the introduction of vaccines and their use requires not only data on vaccine product characteristics (safety, efficacy, and cost), but also information on effective vaccination, their likely impact on disease burden, and cost effectiveness.

Endemic countries must have a rationale for deciding which segments of the population to protect when national resources or vaccine supplies are scarce. Therefore, an understanding of epidemiological differences in infection rates and severity of disease is important for public health control programs. Most published data are available for dengue hospitalizations, but are lacking for dengue non-hospitalized cases. The aim of this study was to describe the epidemiological pattern of dengue patients, both hospitalizations and non-hospitalizations, in a tertiary care teaching hospital in Thailand from 2006 to 2015.

MATERIALS AND METHODS

A retrospective study was conducted among patients with dengue infection who attended Thammasat University Hospital (TUH), Thailand from January 2006 to December 2015. The diagnosis of dengue patients adhered to the criteria established by the WHO (1997). The data were collected from the hospital database of patients diagnosed with DF, DHF or dengue shock syndrome (DSS).

The study protocol was approved by the Ethics Review Committee of the Faculty of Medicine, Thammasat University.

Data were analysed using descriptive statistics including frequency, percentage, range, mean and standard deviation.

RESULTS

Between 2006 and 2015, there were 5,633 dengue patients at TUH including 4,132 (73.4%) with DF, and 1,501 (26.6%) with DHF; 2,947 (52.3%) were males and 2,686 (47.7%) were females (Table 1). There were 3,542 (62.9%) from the outpatient department (OPD) and 2,091 (37.1%) from inpatient department (IPD) (Fig 1).

The number of dengue cases reported during the years 2006 to 2015 varied from a lowest of 425 in 2006 to a highest of 737 in 2008 (Fig 2). Peaks in dengue cases occurred in the years 2008, 2013, and 2015. Dengue patients were reported throughout the year (Fig 3). The incidence was highest during the rainy season and usually peaked between May and September with the exception of 2015, which had a peak during October to December.

Figs 4 and 5 show dengue prevalence by age groups. During the years 2006 to 2015, 1,540 cases (27.3%) were children (<15 years old) and 4,093 (72.2%) were adults. The highest numbers of dengue cases were reported in individuals aged 15-19 years (17.5%), followed by 20-24 years (17.1%), and 10-14 years (13.3%). Rates have constantly been high amongst 10-24 year olds.

According to the data on the age distribution of DHF, the highest numbers of DHF cases were reported in those aged 20-24 years (17.5%), followed by 25-29 years (15.5%), 15-19 years (15.4%), 30-34 years (9.7%), and 10-14 years (9.6%) with adolescents and young adults representing approximate 70% of reported cases (Fig 6). The hospitalization rate was highest in adults aged 25-29 years (46.6%), followed by those who were 10-14 years (41.7%) and 30-34 years (41.3%) of age.

Fig 7 shows the dengue prevalence in children. Reported cases increased in older children, of which the 13-14 year-old age group was most affected.

The overall case fatality rate from dengue was 0.1% (*n*=6). The case fatality rate was higher for children (0.19%) than for adults (0.07%).

DISCUSSION

In our retrospective study among patients with dengue infection from January 2006 to December 2015, the annual incidence rate of dengue varied
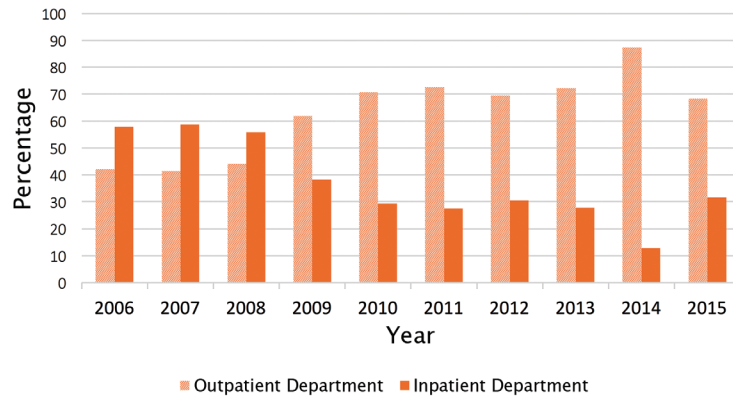
Fig 1–Reported cases of dengue at the outpatient department and the inpatient department, Thammasat University Hospital, 2006-2015.

	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015
DF	258	297	419	365	384	351	372	511	375	584
DHF	167	193	318	201	180	169	140	133	67	149
Total	425	490	737	566	564	520	512	644	442	733
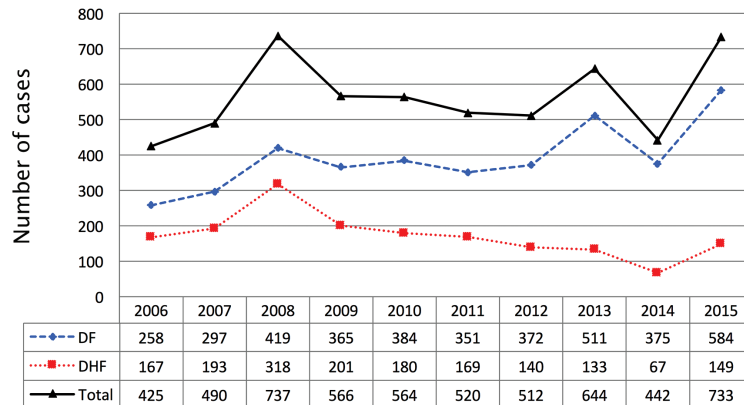
DHF, dengue hemorrhagic fever; DF, dengue fever.

Fig 2–Reported cases of dengue fever and dengue hemorrhagic fever at Thammasat University Hospital, 2006-2015.
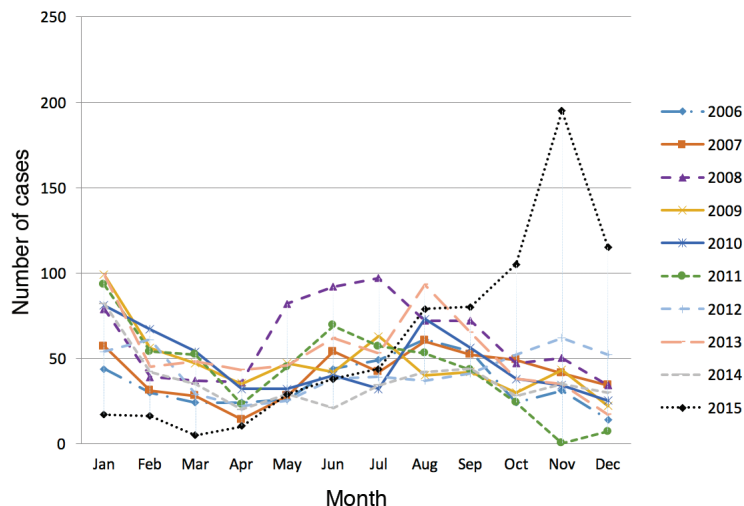
Fig 3–Number of reported cases due to dengue by month at Thammasat University Hospital, 2006-2015.
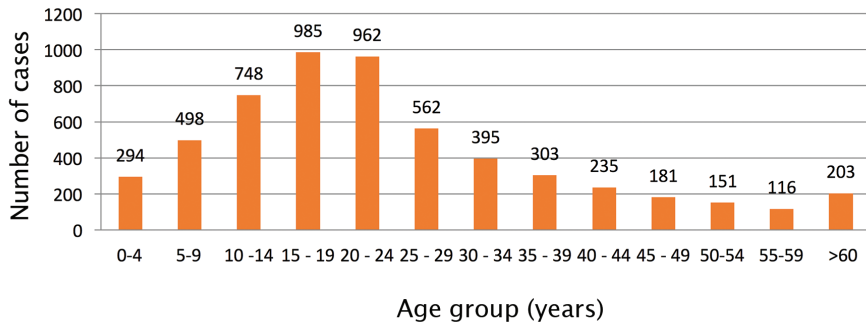
Fig 4–Reported cases of dengue by age group at Thammasat University Hospital during 10 years from 2006-2015.
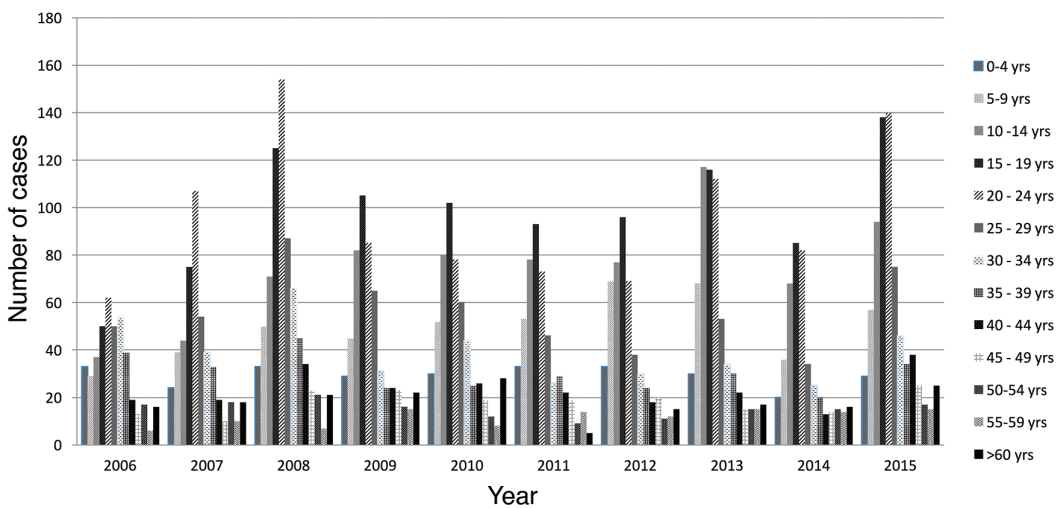
Fig 5–Age distribution of dengue, 2006-2015.
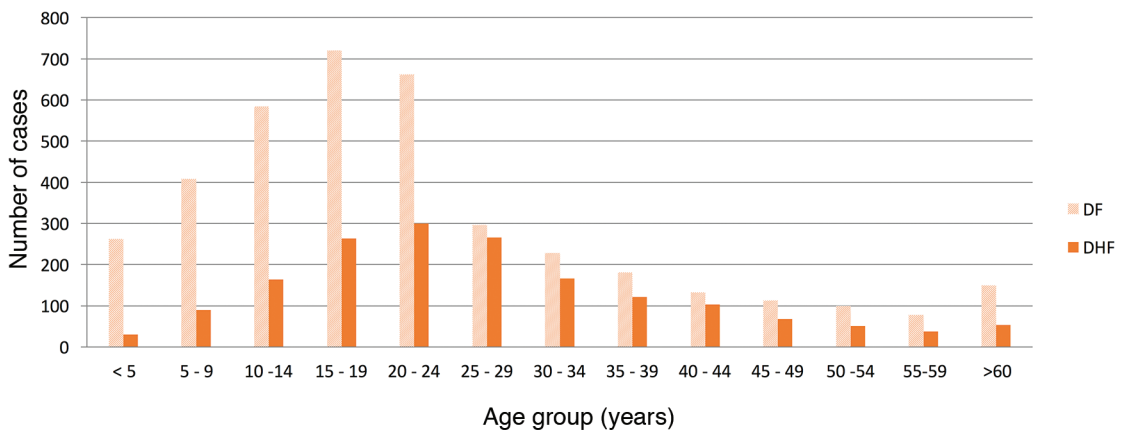
DHF, dengue hemorrhagic fever; DF, dengue fever.

Fig 6–Reported cases of dengue fever and dengue hemorrhagic fever across different age groups during 2006-2015.
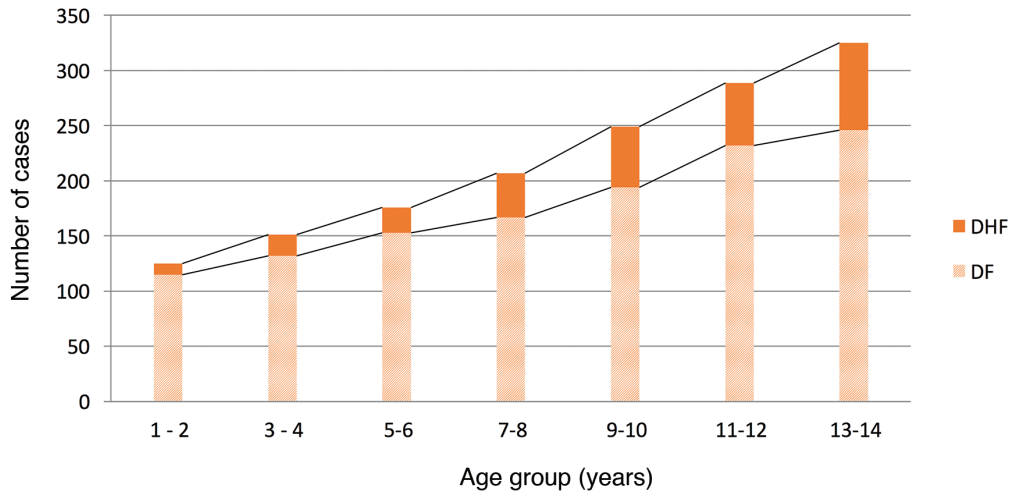
DHF, dengue hemorrhagic fever; DF, dengue fever

Fig 7–Number of dengue fever and dengue hemorrhagic fever in children during 2006-2015.

during the 10-year period. The highest numbers of dengue cases were reported in adolescents and young adults (10-24 years old). Historically, dengue was predominantly a pediatric disease, but the number of adult patients has been increasing in the last decade. Several studies in both Latin America and Southeast Asia have reported a higher association of DHF with older ages (Sapir and Schimmer, 2005; Ooi and Gubler, 2008; Cummings *et al*, 2009; Beatty *et al*, 2010; Martin *et al*, 2010; Bravo *et al*, 2014; Karyanti *et al*, 2014; Mohd-Zaki *et al*, 2014).

A literature survey was conducted by Limkittikul *et al* (2014) to shady the epidemiology of dengue in Thailand between 2000 and 2011. This review showed a shift in age group predominance towards older ages, which also continued through the review period. Disease incidence and deaths remained highest in children aged ≤15 years. Other studies in Thailand showed affected adults >15 years of age comprised 30-40% of dengue cases (Patumanond *et al*, 2003; Simmons *et al*, 2010). Many studies have only concentrated on cases that have hospitalizations. One strength of our study was the inclusion of both hospitalized patients and non-hospitalized patients, which indicated that the dengue was predominantly (73%) in the adult age group.

Comparing the hospitalization for dengue in children and adults, we found that the numbers are still highest in the adult age group. Increased mobility of the adult population in our country, better access to health care facilities, improved reporting, and ease of reporting to physicians might be some of the factors of high incidence of dengue among adults. This changing epidemiology is important for public health control programs. We believe that awareness about the shifting age-pattern is not only essential for clinical and public health vigilance, but also for the efficiency of preventive strategies. Education for the public on dengue awareness in the adult age group could improve timely medical interventions. Furthermore, national dengue immunization programs should consider the epidemiological data about the burden of disease.

Dengue infection depends on the seasonal variation of the climate. Standing water from rainfall provides places for the mosquitoes to lay their eggs and develop to the adult stage. Wu *et al* (2009) showed that with every 1°C increase in the monthly average temperature in Taiwan, the total population at risk for dengue fever transmission would increase by 1.95 times. Dengue is endemic in Thailand; Peak transmission rates occur in

the rainy season, between May and September (Limkittikul *et al*, 2014). Our study confirmed a seasonal pattern of dengue with the majority of cases occurring during the rainy season. However, our results showed an outbreak from October-December only in 2015, which was not the rainy season.

Climatic changes resulting in increased temperature and rainfall together with urbanization may therefore be associated with increased dengue incidence and outbreak risk (Khasnis *et al*, 2005). Thailand is a tropical country with a relatively high temperature and humidity all year-round. These conditions are ideal for *Aedes* mosquitoes to have established themselves. The potential for the dengue transmission requires the following four factors: (1) a number of susceptible humans, (2) a number of mosquitoes, (3) virus transmission potential, and (4) a suitable climate (Polwiang, 2015).

There are several limitations to our study. First, our retrospective study design relies on reports dependent on the clinician's documentation. Also, the number of cases may be over-diagnosed because this study did not use serological laboratory-confirmation for diagnosis. Third, specific incidences of DHF were not classified according to severity. Another limitation was the lack of serotyping of the reported dengue cases. Notably, TUH was closed from October 22, 2011 to November 14, 2011 due to severe flooding in the region. On November 15, 2011, the hospital was only operating at partial capacity for a month; for example, some floors were temporarily shut due to flooding, and some OPDs were moved to another location. This would have affected the patient records during this period of time.

Dengue remains a major public health concern in Thailand. The epidemiology in this region has certainly changed and appears to be shifting from children to adult age group. However, children still remain at risk for infection and death. Epidemiological and virological surveillance of dengue in Thailand should be improved so that we have a better knowledge base on how to control it. Most importantly, more research is needed on affected age groups and vaccine use, as well as specific serotype and weather conditions for mosquito breeding.

ACKNOWLEDGEMENTS

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CHANGING EPIDEMIOLOGY OF DENGUE PATIENTS AT VACHIRA PHUKET HOSPITAL, THAILAND

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Abstract: Between 2009 and 2015, 7,030 dengue patients, 3,580 male and 3,450 female, were admitted to Vachira Phuket Hospital, Phuket Province, Thailand. Among these patients, there were 2,257 with dengue fever (DF), 4,725 with dengue hemorrhagic fever (DHF), and 48 with dengue shock syndrome (DSS) with 22 deaths. The diagnosis of dengue patients adhered to clinical and laboratory criteria for the diagnosis of dengue patients established by the World Health Organization. The disease was seen all year round with higher incidence in the rainy season. A trend of shift in age group towards older children and adults was seen. Our study indicated that admissions of dengue patients to Vachira Phuket Hospital are common, causing a heavy burden on the health system. The trend towards higher age of dengue patients may have implications for further prevention and control of dengue.

Keywords: dengue, epidemiology, Phuket, Thailand

INTRODUCTION

Dengue is the most common arboviral infection in humans and is transmitted by *Aedes* mosquitoes, principally *Aedes aegypti*. There are four antigenically distinct serotypes of dengue virus (DEN 1-4), which can cause a continuum of disease: dengue fever (DF) causes fever, rash, muscle or joint pain, headache, and eye pain; dengue hemorrhagic fever (DHF) causes abnormal hemostasis and increased vascular permeability, with severe cases leading to dengue shock syndrome (DSS) and death. Because of factors such as environmental and climate change and human movement, a global increase in dengue cases has occurred, and there is also the potential spreading of the disease to non-endemic areas.

Main public health preventive interventions consist of mosquito control, which is currently used in endemic countries, and use of vector repellents.

These interventions have generally had limited results. A dengue vaccine is seen as the best hope to fight this disease. In Thailand, dengue patients were first reported in Bangkok in 1958, and then appeared in other parts of the country (Thisyakorn and Thisyakorn, 2015a). The aim of this study was to describe the changes in the epidemiological pattern of dengue patients at Vachira Phuket Hospital, Phuket Province, Thailand.

MATERIALS AND METHODS

Dengue patients admitted to Vachira Phuket Hospital, Phuket Province, Thailand during 2009-2015 were studied. Vachira Phuket Hospital is a provincial hospital in southern Thailand. The diagnosis of dengue patients adhered to clinical and laboratory criteria for the diagnosis of dengue patients as established by the World Health Organization (WHO, 1997). The study was approved by the ethics committee of Vachira Phuket Hospital.

RESULTS

Between 2009 and 2015, 7,030 dengue patients, 3,580 male and 3,450 female, were admitted to Vachira Phuket Hospital, Phuket

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Province, Thailand. Among these patients, there were 2,257 with dengue fever (DF), 4,725 with dengue hemorrhagic fever (DHF), and 48 with dengue shock syndrome (DSS) with 22 deaths. The disease was seen all year round with higher incidence in the rainy season, which begins in June and usually lasts until October (Fig 1).

Fig 2 describes the incidence by age group. Rates were constantly high among children with a trend of increasing mean age with time. Rates in older children and adults increased dramatically throughout the period of study. Fig 3 describes the severity of dengue disease by age group. It shows that all dengue severity can be seen in all age group with the trend of higher DSS cases in older children and young adults.

DISCUSSION

Dengue epidemics are known to have occurred regularly during the past decades in Phuket, Thailand, which causes a heavy burden on the health system. The population growth together with the remarkable degree of urbanization has allowed dramatic expansion of the mosquitoes through an increase of urban breeding sites. This may explain the explosive growth of reported cases. A greater awareness and better reporting could have contributed to some of the increase over time. The reasons for the apparent upsurge in dengue are probably multifactorial.

Feeding efficiency of *Aedes aegypti* vectors increases with increasing temperature (Watts *et al*, 1987; Kuno, 1995). This may explain the increasing dengue patients during the dry hot season. Global warming may also contribute to greater spread of dengue infection (Patz *et al*, 1996). The availability of water and higher humidity, including higher biting rates, may augment an epidemic during the rainy period (Pant and Self, 1993). Weather patterns, with average temperatures and increases in rainfall, are classically seen as factors. Many other factors may influence the epidemiologic patterns of dengue beside climate, such as movements of mosquitoes, the type of circulating dengue viruses, environmental factors such as temperature and humidity, and human behavior and development.

Well-targeted research, such as population-based epidemiological studies with clear operational objectives, is needed to make progress in control and prevention. Dengue remains predominantly a pediatric disease, but the trend towards higher rates in older children and adults during the last
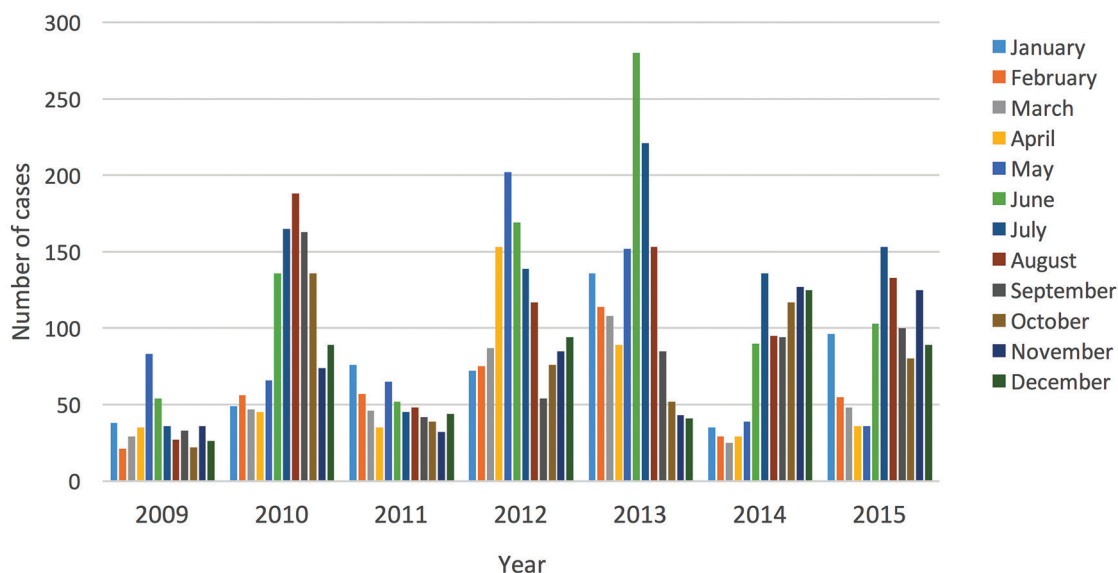
Fig 1–The seasonal distribution of dengue patients at Vachira Phuket Hospital, Thailand between 2009-2015.
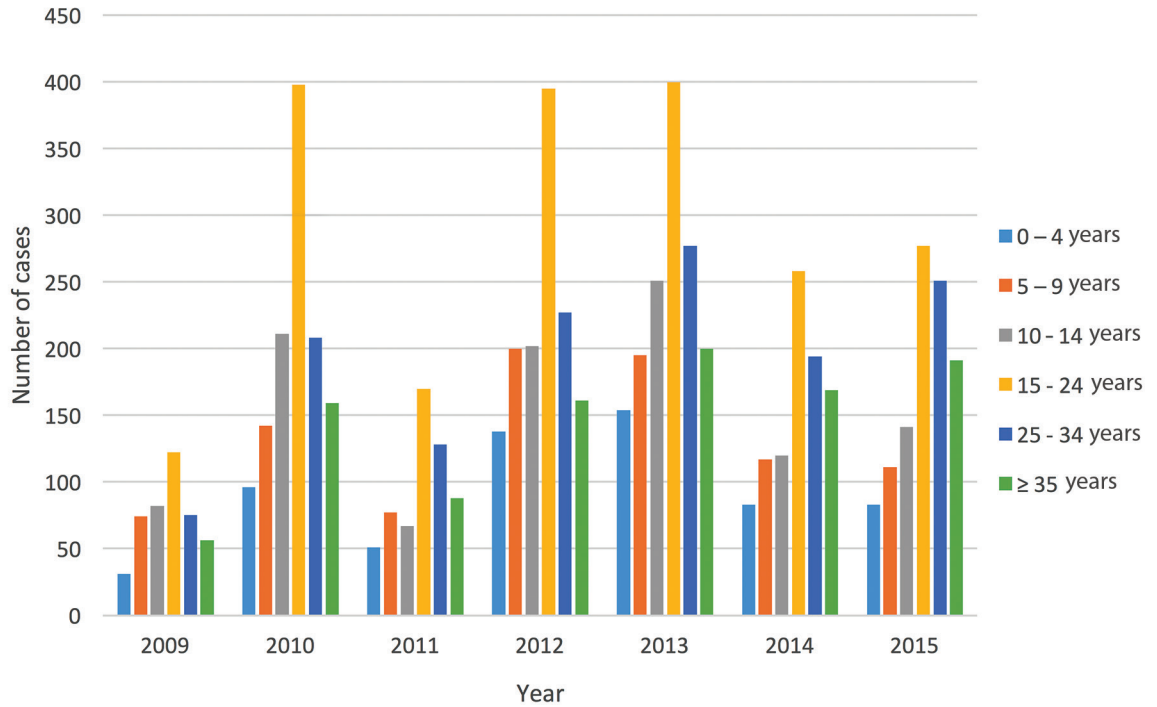
Fig 2–Age distribution of dengue patients at Vachira Phuket Hospital, Thailand between 2009-2015.
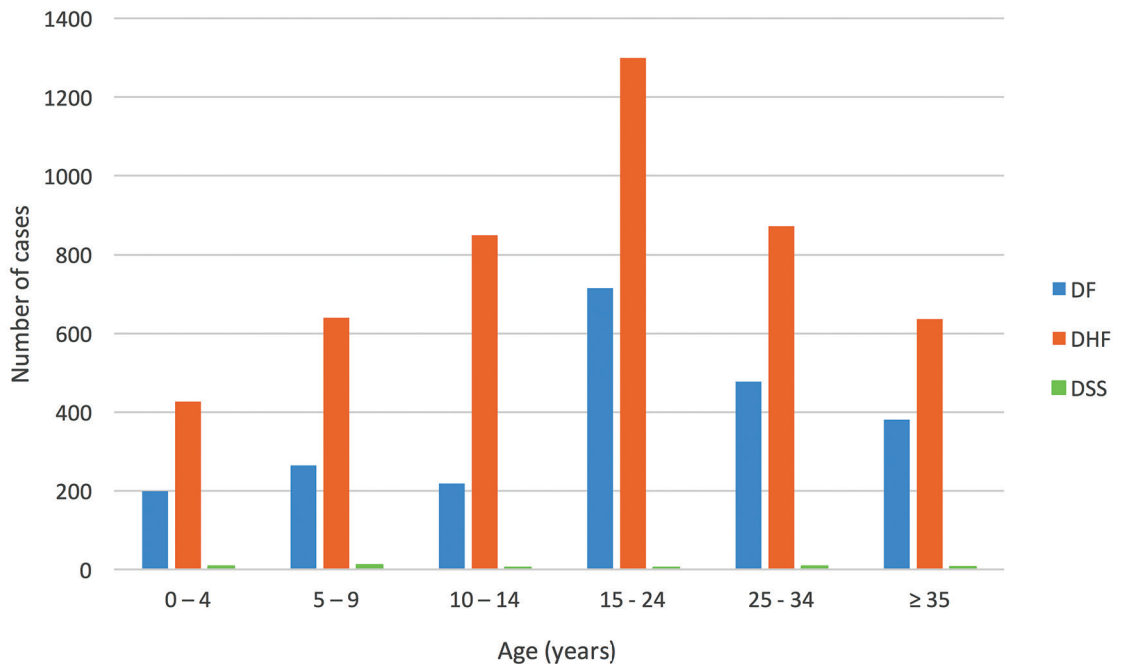
Fig 3–Severity of dengue patients by age group at Vachira Phuket Hospital, Thailand between 2009-2015.

decade is incompletely understood; possibly, it may be the result of less frequent epidemics in the last decades so that second exposure to dengue virus is postponed. Several studies in both Latin America and Southeast Asia have reported this age shift, which indicates an epidemiological change in dengue infection in those locations.

The trend for increased incidence among adults has important implications for effective control and prevention, which involves demographic, economic, behavioral, and social factors (Guha-Sapir, and Schimmer, 1999). Generally, the percentage of DHF in adults is lower than in children. Adults with DHF have a course similar to that in children. However, some studies have mentioned less severe plasma leakage in adult patients. Yet there are some countries where most deaths are seen in adults, which could be explained by the late recognition of the disease. In addition, comorbidities in adult patients such as peptic ulcers disease, preexisting liver disease are more likely to be present in adults than in children and can aggravate the disease severity (Tantawichien, 2015).

In the 1960s the case fatality rate was as high as 6-8% and it has decreased with time (Chareonsook *et al*, 1999). The case fatality rate less than 1% throughout the period of study indicates early recognition and improved management of dengue patients in Vachira Phuket Hospital. Prevention of dengue by vector control has achieved only limited success in reducing the transmission of dengue. The use of a safe and effective dengue vaccine may be a major means to effectively control dengue with the high feasibility of a dengue vaccine (Thisyakorn and Thisyakorn, 2015b). This study shows that dengue patients are common in Vachira Phuket Hospital, Thailand. The low case fatality rate throughout the period of study indicates early recognition and improved management of patients. The trend towards higher age in dengue patients during the past decade has important implications for control and prevention.

Better understanding of new paradigms for a changing dengue epidemiology will not only feed into operational policy for dengue control, but also provide fertile terrain for vaccine application strategies in the future. Epidemiological data of this kind will be both valuable for dengue vaccine efficacy trials and for consideration of age group to be vaccinated, which will lead to universal dengue vaccine implementation in the future.

These data indicated that dengue is common in Vachira Phuket Hospital, which causes a heavy burden on the health system. The low case fatality rate throughout the period of study indicated early recognition and improved management of dengue patients. The trend towards higher age in dengue patients during the past decade is a problem of concern and need further clarification.

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Severe Dengue

CHAPTER 2

Dengue pathogenesis

- Pathogenesis of dengue viral diseases
- Hematologic changes in dengue
- Cytokine-related gene expression in peripheral blood leukocytes and dengue infection severity

PATHOGENESIS OF DENGUE VIRAL DISEASES

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Abstract. In recent efficacy trials of a dengue vaccine candidate, about 7-15% of volunteers in the control group manifested at least one of the three dengue hemorrhagic fever (DHF)-defining abnormalities (hemorrhage, thrombocytopenia, and plasma leakage) during symptomatic dengue virus infection. A high risk of developing DHF observed in secondarily infected persons is related to an increased viral burden. Complex interactions between structurally heterogeneous viral particles and pre-existing antibodies specific for the viral envelope glycoproteins, E and prM, are thought to play a role in enhancing virus replication. Concomitant high level of circulating NS1, a virally encoded glycoprotein that is essential for viral RNA replication, may cause plasma leakage through its direct and indirect effects on the vascular endothelial cells. Cross-reaction of anti-NS1 antibodies generated during dengue virus infection with platelets may lead to thrombocytopenia. Dengue serotype cross-reactive T cells rapidly expand during the secondary infection, and their levels in the circulation are associated with the development of DHF. However, the role of T cells in mediating an increase in vascular permeability at the tissue level remains unclear.

Keywords: dengue, dengue hemorrhagic fever, pathogenesis, virus

INTRODUCTION

Diseases caused by dengue virus infection remain a major public health problem. Recent estimates of the global burden of dengue suggest that about 390 million dengue infections occur every year, and 96 million are symptomatic (Bhatt *et al*, 2013). The great majority of dengue infected persons are found in Asia, where outbreaks of dengue hemorrhagic fever (DHF) have been reported since 1954. There are four serotypes of dengue viruses, which are often co-circulating, particularly after the year 2000 (Messina *et al*, 2014). A highly effective vaccine and a licensed specific antiviral agent are not yet available, necessitating a better understanding of dengue virus biology and the pathophysiologic mechanism.

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This review focuses on selected recent findings, and the readers are referred to the following recent reviews for more information (Diamond and Pierson, 2015; Olagnier *et al*, 2016; Souza *et al*, 2016; Yacoub *et al*, 2016; Yam-Puc *et al*, 2016).

The four dengue virus serotypes are classified by *in vitro* cross-neutralization of virus infectivity by employing sera of infected individuals. There are about 30-40% differences in the nucleotide sequence of the viral genome among these serotypes. Infection by a dengue serotype induces prolonged immunity against the infecting serotype, while cross-protective immunity against other serotypes is of limited duration. In the localities where multiple dengue serotypes co-circulate, repeated infections may occur and can result in diverse clinical outcomes.

Following an initial dengue virus infection, the plaque-reduction neutralization test (PRNT) antibody titer determined early after the infection episode provides an indicator of protection against symptomatic re-infection (Katzelnick *et al*, 2016). Among Thai children, the PRNT titers required for protection against local strains that circulated

during 1994-1997 appear to be uneven between persons who had been infected with each of the four dengue virus serotypes, ranging from about 100 for serotype 3-to-300 for serotype 4 and 600 for serotypes 1 and 2 (Clapham et al, 2016). The finding that, following an initial infection by serotypes 1 or 2, higher antibody titers are required for the protection against subsequent infection is consistent with comparatively lower efficacies of a tetravalent chimeric yellow fever-dengue vaccine in the prevention of symptomatic dengue caused by these two serotypes in Asian and Latin American children (Capeding et al, 2014; Villar et al, 2015).

HOW COMMON ARE DHF-DEFINING ABNORMALITIES IN CHILDREN WITH DENGUE?

A range of illnesses that occur during dengue virus infection have been classified originally as undifferentiated fever, dengue fever (DF), DHF of various severities, and expanded dengue syndrome/ isolated organopathy (unusual manifestations) (WHO SEARO, 2011). This classification does not reflect differences in disease severity, particularly between DF and DHF, but represents a syndromic approach in grouping clinical manifestations with distinct underlying pathophysiologic bases.

The pathological abnormalities in DHF include hemorrhage, thrombocytopenia, and plasma leakage. In recent phase III clinical trials of a tetravalent live-attenuated chimeric dengue vaccine candidate in children in Asia and Latin America, a number of volunteers in the non-vaccinated control group experienced symptomatic dengue virus infection during a two-year prospective follow-up period. Among these infected volunteers, up to 15% manifested at least one of the three DHF-defining abnormalities (Capeding et al, 2014; Villar et al, 2015).

Plasma leakage with clinical signs and marked thrombocytopenia were found at about 4-9% of infected Asian control volunteers. The proportions of children with these abnormalities are consistently lower among infected Latin American volunteers compared with their Asian counterparts (Capeding

et al, 2014; Villar et al, 2015). The disparity may reflect a higher mean age and the greater proportion of children with previous exposure to dengue viruses that manifested as higher baseline PRNT antibody titers among Latin American volunteers. However, other factors may also be involved. These factors include genetic predispositions of the human host, difference in the proportion of circulating dengue virus serotypes, variations in the virus replicative ability, and associated viral burden, as well as virus-host interactions.

DENGUE VIRUS HETEROGENEITY

Recent studies on the intratypic and intertypic diversities of dengue viruses indicate that the four canonical dengue virus serotypes are antigenically more heterogeneous than was previously thought (Katzelnick et al, 2015). A series of neutralization test employing large panels of human sera along with temporally and geographically diverse dengue viruses indicated that some dengue virus strains are as different from other strains of the same serotype in their susceptibility to antibody-mediated neutralization as a number of strains of different serotypes (Katzelnick et al, 2015). Such diversity is observed despite a clear clustering of members of the same serotype when nucleotide sequence differences were compared. Drastic changes in the susceptibility to antibody-mediated neutralization of virus infectivity, therefore, can occur with non-exceptional levels of sequence variation. This finding may explain why some persons can be infected twice with dengue viruses of the same serotype (Waggoner et al, 2016), and why different sequential infections contribute dissimilarly to an altered risk of developing DHF in diverse localities (OhAinle et al, 2011).

Structural analyses of dengue virus particles reveal a mixture of immature, partially mature, and mature particles that co-exist in the extracellular compartment, particularly during virus replication in mosquito cells (reviewed in Lok, 2016). An ineffective cleavage of prM by cellular furin enzyme during virus export results in particles with differences in the arrangement of the surface glycoproteins, E and prM. In immature particles and

the 'immature' patch of partially mature particles, three non-covalently linked prM-E heterodimers assume a knob-like protrusion with the receptor-binding domain III of the E protein at its base and the fusion loop at the tip of E domain II hidden by the pr portion of prM. Following cleavage of prM and the release of the pr peptide from extracellular particles, mature particles and the 'mature' patch of partially mature particles display a flat orientation of the head-to-tail E homodimers in which the E domain III is more readily accessible. Association of the E proteins in the homodimeric complex results in formation of E dimer-dependent epitope at the former pr-binding site of E dimer that can be recognized by broadly neutralizing antibodies (Dejnirattisai *et al*, 2015; Rouvinski *et al*, 2015). Also, other epitopes are present in mature particles that are dependent on the quaternary structure formed between adjacent E dimers (Lok, 2016). Particles of different maturation levels are, therefore, variably recognized by antibodies specific to the two surface glycoproteins and differ in their susceptibility to the neutralizing and infection-enhancing potentials of antibodies.

HIGH VIRAL BURDEN IN DHF

Early studies demonstrate higher viremia during the febrile phase in children with DHF than those with DF (Vaughn *et al*, 2000; Libraty *et al*, 2002). Similarly, higher NS1 antigenemia in a period prior to defervescence correlates with subsequent development of DHF (Libraty *et al*, 2002). These findings raise a possibility that virus and/or virus-encoded product(s) are directly involved in the pathogenesis of DHF. Conversely, different components of the immune system may be involved by contributing to an increase in viral burden, or by responding to the high viral burden in such a way that leads to DHF.

Epidemiological studies suggest that secondary infection is a risk factor for DHF (reviewed in Guzman *et al*, 2013). The role of antibody-dependent enhancement of dengue virus infection in increasing viral burden and virus-infected cell mass during secondary infection has been proposed (Screaton *et al*, 2015). Recent studies have found structural heterogeneity of dengue virus particles that affect their inherent ability to infect receptor-expressing host cells as well as 'enhanced' infections of FcγR-expressing leukocytes mediated by IgG antibodies recognizing different viral envelope proteins (reviewed in Flipse *et al*, 2013). In addition to the well-known role of anti-E antibodies in infection enhancement, cross-reactive anti-prM antibodies may contribute to an enhancement of virus infection during natural infection as they are commonly detected in sera of dengue virus-infected persons, are generally non-neutralizing, and are able to enhance infection of FcγR-expressing leukocytes by prM-containing immature and partially mature viral particles (Dejnirattisai *et al*, 2010).

ROLE OF T CELLS IN THE "IMMUNE-MEDIATED" PATHOGENESIS

Adaptive immunological responses to viral antigens and virus-infected cells have been proposed to underlie pathophysiologic derangements observed in DHF. As dengue viruses share common B and T cells epitopes in many viral proteins, cross-reactive T cells that have been primed during the primary dengue virus infection readily expand during the secondary infection. Higher viral burden in DHF cases is likely to result in greater magnitude of activated B and T lymphocytes independent of the type of infection. Indeed, during both primary and secondary dengue virus infections, activated virus-specific T cells are detected in the circulation of DHF cases at higher frequencies than that of their DF counterparts (reviewed in Screaton *et al*, 2015).

Higher proportions of T cells secreting interferon gamma and tumor necrosis factor are found in DHF patients, whereas T cells expressing the degranulation phenotype are more common in DF cases (Duangchinda *et al*, 2010). These results indicate that, in addition to an expected quantitative difference, there is also qualitative difference in dengue virus-specific T cell activity between these two disease entities. However, the current evidence for the temporal association between elevated level of circulating activated T cells and the onset of hemoconcentration is

still lacking (Dung *et al*, 2010). A recent study found that dengue virus-specific T cells migrate to skin during the acute phase of dengue virus infection, but their levels did not correlate the development of DHF or severe dengue (Rivino *et al*, 2015). Whether such activated, virus-specific T cells causally mediate an increase in vascular permeability at the local tissue level in DHF cases remains to be established.

Many cytokines and chemokines are found at different levels in DHF and DF cases, but their direct role in the pathogenesis of DHF is far from clear. More recently, serotonin, known to be involved in platelet aggregation and activation, is found to be decreased significantly in DHF, whereas kynurenine, an immunomodulator, increases significantly in DHF (Cui *et al*, 2016). This is consistent with their proposed roles in causing thrombocytopenia and immunopathology in severe cases of dengue virus infection.

ROLE OF NS1 IN THE 'VIRUS-MEDIATED' PATHOGENESIS

NS1 is a virally encoded nonstructural glyco-protein involved in viral RNA replication. Structural analysis reveals distinct domains with affinity for lipid bilayer, complement components, and homotypic interaction (Akey *et al*, 2014). In dengue virus-infected cells, NS1 dimers localize to viral replication complexes within the cytoplasm and on the cell surface. NS1 is secreted from infected mammalian and mosquito cells into extracellular compartment as homohexameric complexes (Alcala *et al*, 2016) that can bind a number of lipids and serum proteins, including complement components, as well as cell surface molecules, but the role of extracellular NS1 in virus multiplication *in vivo* remains unknown.

In dengue virus-infected persons, variable levels of circulating NS1 are detected initially during the febrile phase, persisting for up to several days, and can be modulated by the serotype of infecting dengue viruses and the sequence of infection (primary *vs* secondary) (Duyen *et al*, 2011). A high level of circulating NS1 early in the illness correlates with subsequent development of DHF during dengue type 2 virus infections (Libraty *et al*, 2002). It is likely that NS1 plays an important role in the pathogenesis of dengue as it is well established that active immunization with NS1 prevents illness in virus-infected mice, and antibodies to NS1 given passively protect mice against lethal virus challenge (reviewed in Muller and Young, 2013; Amorim *et al*, 2014; Akey *et al*, 2015). Partial protection against dengue in recipients of the chimeric yellow fever-dengue virus vaccine observed in Phase III clinical trials is thought to reflect in part a lack of dengue virus NS1 in this chimeric vaccine, which may be unable to induce adequate level of protective immunity against dengue (Screaton *et al*, 2015; Halstead, 2016).

During dengue virus infection, circulating antibodies to NS1 are frequently detected. NS1 shares common epitopes with many cellular proteins, and anti-NS1 antibodies can bind platelets, proteins of the coagulation cascade, and endothelial cell surface (reviewed in Amorim *et al*, 2014). Interference of the function of platelets and coagulation system by anti-NS1 antibodies has been proposed to represent an autoimmune mechanism that leads to thrombocytopenia and bleeding tendency that are observed in dengue cases. Recently, Wang *et al* (2017) reported an increase in the proportion of anti-dengue ENV and anti-NS1 IgG1 antibodies that lack fucosylated glycans in their Fc portion during the early phase of dengue virus infection.

While the proportion of afucosylated anti-dengue ENV IgG1 antibodies was higher in DHF cases as compared with DF cases and their levels correlated with the extent of thrombocytopenia, these anti-ENV antibodies did not bind platelets. Instead, anti-NS1 IgG antibodies cross-reacted with platelets and likely mediated a reduction of circulating platelets upon transfer of IgG from thrombocytopenic patients into FcR-humanized mice. This platelet-lowering effect was dependent on two leukocytes' Fc receptors, FcγRIIA and FcγRIIIA; the latter is known to bind afucosylated IgG1 antibodies more strongly than other IgG molecules (Wang *et al*, 2017). In addition, induction of endothelial cell apoptosis initiated by

direct anti-NS1 antibody binding to endothelial cell surface and/or complement-mediated endothelial cell damage following anti-NS1 antibody binding to surface-bound NS1 molecules may result in an increased vascular permeability. However, the significance of these autoimmune mechanisms needs to be further substantiated by *in vivo* experiments.

Recent studies have revealed a direct stimulatory effect of NS1 on monocytes/ macrophages and vascular endothelial cells (Beatty *et al*, 2015; Modhiran *et al*, 2015). Purified NS1 that is derived from over-expressing insect cells and that is devoid of bacterial lipopolysaccharide interacts directly with Toll-like receptor 4, inducing the secretion of pro-inflammatory cytokines from monocytes/ macrophages. NS1 also disrupts endothelial cell monolayer integrity *in vitro* and triggers an increase in endothelial permeability and vascular leakage in the mouse model (Beatty *et al*, 2015; Modhiran *et al*, 2015). Alteration of vascular permeability and shock in this model can be prevented by vaccination with NS1, the injection of anti-NS1 antibodies, or a Toll-like receptor 4 antagonist (Beatty *et al*, 2015). Moreover, dengue virus NS1 may cause vascular leakage via the induction of autophagy in endothelial cells following the release of macrophage migration inhibitory factor (Chen *et al*, 2016). These results implicate a direct effect of NS1 glycoprotein as a virus-mediated mechanism underlying the pathogenesis of dengue.

CONCLUSION

DHF occurring during secondary dengue virus infection may result from a complex interaction between primarily and secondarily infecting dengue viruses and the host immune system. Low level of cross-reactive antibody from the primary dengue virus infection could enhance virus replication during the secondary infection, leading to a higher viral burden and an increased risk of developing DHF. Rapid expansion of cross-reactive T cells in response to secondarily infecting virus may potentially contribute to an enhanced local production of inflammatory cytokines. Circulating NS1 affects vascular permeability and induces pro-

inflammatory cytokines secretion from leukocytes via the interaction with Toll-like receptor 4, and NS1 may contribute directly to plasma leakage in DHF patients. Additionally, cross reactivity of anti-NS1 antibodies with platelets in the presence of infection-associated modification of Fc-linked glycans represents a mechanism that could lead to thrombocytopenia.

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HEMATOLOGIC CHANGES IN DENGUE

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Abstract. The pathogenesis of hemostatic changes in dengue patients is not clearly understood. There are evidences suggesting that dengue virus causes pathophysiological changes involving all of the component of hemostasis that result in vasculopathy, thrombocytopenia, thrombopathy, abnormal von Willebrand factor (VWF) multimers, reduction of several coagulation factors, increased antifibrinolytic factors, and consumption of natural anticoagulants. Profound disseminated intravascular coagulation may occur only in severe dengue cases, and this complication leads to uncontrolled bleeding and death. Increased plasma VWF antigen (VWF:Ag) at the febrile phase was found to be the best indicator of progression to severe dengue disease.

Keywords: dengue, hemostatic studies

INTRODUCTION

Dengue infection, one of the most devastating mosquito-borne viral diseases in humanity, is now an expanding global threat. The disease ranges from asymptomatic infection to undifferentiated fever, dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS) (Thisyakorn and Thisyakorn, 2015). The common hemorrhagic manifestations in DHF/DSS are epistaxis, gingival bleeding, gastrointestinal bleeding, hematuria, and menorrhagia. Although severe hemorrhage remains the major cause of death, the pathogenesis of bleeding in dengue patients is poorly understood (Sosothikul *et al*, 2007).

HEMOSTATIC STUDIES IN DENGUE PATIENTS

The hemostatic changes occurring early in the course of the illness in all severities of dengue infection include the clinical syndromes and their manifestations below.

Vasculopathy manifests as generalized petechiae and a positive tourniquet test.

Platelet abnormalities manifest as thrombocytopenia, one of the most consistent abnormal hemostatic tests, and this occurs in the febrile phase to reach its lowest levels in the defervescence phase. The platelet count then increases during the convalescent stage to reach its normal values. Many cases have higher platelet counts than the normal ranges during the second week of illness. The clinical severity also correlates with the degree of thrombocytopenia.

The possible mechanisms of thrombocytopenia include decreased production in bone marrow and increased platelet destruction or increased utilization. The decreased production is evidenced by decreased number of marrow megakaryocytes in the early febrile phase. The megakaryocyte number is normal or increased later. The increased platelet destruction is shown by a shortened platelet half-life survival time during the course of illness, which becomes normal later on. Surface counting of radiolabeled platelets revealed increased pooling of platelets in the liver more than in the spleen. In addition to thrombocytopenia, platelet dysfunction is manifested by impaired platelet aggregation to adenosine diphosphate and a concurrent increase in plasma thromboglobulin and platelet factor 4 levels (Srichaikul *et al*, 1989).

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Coagulopathy is shown by mild to moderately prolonged partial thromboplastin time and prothrombin time, resulting in reduction of coagulation factors. Fibrinogen is the only factor that almost always decreases mildly to moderately due to increased consumption. Minimal increases of fibrin degradation products are noted intermittently throughout the course of illness. In addition, euglobulin lysis time was reported to be normal (Mitrakul and Thisyakorn, 1989). It has been suggested that endothelial cells can be a target for dengue virus infection, leading to alterations of the production of cytokines in those cells and alterations of barrier functions, both of which may play a central role in dengue pathogenesis (Dalrymple and Mackow, 2011).

A prospective cohort study was designed to determine the extent of activation of endothelial cells and the hemostatic system in correlation with dengue clinical severity, as well as to detect the best prognostic factor for severe dengue infection. Endothelial cell activation, coagulation, anticoagulant, and fibrinolysis parameters were measured in 42 children with dengue infections (20 with DF, and 22 with DHF) during the three phases of the illness. In DHF patients during the febrile phase, von Willebrand factor antigen (VWF:Ag), tissue factor, and plasminogen activator inhibitor (PAI-1) were significantly elevated while platelet counts and ADAMTS 13 (a disintegrin and metalloprotease with thrombospondin repeats) were significantly lower compared with those in DF patients.

In DHF patients during the toxic phase, soluble thrombomodulin, tissue plasminogen activator, and PAI-1 were also significantly increased while ADAMTS 13 and thrombin activatable fibrinolysis inhibitor were significantly lower compared with those in DF patients. Abnormal VWF multimers were seen only in DHF patients. For endothelial cell injury and release of procoagulant components, activation of the coagulation cascade with thrombin generation increased antifibrinolytic factors and consumption of natural anticoagulants. Each appeared to play an important role in the development of hemorrhage in dengue patients.

The level of VWF:Ag is the most important prognostic indicator of dengue severity. (Sosothikul et al, 2007).

Besides hematopoietic suppression during dengue infection, there was evidence of the hemophagocytosis of erythroid, myeloid cells, and platelets in bone marrow (Srichaikul, 2014).

CONCLUSION

Dengue virus is the causative agent of a wide spectrum of clinical manifestations, ranging from mild acute febrile illness to classical DF, DHF, and DSS. The major pathophysiologic changes in severe dengue include leakage of plasma and abnormal hemostasis. Vasculopathy, platelet abnormalities, and coagulopathy are responsible for abnormal hemostasis in dengue patients.

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CYTOKINE-RELATED GENE EXPRESSION IN PERIPHERAL BLOOD LEUKOCYTES AND DENGUE INFECTION SEVERITY

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Abstract. Dengue virus (DENV) can produce a wide spectrum of clinical manifestations ranging from mild severity to a severe form with plasma leakage and shock. The mechanism underlying the increased disease severity is not fully understood. However, it is thought to be mediated by various host factors, particularly cytokine-related gene expression. This study aimed to elucidate the cellular gene responses to dengue infection at the transcriptional level and to correlate expression levels with disease activity. Patients with confirmed dengue infection and controls were enrolled during a calendar year at King Chulalongkorn Memorial Hospital. RNA was extracted from peripheral blood leukocytes and was analyzed on the day of defervescence. The mRNA expression levels of interleukin (IL)-8, IL-1ß and matrix metalloproteinase-9 (MMP-9) were assayed in 30 children with DF, 19 children with DHF and 10 unaffected controls by real time reverse transcription quantitative polymerase chain reaction. A level of $p < 0.05$ was considered to be statistically significant. All expression data were analyzed using the Q-gene software. IL-8 and IL-1ß mRNA levels were not significantly different between children with DF and DHF, but those with DHF had significantly higher levels of MMP-9 mRNA. MMP-9 might have an important role in dengue pathogenesis. To gain further insight into the pathogenesis of dengue disease severity, serial transcription profiling of additional selected genes in peripheral blood mononuclear cells (PBMCs) might serve as a predictor of dengue infection as well as disease activity.

Keywords: cytokine-related gene expression, IL-8, IL-1ß, MMP-9, dengue infection severity

INTRODUCTION

Dengue virus (DENV) is the causative agent of the mosquito-borne viral diseases and has become a serious public health problem worldwide. This virus has four major serotypes (DENV-1, DENV-2, DENV-3 and DENV-4) and can produce a wide spectrum of clinical manifestations ranging from mild acute febrile illness, classical dengue fever (DF), dengue hemorrhagic fever (DHF) to a severe form with plasma leakage and shock (dengue shock syndrome, DSS) (Hemunkorn *et al*, 2007). Current hypotheses have been proposed to explain the pathogenesis of DHF/DSS including immune enhancement, virus virulence, autoimmune responses against dengue non-structural 1 (NS1) protein and host genetic predisposition (Prommalikit *et al*, 2004; Prommalikit and Thisyakorn, 2015). The mechanism underlying the increased disease severity is however not fully understood. It is thought to be mediated by various host factors, particularly cytokine-related gene expression. Previous studies have suggested an involvement of immune response mediators in the severity of dengue infection (Basu and Chaturvedi, 2008). Elevated levels of serum interleukin (IL)-8, IL-1ß and matrix metalloproteinase-9 (MMP-9) are associated with the feature of severe dengue infection (Talavera *et al*, 2004; Luplertlop *et al*, 2006; Bozza *et al*, 2008).

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This study aimed to elucidate the cellular gene responses to dengue infection at the transcriptional level and to correlate expression levels with disease activity.

MATERIALS AND METHODS

Patients with serologically and virologically confirmed dengue infection by an enzyme linked immunosorbent assay (ELISA) and real time polymerase chain reaction (RT-PCR) were enrolled at the Department of Pediatrics, King Chulalongkorn Memorial Hospital during a calendar year. Clinical diagnosis of dengue infection and its severity were based on the 1997 World Health Organization criteria (WHO, 1997). Informed consent was obtained from the parents of the subjects and controls recruited into the study. After informed consent, whole blood was drawn. RNA was extracted from 3 ml of peripheral blood leukocytes using the QIAamp RNA Blood Mini Kit® (Qiagen, Hilden, Germany) and was analyzed on the day of defervescence.

The expression levels of IL-8, IL-1ß and MMP-9 were assayed in 30 children with DF, 19 children with DHF and 10 controls with other febrile illness (OFI) by real time reverse transcription quantitative polymerase chain reaction. Data on patients' characteristics and laboratory results were compared between two groups by using the Mann-Whitney U test. A $p < 0.05$ was considered to be statistically significant. All expression data were analyzed using the Q-gene software (http://www.biotechniques.com/softlib/qgene.html).

RESULTS

The mRNA expression levels of IL-8, IL-1ß and MMP-9 were examined in 30 DF patients, 19 DHF patients and 10 controls.

There was no significant difference among the characteristics of children with dengue infection at the time of admission, except for white blood cell count and hematocrit which were significantly higher among children with DHF (Table 1).

Concerning gene expression pattern in dengue infection, mRNA levels analyzed on the day of defervescence (arbitrary units relative to 18SrRNA expression; presented as mean±SEM) showed that IL-8 and IL-1ß mRNA levels were not significantly different between children with DF and DHF, but those with DHF had significantly higher levels of MMP-9 mRNA (Figs 1-3).

DISCUSSION

Dengue virus infection is a systemic and dynamic disease with a wide spectrum of clinical manifestations. The exact pathogenesis of DHF/DSS

Table 1. Characteristics of children with dengue infection at the time of admission.

Variables	DF (*n*=30)	DHF (*n*=19)	*p*-value
Age; median years (range)	11 (3-14)	11 (4-11)	>0.05
Sex (M:F)	14:16	11:8	
Illness day prior to study entry	4.5 days	4.9 days	>0.05
Temperature at study entry (range)	38.9°C (36.4-41.0)	38.5°C (37.0-41.0)	>0.05
The median white blood cell count at study entry (cells/mm³)	2,445	3,530	<0.05
The median hematocrit at study entry (range)	39.5% (30.9-45.0)	42.5% (35.0-49.8)	<0.01
The median platelet count at study entry (range) (cells/mm³)	88,500 (38,000-294,000)	76,000 (14,000-199,000)	>0.05
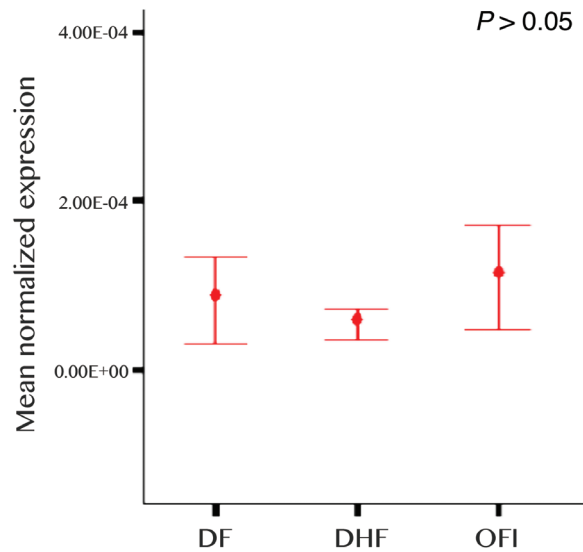
Fig 1–The mRNA expression levels of IL-8 in dengue patients and controls. DF, dengue fever, DHF, dengue hemorrhagic fever, OFI, other febrile illness.
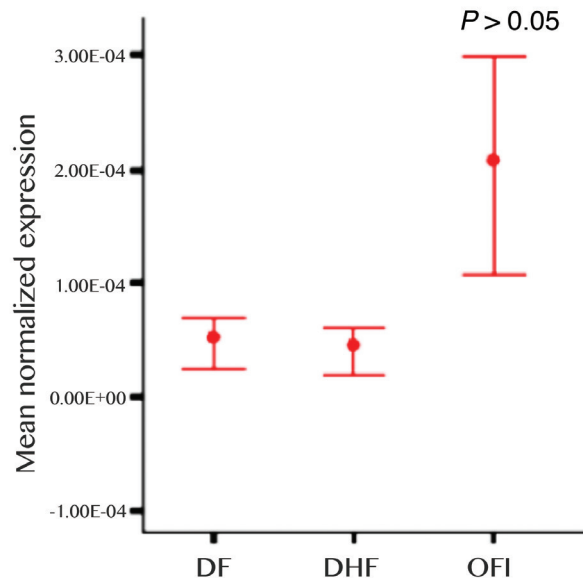
Fig 2–The mRNA expression levels of IL-1ß in dengue patients and controls.

is not well understood. However, the risk factors are ever mentioned for severe dengue including age, the genetic background of the host, dengue virus virulence (viral serotype and genotype), autoimmune responses and antibody-dependent enhancement (Sangkawibha *et al*, 1984; Rico-Hesse *et al*, 1997; Gubler, 1998; Guzman *et al*, 2002). Based on previous evidence, the disease symptoms in dengue may be a consequence of the immune response against the virus (Basu and Chaturvedi, 2008). Elevated serum levels of many different cytokines including IL-8, IL-1ß and
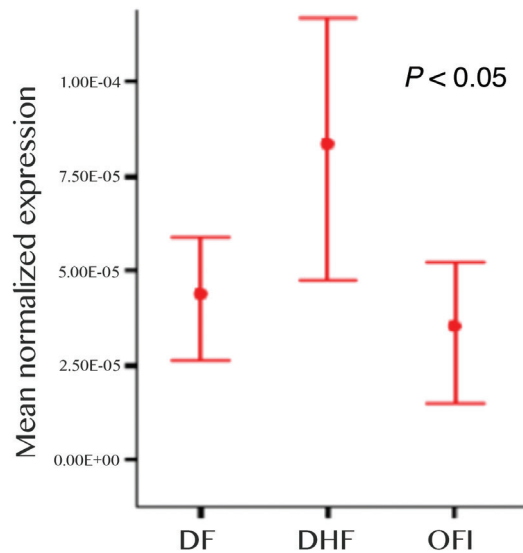
Fig 3- The mRNA expression levels of MMP-9 in dengue patients and controls.

also MMP-9 are associated with severe dengue pathology (Talavera *et al*, 2004; Bozza *et al*, 2008; Luplertlop and Missé, 2008). However, it is not fully understood how these cytokines and MMP-9 cause abnormal pathology in severe dengue virus infection (Bäck and Lundkvist, 2013).

Elevated serum levels of IL-8 are associated with secondary DENV infections, deregulated coagulation and fibrinolysis, and autoimmune responses in dengue virus infection which are correlated with disease severity (Bäck and Lundkvist, 2013). IL-1ß is a potent cytokine that is regulated and can be induced by DENV in macrophages and monocytes (Dinarello, 1997). Elevated serum levels of IL-1ß increase vascular permeability that can lead to leakage of plasma as described in a mouse model of severe dengue pathology (Martin *et al*, 1988; Bozza *et al*, 2008). But in the present study, the mRNA expression levels of IL-8 and IL-1ß were not significantly different between children with DF and those with DHF.

MMP-9 is a matrixin, a class of enzymes that belong to the zinc-metalloproteinases family involved in the degradation of the extracellular matrix. This enzyme is concerned in embryonic development, reproduction, angiogenesis, bone development and wound healing. *In vitro* studies and mouse model experiments have found that MMP-9 could induce vascular leakage in dengue virus infection (Luplertlop *et al*, 2006). MMP-9 may play a role in the mechanism of plasma leakage in DHF. Our study has demonstrated that children with DHF have significantly higher levels of the MMP-9 mRNA expression than children with DF. This mediator might have an important role in dengue pathogenesis. However, Voraphani *et al* (2010) reported that there was no significant difference between serum MMP-9 levels in patients with DHF and those with DF at any stage of the disease.

To gain further insight into the pathogenesis of dengue disease severity, serial transcription profiling of additional selected genes in PBMCs might serve as a predictor of dengue infection as well as disease activity.

ACKNOWLEDGEMENTS

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Severe
Dengue

CHAPTER 3

Diagnosis of dengue virus infection

- WHO 2009 guidelines and case classification: an update and short report
- Comparison of the 1997 and 2009 WHO classifications for determining dengue severity in Thai patients
- Laboratory diagnosis of dengue virus infections

WHO 2009 GUIDELINES AND CASE CLASSIFICATION: AN UPDATE AND SHORT REPORT

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INTRODUCTION

In 2009, the World Health Organization (WHO) issued new dengue guidelines (WHO/TDR, 2009), in collaboration with the Special Program for Research and Training in Tropical Diseases (WHO/TDR), for WHO regional offices and many dengue researchers and program planners. One of the key recommendations was the introduction of the 2009 WHO dengue case classification. This classification describes dengue as 'dengue and severe dengue (D/SD).' Warning signs (WS) have been developed for triage, helping medical staff with symptomatic dengue cases to facilitate the decision of closer surveillance and/or hospitalization (dengue with warning signs (D+WS)) (Fig 1).
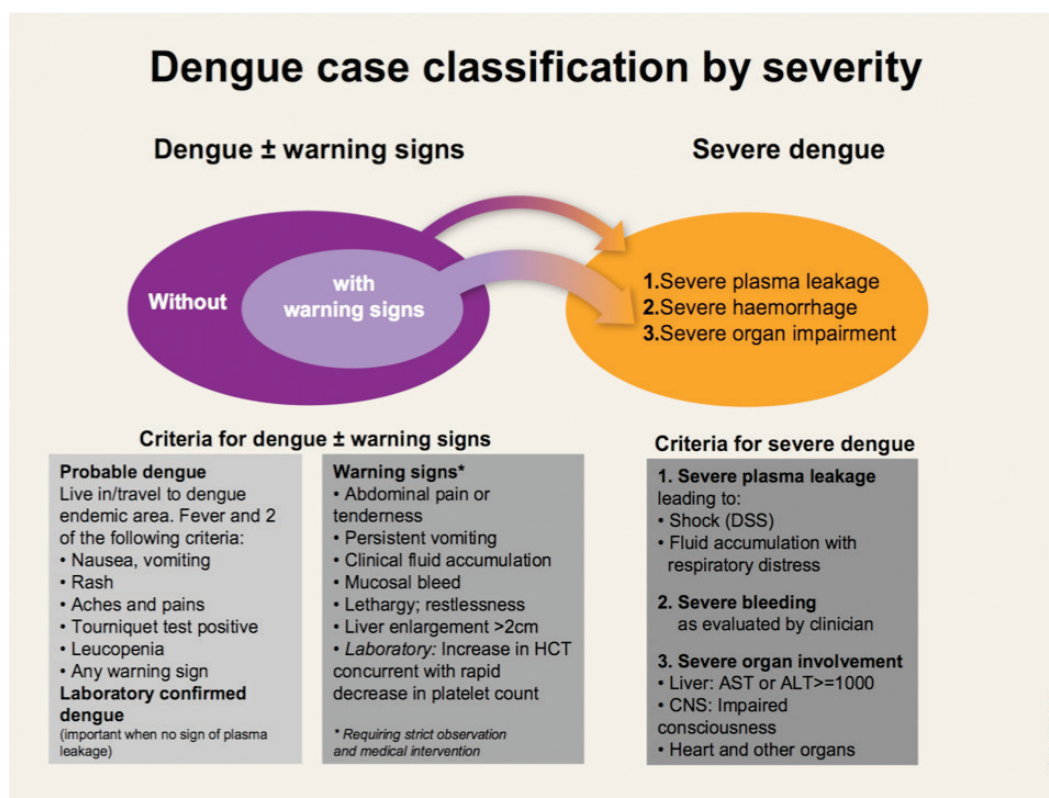
Fig 1– 2009 WHO dengue case classifications.

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The 2009 WHO dengue case classification has been developed with a series of studies, in step–by–step procedures, including quantitative and qualitative data, to produce: 1) the largest ever collection of dengue patient data prospectively and globally (Alexander *et al*, 2011); 2) systematic reviews to describe the problems with the previous classification (Bandyopadhyay *et al*, 2006), and studies comparing both classifications (Horstick, 2014a); 3) mixed methods to describe necessities for the 2009 WHO dengue case classification (Santamaria *et al*, 2009) and comparing both classifications (Barniol *et al*, 2011); 4) qualitative methods with experts comparing both classifications (Horstick *et al*, 2015a); and 5) descriptions of the process (Horstick *et al*, 2012, 2015b), detailing the step-by-step procedure.

Furthermore, clinical algorithms have been developed, based on the 2009 WHO case classification (Fig 2) and a clinical handbook (WHO/TDR, 2012).

The question now arises, is the 2009 WHO dengue case classification implemented? There has been controversy about the 2009 WHO dengue case classification, between different research groups (Halstead, 2012; Farrar *et al*,

2013). Advocates for the 1997 WHO dengue case classification continue to use the model dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), developed in 1975 by expert consensus, based on studies on Thai children in the 1950′s and ~60′s, with modifications in 1986 and 1997 (Bandyopadhyay *et al*, 2006). Key differences between the two classifications are summarized in Table 1.

This review aims to update on the implementation process. As a simple review, no formal methods have been used, but including a description of the definitions used by key organizations involved in dengue control (WHO, WHO regional offices, ECDC, CDC, NIH, and including research and clinical support material, as the BMJ) and recommendations including for research.

DISCUSSION

Descriptions of the definitions are used by key organizations involved in dengue control. The International Classification of Diseases 11 update (ICD 11) in its currently available beta-draft uses the 2009 WHO dengue case classification, coding dengue as 1D60 Dengue without warning signs, 1D61 Dengue with warning signs, 1D62 Severe

Table 1. Summary of differences between the 2009 WHO dengue case classification (DCC) and the previous case classification.

WHO DCC	2009 WHO DCC	1997 WHO DCC
Development	Series of studies, both quantitative and qualitative	Expert consensus, based on pervious studies and clinical experience
Validation	Tested in many different countries	No formal validation process
Focus	Towards severity of disease and early detection of severe cases	No relation to severity (especially DHF)
Usefulness	Especially for clinical management, but also for improved surveillance	Developed for both clinical management and research
Strength	Inclusion of all severe clinical pictures of dengue Helpful for clinical management without laboratory facilities	Medical staff is trained to use this model
ICD	ICD 11	Previous ICDs
Outlook	Further studies soon available on warning signs and case definitions	

DENGUE CASE

ASSESSMENT

PRESUMPTIVE DIAGNOSIS
Live in/travel to dengue endemic area.
Fever and two of the following criteria:
- Anorexia and nausea
- Rash
- Aches and pains
- Warning signs
- Leukopenia
- Tourniquet test positive

Laboratory confirmed dengue
(important when no sign of plasma leakage)

WARNING SIGNS*
- Abdominal pain or tenderness
- Persistent vomiting
- Clinical fluid accumulation
- Mucosal bleed
- Lethargy, restlessness
- Liver enlargment >2 cm
- Laboratory: increase in HCT concurrent with rapid decrease in platelet count

*(requiring strict observation and medical intervention)

CLASSIFICATION

NEGATIVE

Co-existing conditions
Social circumstances → **POSITIVE**

NEGATIVE

DENGUE WITHOUT WARNING SIGNS

DENGUE WITH WARNING SIGNS

MANAGEMENT

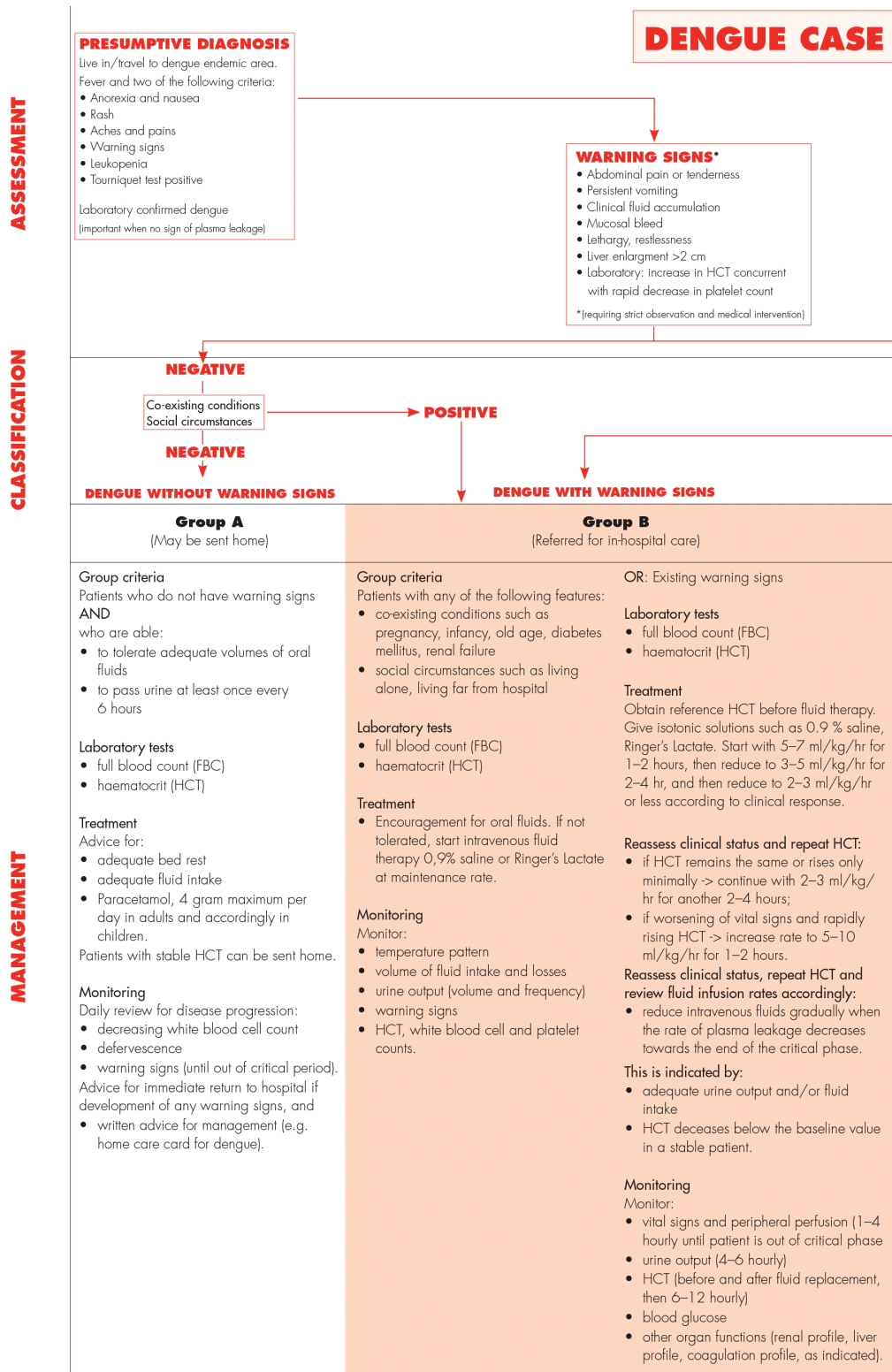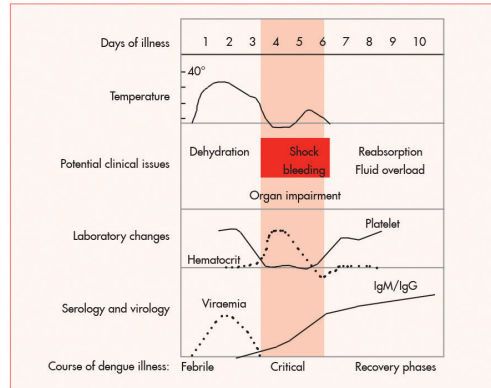| Group A (May be sent home)	Group B (Referred for in-hospital care)	
Group criteria Patients who do not have warning signs AND who are able: • to tolerate adequate volumes of oral fluids • to pass urine at least once every 6 hours **Laboratory tests** • full blood count (FBC) • haematocrit (HCT) **Treatment** Advice for: • adequate bed rest • adequate fluid intake • Paracetamol, 4 gram maximum per day in adults and accordingly in children. Patients with stable HCT can be sent home. **Monitoring** Daily review for disease progression: • decreasing white blood cell count • defervescence • warning signs (until out of critical period). Advice for immediate return to hospital if development of any warning signs, and • written advice for management (e.g. home care card for dengue).	**Group criteria** Patients with any of the following features: • co-existing conditions such as pregnancy, infancy, old age, diabetes mellitus, renal failure • social circumstances such as living alone, living far from hospital **Laboratory tests** • full blood count (FBC) • haematocrit (HCT) **Treatment** • Encouragement for oral fluids. If not tolerated, start intravenous fluid therapy 0,9% saline or Ringer's Lactate at maintenance rate. **Monitoring** Monitor: • temperature pattern • volume of fluid intake and losses • urine output (volume and frequency) • warning signs • HCT, white blood cell and platelet counts.	**OR**: Existing warning signs **Laboratory tests** • full blood count (FBC) • haematocrit (HCT) **Treatment** Obtain reference HCT before fluid therapy. Give isotonic solutions such as 0.9 % saline, Ringer's Lactate. Start with 5–7 ml/kg/hr for 1–2 hours, then reduce to 3–5 ml/kg/hr for 2–4 hr, and then reduce to 2–3 ml/kg/hr or less according to clinical response. **Reassess clinical status and repeat HCT:** • if HCT remains the same or rises only minimally -> continue with 2–3 ml/kg/hr for another 2–4 hours; • if worsening of vital signs and rapidly rising HCT -> increase rate to 5–10 ml/kg/hr for 1–2 hours. **Reassess clinical status, repeat HCT and review fluid infusion rates accordingly:** • reduce intravenous fluids gradually when the rate of plasma leakage decreases towards the end of the critical phase. **This is indicated by:** • adequate urine output and/or fluid intake • HCT deceases below the baseline value in a stable patient. **Monitoring** Monitor: • vital signs and peripheral perfusion (1–4 hourly until patient is out of critical phase • urine output (4–6 hourly) • HCT (before and after fluid replacement, then 6–12 hourly) • blood glucose • other organ functions (renal profile, liver profile, coagulation profile, as indicated).

Fig 2– Dengue treatment algorithm, for local adaptation.

MANAGEMENT

POSITIVE

SEVERE DENGUE

Group C
(Require emergency treatment)

Group criteria
Patients with any of the following features:
- severe plasma leakage with shock and/or fluid accumulation with respiratory distress
- severe bleeding
- severe organ impairment

Laboratory tests
- full blood count (FBC)
- haematocrit (HCT)
- other organ function tests as indicated

Treatment of compensated shock
Start IV fluid resuscitation with isotonic crystalloid solutions at 5–10 ml/kg/hr over 1 hour. Reassess patients' condition.

If patient improves:
- IV fluids should be reduced gradually to 5–7 ml/kg/hr for 1–2 hours, then to 3–5 ml/kg/hr for 2–4 hours, then to 2-3 ml/kg/hr for 2–4 hours and then reduced further depending on haemodynamic status;
- IV fluids can be maintained for up to 24–48 hours.

If patient is still unstable:
- check HCT after first bolus;
- **if HCT increases**/still high (>50%), repeat a second bolus of crystalloid solution at 10–20 ml/kg/hr for 1 hour;
- if there is improvement after second bolus, reduce rate to 7–10 ml/kg/hr for 1–2 hours and continue to reduce as above;
- **if HCT decreases**, this indicates bleeding and need to cross-match and transfuse blood as soon as possible.

Treatment of hypotensive shock
Initiate IV fluid resuscitation with crystalloid or colloid solution at 20 ml/kg as a bolus for 15 minutes.
If patient improves:
- give a crystalloid/colloid solution of 10 ml/kg/hr for 1 hour, then reduce gradually as above.
If patient is still unstable:
- review the HCT taken before the first bolus;
- **if HCT was low** (<40% in children and adult females, <45% in adult males) this indicates bleeding, the need to cross-match and transfuse (see above);
- **if HCT was high** compared to baseline value, change to IV colloids at 10–20 ml/kg as a second bolus over 30 minutes to 1 hour; reassess after second bolus.
- **If patient is improving** reduce the rate to 7–10ml/kg/hr for 1–2 hours, then back to IV cystalloids and reduce rates as above;
- **if patient's condition is still unstable**, repeat HCT after second bolus.
- **If HCT decreases**, this indicates bleeding (see above);
- **if HCT increases**/remains high (>50%), continue colloid infusion at 10–20 ml/kg as a third bolus over 1 hour, then reduce to 7–10 ml/kg/h 1–2 hours, then change back to crystalloid solution and reduce rate as above.

Treatment of haemorrhagic complications
Give 5–10 ml/kg of fresh packed red cells or 10–20 ml/kg of fresh whole blood.

Dengue and 1DGZ Dengue fever unspecified; furthermore as XB01.81 Dengue virus, QA23.6 Special screening examination for viral diseases other than human immunodeficiency virus, screening for dengue fever, 8D70.2Y Other specified viral encephalitis, encephalitis due to dengue fever and 8D72.2 Viral Myelitis, myelitis due to dengue virus.

With the update, the 2009 WHO dengue case classification should enter into all surveillance systems, which will facilitate reporting for dengue, but also to improve estimates for dengue disease burden, including severity of dengue and costs. This could help in the health policy context as well, underlining the importance to include dengue control in health programs in all affected countries (Horstick, 2014b).

The inclusion of the 2009 WHO dengue case classification in the ICD is closely mirrored by the bigger health organizations globally, WHO reporting for example on dengue fact sheets as "dengue and severe dengue" (WHO, 2016). For the WHO regional offices, the office for Africa, AFRO, follows the WHO dengue fact sheet, but quotes "dengue haemorrhagic fever" on its website (WHO/AFRO, 2017). The Pan American Health Organization, with the regional office for WHO in the Americas (AMRO), uses consistently the 2009 WHO dengue case classification (PAHO, 2017). In the South East Asian Region for WHO however, a separate guideline has been issued in 2011, using DF/DHF/DSS (WHO/SEARO, 2011). For the Western Pacific Regional Office, the 2009 WHO dengue case classification is used, including newly developed training material to explain the differences to the previous classification (WHO/WPRO, 2017).

As for the surveillance centers, the Centers for Diseases Control (CDC) explain in detail the 2009 WHO dengue case classification (CDC, 2015),

…in 1997 the dengue case definition was limitated in terms of its complexity and applicability. This recognition of the limitations led to a multicenter study in seven countries in Asia and Latin America and a new case

definition emerged from this study. The new WHO classification for dengue severity is divided into Dengue without Warning Signs, Dengue with Warning Signs, and Severe Dengue.

The European Centre for Disease Control (ECDC), on its websites, uses the terminology of the 2009 WHO dengue case classification, however explaining also, *Severe dengue — commonly referred to as 'Dengue haemorrhagic fever/Dengue shock syndrome (DHF/DSS)' to distinguish it from 'classic' dengue fever (DF)* (ECDC, nd).

As a support system for research and clinicians, the British Medical Journal shows in their Best Practice Guideline series the use of the 2009 WHO dengue case classification (BMJ, 2016), quoting:

The 1997 dengue case definition…. is limited in terms of its complexity and applicability. This led to a new WHO classification where dengue severity is divided into dengue without warning signs, dengue with warning signs, and severe dengue. While WHO still support both case definitions, there is a move towards using the 2009 case definition due to its ease of use".

One of the areas that were criticized of the 2009 WHO dengue case classification is that research endpoints are not well enough defined, since research may require further clinical endpoints, as also witnessed during the trials of the first available — and partially effective — vaccine (Hadinegoro, 2015), these endpoint measures are now being defined in an empirical process (NIH, 2015). Furthermore, and referring to this argument, the 2009 WHO dengue case classification has been particularly developed to help clinical management, in the crucial area to reduce case fatalities.

In an expert consensus in Havana, Cuba in 2014 (Horstick, 2015a), dengue experts agreed on this argument and described, 1) the need to update ICD10, 2) include D/SD in country epidemiological reports and 3) implement studies improving sensitivity/specificity of the dengue case definition. Most importantly, the group of experts favored

largely the 2009 WHO dengue case classification, with the clinical management implications, since it 1) standardizes clinical management, 2) raises awareness about unnecessary interventions, 3) matches patient categories with specific treatment instructions and 4) makes the key messages of patient management understandable for all health care staff.

In conclusion, with the inclusion of the 2009 WHO dengue case classification in the ICD11 and the adaptation of the classification in most regions in the world, and organizations working in this field, a consensus for its global use is overdue, for the few remaining countries not using this classification model. This could be stimulated by WHO. If a process of updating the 2009 WHO dengue case classification is envisaged, it should however be based on data, this could be quantitative and qualitative, however not based on single expert opinion only. Furthermore, research is ongoing to "fine-tune" the warning signs, and results should become available in the next couple of years. The NIH-lead process to define research endpoints is most welcome, particularly since the next vaccines are entering or coming out of clinical phase 3 trials.

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COMPARISON OF THE 1997 AND 2009 WHO CLASSIFICATIONS FOR DETERMINING DENGUE SEVERITY IN THAI PATIENTS

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Abstract. The World Health Organization (WHO) proposed a revised dengue classification in 2009 to facilitate a more effective identification of severe dengue cases. We compared the two systems of dengue severity classification, 1997 and 2009 WHO guidelines, at a Thai tertiary-care teaching hospital. A total of 765 patients with dengue infection were studied: 510 (66.7%) were adults, and 496 (64.8%) were from the outpatient department. According to the WHO 2009 guidelines, 61.7%, 33.5%, and 4.8% were classified as having dengue without warning signs, dengue with warning signs, and severe dengue, respectively. When the WHO 1997 classification was applied, 87.2%, 11.4%, and 1.4% were classified as dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS), respectively. Seven cases (1%) of DF patients were categorized as severe dengue by severe bleeding. Of DHF patients, 10.3% had severe bleeding, and 10.3% had severe organ impairment. Overall, we observed that the 2009 WHO classification stratifies a much larger proportion of patients into a category requiring a higher level of medical and nursing care (dengue with warning signs or severe dengue) than the 1997 classification (DHF or DSS). However, DHF patients had a significantly higher frequency of in-patient treatment than dengue with warning signs patients (92% *vs* 53.1%; *p*<0.001). The 1997 classification appeared to identify truly severe cases while the 2009 guidelines were more useful in detecting a broad range of severe clinical manifestations such as severe bleeding. Further studies are needed to assess the utility of the WHO dengue severity classification guidelines and to identify areas that require modification.

Keywords: dengue, severity, severe dengue, WHO classification

INTRODUCTION

Dengue remains the most prolific mosquito-borne infection worldwide. Data from the World Health Organization (WHO) noted a significant increase in the number of cases from 0.4 million cases in 1996 to 3.2 million cases in 2015 (WHO, 2012, 2016). Dengue infection is a systemic and dynamic disease with a wide clinical spectrum that includes both severe and non-severe clinical manifestations. While most patients recover following a self-limiting and non-severe clinical course, a small proportion progress to severe disease, mostly characterized by plasma leakage with or without hemorrhage (Thisyakorn and Thisyakorn, 2015). The reasons for some patients progressing from non-severe to severe disease are yet to be determined. However, identifying such patients early is critical to provide appropriate treatment and to prevent the development of severe clinical conditions.

In the 1997 WHO guidelines, patients are classified in three separate categories: dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS) (WHO, 1997). The

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diagnosis of DHF was restricted to those patients with the collective presence of fever, hemorrhagic tendency, thrombocytopenia, and signs of plasma leakage. DHF with signs of shock was classified as DSS. In 2009, a new classification of dengue proposed by WHO Tropical Disease Research was published. The new guidelines classify dengue into dengue without warning signs, dengue with warning signs and severe dengue (WHO, 2009).

Abdominal pain or tenderness, persistent vomiting, clinically manifesting fluid accumulation, mucosal bleeding, lethargy and restlessness, hepatomegaly, and an increase in hematocrit with a drop in platelet count are all listed as warning signs. Severe dengue is defined by the occurrence of plasma leakage and/or fluid accumulation leading to shock or respiratory distress; and/or severe bleeding; and/or severe organ impairment (Table 1).

Although the revised scheme is more sensitive to the diagnosis of severe dengue and beneficial to triage and case management, there remain issues with its applicability. It is considered by many to be too broad, requiring more specific definition of warning signs. Quantitative research into the predictive value of these warning signs on patient outcomes and the cost effectiveness of the new classification system is required to ascertain whether the new classification system requires further modification, or whether elements of both classification systems can be combined (Hadinegoro, 2012).

This study aimed to compare the two systems of dengue severity classification, 1997 and 2009 WHO guidelines at a tertiary-care teaching hospital in Thailand.

MATERIALS AND METHODS

Study designs and setting

A retrospective cross-sectional study was conducted among 840 patients who were diagnosed with dengue infection at a tertiary care hospital in Thailand during 2014 to 2015. Patient records were reviewed. The diagnosis of dengue patients adhered to the criteria established by the WHO 2009 (WHO, 2009). After medical record review, 31 cases were excluded due to misdiagnosis of dengue, 44 cases were excluded due to incomplete data; they were referred to another hospital in compliance with their health insurance. Finally there were 765 dengue patients; each patient was classified/graded according to both the 1997 (DF, DHF, and DSS) and the 2009 WHO guidelines (dengue without warning signs, dengue with warning signs and severe dengue) (Table 1). This study was approved by the Ethics Committee of the Faculty of Medicine, Thammasat University.

Statistical analysis

Descriptive statistics including frequency, percentage, range, mean, and standard deviation were calculated for the demographic and clinical data as appropriate. Treatment and outcomes of dengue using the 1997 and 2009 WHO classifications were analyzed. Categorical variables were compared using chi-square or Fisher's exact test as appropriate. Continuous variables were compared using the Student's t-test. Significance level was set at a p-value < 0.05.

RESULTS

Of the 765 patients with dengue infection during the study period, 510 (66.7%) were adults and 394 (51.5%) were males. The mean age was 23.5 years (range 0-77 years). There were 496 (64.8%) patients treated in the outpatient department (OPD), and 269 (35.2%) were treated in the inpatient department (IPD).

According to the 2009 WHO classification, 472 patients (61.7%) were dengue without warning signs, 256 patients (33.5%) were dengue with warning signs, and the remaining 37 patients (4.8%) were severe dengue (Fig 1). Of the 37 patients with severe dengue, 14 (37.8%) had severe plasma leakage, 19 (51.4 %) had severe clinical bleeding, and 14 (37.8%) had severe organ involvement. Of the 14 patients with severe organ involvement, 11 patients had AST >1000 IU/l and/or ALT >1000 IU/l, 8 patients had alteration of consciousness, 4 patients had serum creatinine ≥3 times above baseline, and 3 patients had respiratory failure.

Table 1.   The WHO 1997 and 2009 classifications for dengue severity.

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WHO 1997 classification for dengue severity

Dengue Fever
Acute febrile illness with two or more of the following:
    Headache
    Retro-orbital pain
    Myalgia
    Leukopenia
    Arthralgia
    Rash
    Hemorrhagic manifestations
    Supportive serology or occurrence at the same location and time as other confirmed cases of dengue fever

Dengue Hemorrhagic Fever
All of the following must be present:
    Fever or history of acute fever, lasting 2–7 days, occasionally biphasic.
    Hemorrhagic manifestations: Positive tourniquet test;
        Petechiae, equimosis, purpura or bleeding from mucosa, gastrointestinal tract, injection sites or other locations;
        or hematemesis/melena.
    Thrombocytopenia (<100,000 platelets per mm^3)
    Evidence of plasma leakage due to increased vascular permeability

Dengue Shock Syndrome
DHF with hypotension for age or narrow pulse pressure (>20 mmHg), plus one of the following:
    Rapid and weak pulse
    Cold, clammy skin, restlessness

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WHO 2009 classification for dengue severity

Dengue without Warning Signs
Fever and two of the following:
    Nausea, vomiting
    Rash
    Aches and pains
    Leukopenia
    Positive tourniquet test

Dengue with Warning Signs
Dengue as defined above with any of the following:
    Abdominal pain or tenderness
    Persistent vomiting
    Clinical fluid accumulation
    Mucosal bleeding
    Lethargy, restlessness
    Liver enlargement >2 cm
    Laboratory: increase in HCT concurrent with rapid decrease in platelet count

Severe Dengue
Severe Dengue with at least one of the following criteria:
    Severe Plasma Leakage leading to:
        Shock
        Fluid accumulation with respiratory distress
    Severe bleeding as evaluated by clinician
    Severe organ involvement
        Liver: AST or ALT ≥1,000
        CNS: impaired consciousness
        Failure of heart and other organs

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According to the 1997 WHO classification, 667 patients (87.2%) were DF, 87 (11.4 %) were DHF, and 11 (1.4%) were DSS (Fig 1).

When comparing the 1997 classification to the 2009 classification, 70.8% of DF patient were categorized as dengue without warning signs, 28% as dengue with warning signs, and 1.0% as severe dengue. Of the DHF patients, 78.2% were categorized as dengue with warning signs and 21.8% as severe dengue. All of DSS were categorized as severe dengue. All dengue without warning signs were categorized as DF. Among dengue with warning signs patients, 73.4% were categorized as DHF and 26.6% as DSS. Of all severe dengue patients, 18.9% were categorized as DF, 51.4% as DHF, and 29.7% as DSS (Table 2).

Type of severe dengue by the 2009 WHO classification and disease severity by the 1997 WHO classification are shown in Fig 2. Seven cases (1%) of DF patients were categorized as severe dengue by severe bleeding. Of DHF patients, 10.3% had severe bleeding and 10.3% had severe organ impairment. Of DSS patients, 100% had severe plasma leakage, 27.3% had severe bleeding, and 27.3% had severe organ impairment.

Table 3 demonstrates types of treatment and outcomes among dengue patients classified by the two guidelines. Six cases required intensive care, and one died. In the 1997 classification, the majority of DF cases (73.3%) were treated as outpatients, and 26.7% were hospitalized, receiving some type of intravenous (IV) rehydration. Most DHF patients (92.0 %) were hospitalized, and 2.3% required intensive care unit (ICU). All DSS were hospitalized and 36.4% required ICU. In the revised 2009 classification, 20.8% of patients with dengue without warning signs and 53.1% of dengue with warning signs were hospitalized.
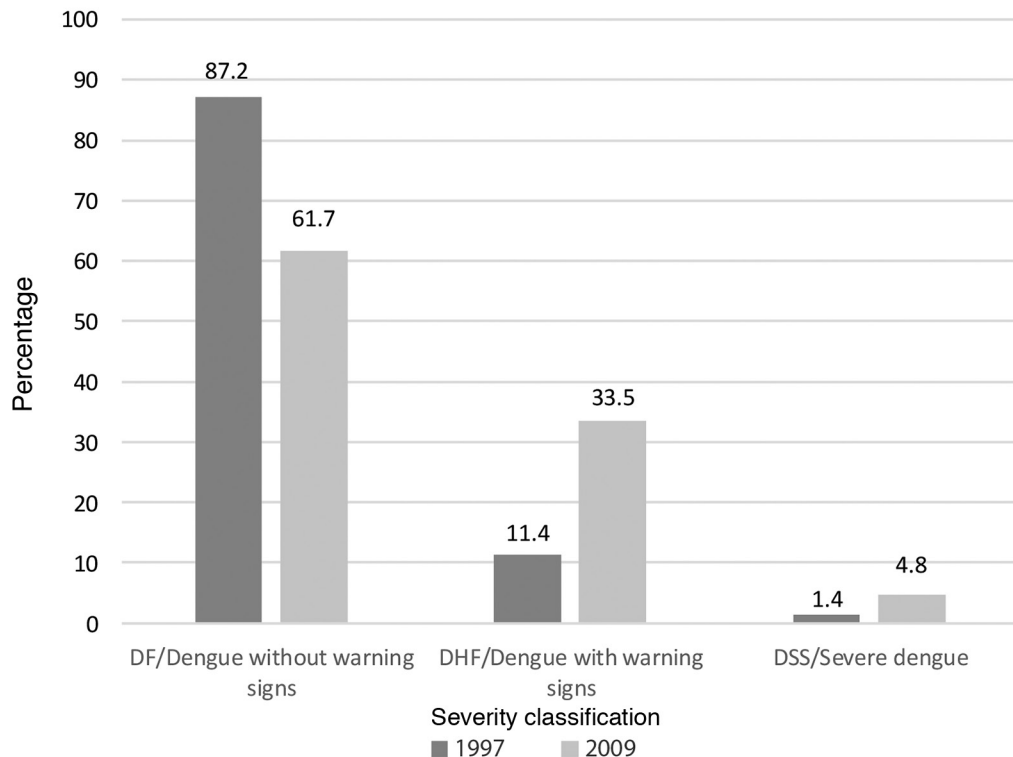
Fig 1–The 1997 and 2009 WHO classifications for dengue severity. DF, dengue fever; DHF, dengue hemorrhagic fever; DSS, dengue shock syndrome.

Among patients with severe dengue, 94.6% were hospitalized, and 5.4% (2 patients) were treated as outpatients: these two patients had vaginal bleeding. Patients with DF had a significantly higher frequency of whole blood/pack red cell transfusion and hospitalization than patients with dengue without warning signs (p=0.023 and p=0.013, respectively). Patients with DHF had a significantly higher frequency of platelet transfusion and hospitalization than patients with dengue with warning signs (p=0.015 and

p=<0.001, respectively). Patients with DSS were significantly more likely to receive colloid for fluid resuscitation than severe dengue (p=0.017).

DISCUSSION

There has been considerable debate on the application of both the 1997 and 2009 WHO dengue classification guidelines for diagnosis and management of dengue infection. Previous studies have shown that the 2009 guidelines, which focus

Table 2.   Distribution of dengue severity between the 1997 and 2009 WHO classifications.

Disease severity by WHO 2009	Disease severity by WHO 1997, n (%)			Total n (%)
	DF	DHF	DSS	
Dengue without warning signs	472 (70.8)	0 (0)	0 (0)	472 (61.7)
Dengue with warning signs	188 (28.2)	68 (78.2)	0 (0)	256 (33.5)
Severe dengue	7 (1.0)	19 (21.8)	11 (100)	37 (4.8)
Total n (%)	667 (87.2)	87 (11.4)	11 (1.4)	765 (100)

DF, dengue fever; DHF, dengue hemorrhagic fever; DSS, dengue shock syndrome.
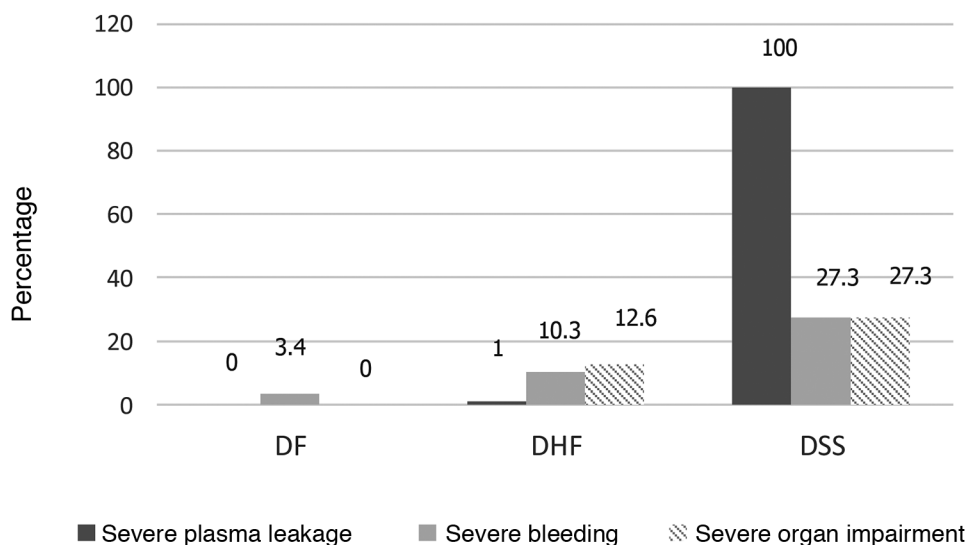
Fig 2–Severe dengue classified by the 2009 WHO guidelines and disease severity classified by 1997 WHO guideline. DF, dengue fever; DHF, dengue hemorrhagic fever; DSS, dengue shock syndrome.

Table 3. Comparison of treatment and outcomes of dengue between the 1997 and 2009 WHO classifications.

Treatment and outcomes	1997 WHO classification, n (%)			2009 WHO classification, n (%)			p-value		
	DF	DHF	DSS	Dengue without warning signs	Dengue with warning signs	Severe dengue	DF vs Dengue without warning signs	DHF vs Dengue with warning signs	DSS vs Severe dengue
	(n=667)	(n=87)	(n=11)	(n=472)	(n=256)	(n=37)			
Colloid use	0 (0)	3 (3.4)	7 (63.6)	0 (0)	2 (0.8)	8 (21.6)	NA	0.212	0.017*
Whole blood/Pack red cell transfusion	7 (1.0)	2 (2.3)	2 (18.2)	0 (0)	6 (2.3)	5 (13.5)	0.023a	1.341	1.626
Platelet transfusion	1 (0.1)	7 (8.0)	2 (18.2)	0 (0)	4 (1.6)	6 (16.2)	0.586	0.015a	1.483
Inotropic use	0 (0)	0 (0)	1 (9.1)	0 (0)	0 (0)	1 (2.7)	NA	NA	0.819
Ventilator/respiratory support	0 (0)	0 (0)	3 (27.3)	0 (0)	0 (0)	3 (8.1)	NA	NA	0.248
Chest drainage	0 (0)	0 (0)	1 (9.1)	0 (0)	0 (0)	1 (2.7)	NA	NA	0.819
Exchange transfusion	0 (0)	0 (0)	1 (9.1)	0 (0)	0 (0)	1 (2.7)	NA	NA	0.819
Hemodialysis	0 (0)	0 (0)	1 (9.1)	0 (0)	0 (0)	1 (2.7)	NA	NA	0.819
Hospitalization	178 (26.7)	80 (92.0)	11 (100)	98 (20.8)	136 (53.1)	35 (94.6)	0.013a	<0.001a	1.181
ICU admission	0 (0)	2 (2.3)	4 (36.4)	0 (0)	0 (0)	6 (16.2)	NA	0.128	1.928
Death	0 (0)	0 (0)	1 (9.1)	0 (0)	0 (0)	1 (2.7)	NA	NA	0.819

DF dengue fever; DHF, dengue hemorrhagic fever; DSS, dengue shock syndrome; NA, not analyzed.a p<0.05).

on the severity level, are considered to be more sensitive in capturing severe disease compared to the 1997 guidelines (Basuki *et al*, 2010; Narvaez *et al*, 2011; Horstick *et al*, 2014). However, problems with using the 2009 classification have also been noted. These include the requirements of additional training and dissemination of the guidelines for healthcare workers to remedy any confusion over the changes to the system (Barniol *et al*, 2011).

There was an increase in the diagnosis of the severe form of dengue infection using the 2009 guidelines compared to the 1997 guidelines. The proportion of patients with severe dengue was lower by the WHO 1997 guideline classification (12.8% with DHF or DSS) compared with the 2009 guidelines classification (38.3% with dengue with warning signs or severe dengue).

The 2009 classification captured a higher number of cases with severe dengue than those captured as DSS by the 1997 classification. Moreover, 7 (1.0 %) of DF cases (non-severe form) classified by the 1997 guidelines were classified as severe dengue (severe form) due to severe bleeding. Previous studies have demonstrated the overlap between case definitions of DF, DHF, and DSS (Phuong *et al*, 2004; Deen *et al*, 2006) while another study found that the 1997 classification did not detect severe dengue manifestations in some patients, particularly in adults (Balmaseda *et al*, 2005). Some manifestations of severe dengue, such as severe bleeding or organ failure were not included in the 1997 classification.

However, our study found that patients who had DHF by the 1997 classification were more likely to be hospitalized than patients who had dengue with warning signs by the 2009 classification (*p*<0.001). These results suggest that the 1997 WHO classification is more likely to identify clinically severe cases than the 2009 WHO classification.

The WHO 2009 guidelines recommend that all cases of severe dengue and dengue with warning signs should be hospitalized; this led to a 38.3% hospitalization rate among dengue patients in our

study. In comparison, based on the 1997 guidelines that recommend hospitalization among DHF and DSS cases, the rate would have been 12.8%. This raises a concern regarding the increasing workload for healthcare workers caring for hospitalized patients with dengue infection if the 2009 classification is used in Thailand (Kalayanarooj, 2011). This would have a significant impact on the utilization of hospital resources in the region.

We discovered a higher proportion of patients with dengue with warning signs compared with patients with DHF. This may have been due to the less stringent and non-specific classification of dengue with warning signs that allowed the capturing of more patients potentially at risk of developing severe manifestations. However, such a large number of patients classified as dengue with warning signs may lead to an increased burden on the healthcare system in resource-limited settings. Revision of the definitions of the warning signs is needed to accurately identify patients who actually require hospitalization.

To give a more complete overview, our study included both hospitalized patients and non-hospitalized patients with dengue infection. However, there are several limitations to note. First, the retrospective design of the study may be associated with incomplete data recording and misclassification bias. Forty-four cases were excluded due to incomplete data. Furthermore, 9 cases were originally diagnosed as DF but we reclassified them to DHF. In addition, 26 cases were also first diagnosed with DHF but reclassified as DF. This was in accordance with 1997 WHO classification. Second, there were few severe cases requiring intensive care and only one fatality, limiting our ability to assess the clinical relevance of both dengue classifications for detecting life-threatening situations.

In conclusion, we observed that the 2009 WHO classification stratifies a much larger proportion of patients into a category that requires a higher level of medical and nursing care (dengue with warning signs and severe dengue) than the 1997 classification (DHF or DSS). However, DHF

patients had a significantly higher frequency of hospitalization than dengue with warning signs patients. The 1997 WHO classification tended to identify truly severe cases versus the 2009 WHO classification; the use of the 2009 classification to determine dengue severity and guide management may result in increased unnecessary hospitalizations and an increased burden on resources in our setting where dengue infection is endemic. Nevertheless, the 2009 guidelines were more useful in detecting a broad range of severe clinical manifestations such as severe bleeding. Further studies are needed to assess the utility of the 2009 WHO classification guidelines and to identify areas that require modification.

ACKNOWLEDGEMENTS

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LABORATORY DIAGNOSIS OF DENGUE VIRUS INFECTIONS

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Abstract: Accurate and efficient diagnosis of dengue is important for clinical care, surveillance support, pathogenesis studies, and vaccine research. Laboratory diagnosis is also important for case confirmation. Here is presented a review of the current state of dengue virus diagnosis. Laboratory dengue diagnosis can be performed through virus isolation, genome and antigen detection and serological studies. For virus detection, dengue viremia is short, usually observed two or three days before onset of fever and lasts four to five days later. Therefore, samples for virus detection must be taken in the first four to five days of disease during the febrile phase. In recent years, PCR (polymerase chain reaction) has become an important tool as a quick method for diagnosis of dengue. Another method of diagnosis is detection of NS1 antigen, using a commercial ELISA kit. Although PCR is the most sensitive and rapid method for the detection of dengue virus in early stage of disease, classical dengue virus culture using dengue tissue culture seeds also has important benefit, especially for genotype studies. In serologically based diagnosis of primary infection, the dominant immunoglobulin isotype is IgM. Anti-IgM may appear during the febrile phase in 50% of cases, in the other half, it appears within 2-3 days following defervescence. Once detectable, IgM levels rise quickly and appear to peak about 2 weeks after the onset of symptoms, and then they decline to an undetectable level over 2-3 months. Anti-IgG appears shortly afterwards at a very low level. The physiological definition of a primary infection is therefore characterized by a high molar fraction of anti-dengue IgM and low molar fraction of IgG. Secondary dengue infections are characterized by a rapid increase in IgG antibodies and when anti-dengue IgM appears in most instances, the levels are dramatically lower.

Keywords: dengue infection, dengue virus, laboratory diagnosis

INTRODUCTION

Dengue virus (DENV) consists of four antigenically distinct serotypes (DENV-1, DENV-2, DENV-3, and DENV-4) that display a high degree of antigenic cross-reactivity with each other as well as with other mosquito and tick-borne flaviviruses such as Japanese encephalitis (JE), yellow fever, West Nile, and tick-borne encephalitis viruses (Calisher *et al,* 1989; Innis, 1995). In countries where dengue is now or is becoming endemic, the cocirculation of two or more flaviviruses such as dengue and JE in Asia, dengue and JE and West Nile in India, and dengue and yellow fever in the Americas makes the serologic diagnosis of acute DENV infection a difficult task. From a public health and clinical care perspective, the accurate diagnosis of DENV infection as well as the identification of circulating DENV serotypes affects public health policy and action for vector control as well as individual case management.

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The views expressed in this article are those of the author and do not reflect the official policy of the Department of the Army, Department of Defense, or the US Government.

By 1964, dengue fever (DF) and dengue hemorrhagic fever (DHF) were recognized as a serious public health problem in most of Southeast Asia. Hammon and Sather (1964) summarized the difficulties of making a serotype-specific diagnosis due to the serologic cross reactivity between dengue and other flaviviruses. Three assays were available in 1964 to distinguish DENV serotypes: virus neutralization using a suckling mouse model (Sabin, 1950), complement fixation (Casals, 1949) and hemagglutination inhibition (HI) (Clarke and Casals, 1958). The latter two methods were plagued with a high degree of serologic cross-reactivity, and the former complicated by the use of suckling mice. By 1990, several milestones in dengue diagnosis were achieved, to include the use of mosquitoes to propagate DENV, the development of mosquito cell lines for virus isolation, monoclonal antibodies to all four DENV serotypes, the adaptation of the enzyme immunoassay (EIA) format to dengue diagnosis, and the first genome-based diagnostic efforts (Shope, 1990).

The purpose of this chapter is to detail assays currently available to diagnose DENV infection. Understanding a diagnostic assay's advantages and limitations is essential to proper interpretation and application whether that application is at the level of the patient or population.

ANTIBODY AND VIRUS PATTERNS IN DENGUE VIRUS INFECTION

Upon the bite of an infected mosquito, DENV is introduced intradermally and replicates within skin dendritic cells (Wu *et al*, 2000). From the time of inoculation, DENV replicates and disseminates through the lymphatic system producing measurable viremia approximately 3 days after inoculation lasting, approximately another 4 days (Fig 1). The onset of fever and symptoms occurs approximately 24 hours after the onset of measurable viremia. Fever will last 4 days on average followed by a sudden defervescence (Chandler and Rice, 1923; Siler *et al*, 1926; Simmons, 1931; Sabin, 1955; Kalayanarooj *et al*, 1997; Vaughn *et al*, 1997). The day of defervescence is an immunologically important

landmark in the course of DENV infection as it defines the approximate onset of plasma leakage in patients with DHF (WHO, 1997; Green *et al*, 1999).

Primary dengue

Primary DENV infection occurs when a patient lacking previous exposure to a flavivirus develops an acute DENV infection that results in dengue-specific antibody production. The patient with a primary infection rarely develops DHF and anti-dengue antibody evolves slowly during the course of the clinical illness with high production of IgM antibody (Vaughn *et al*, 1997). The molar ratio of IgM to IgG is high ($\geq 1.8:1.0$) for at least three weeks following infection (Innis *et al*, 1989). Using HI and neutralizing antibody, a primary infection is defined by low titers of antibody that develop slowly (Russell *et al*, 1967; WHO, 1997).

Secondary dengue

Secondary dengue implies previous flavivirus exposure (secondary flavivirus infection) or a previous DENV infection with a different serotype (secondary DENV infection). The IgG antibody response occurs early and vigorously during the clinical illness; nearly all patients have diagnostic levels of antibody by EIA within 24 hours of defervescence (Innis *et al*, 1989; Vaughn *et al*, 1997). The rapid increase in antidengue antibody to high levels indicates an anamnestic (memory) response (Halstead *et al*, 1983). The dengue-specific IgM response is more variable with a IgM to IgG ratio ≤ 1.8 as measured by IgM capture ELISA (Innis *et al*, 1989).

The level of IgM response may relate to the number of new epitopes present on the current infecting virus compared to the previous flavivirus. That is, previous infection with a more distantly related flavivirus (*eg*, JE virus) will result in higher IgM levels by EIA when infected by a DENV than if the current infection follows a previous DENV infection with another serotype. HI and neutralizing antibody levels elevate quickly to high titers in secondary infections (HI titer $\geq 1:2560$) (WHO, 1997). Again, the degree of shared epitopes may influence the degree of anamnestic antibody response.

Viremia pattern

Due to antibody cross-reaction, definitive diagnosis of DENV infection requires isolation of the virus or detection of virus genome. The pattern of viremia in dengue patients correlates closely with fever with peak levels (titers up to 10 logs/ml) occurring 2 to 3 days following the onset of illness, which is typically 2 to 3 days prior to defervescence (Vaughn *et al,* 2000). For DENV-1, DENV-2, and DENV-3, the viremia titer correlates with dengue disease severity (Vaughn *et al,* 2000; Libraty *et al,* 2002). This finding is of more than theoretical interest as it provides a potential tool for the clinician to assess the risk of severe disease (DHF) prior to the onset of plasma leakage.

With the development of new and rapid tools to measure viremia titer, this may prove to be a potentially important measurement in defining persons at risk for DHF. Viremia level, as originally determined by Vaughn and colleagues (2000), used serial titrations of viremic plasma from dengue patients inoculated into *Toxorhynchites splendens* mosquitoes. The titer at which infection occurred in half of the *T. splendens* was determined using probit analysis resulting in the mean infectious

dose 50% (MID_{50}). This is a time consuming and tedious process requiring serial specimen dilution and numerous mosquito inoculations followed by a two-week incubation period and virus detection in each mosquito by indirect immunofluorescence or RT-PCR.

New molecular tools are proving useful to measure dengue viral load (Houng *et al*, 2001; Libraty *et al,* 2002). However, where mosquito or cell culture inoculation measures viable virus, molecular approaches detect only the presence of genome. RT-PCR methodologies may allow a virus-based diagnosis extending many hours past the detection of replicating virus due to its detection of viral genome in virus-antibody complexes (neutralized virus) (Wang *et al,* 2003). How rapid molecular diagnostic tool will affect the clinical management of DHF patients needs to be determined in prospective studies.

DIAGNOSTIC PATHWAY IN PATIENTS WITH SUSPECTED ACUTE DENGUE

Fig 1 illustrates the diagnostic options currently available to diagnose an acute DENV infection
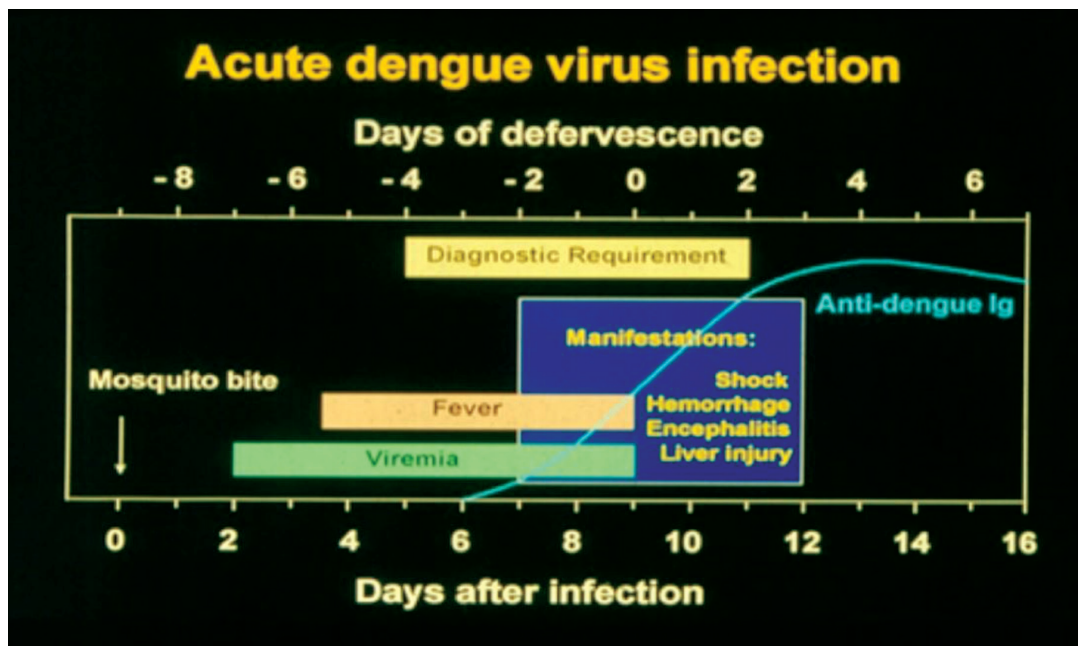

Fig 1–Diagnostic pathway in patients with suspected dengue.

in patients with suspected dengue. Diagnostic options are divided first into assays that detect the presence of the virus (including virus antigen and virus genome) versus assays that detect the host's response to the virus (antibody). Virus detection methods can be divided between time-consuming virus culture methods using animals, mosquitoes, or cell culture versus rapid antigen or genome detection methods. Likewise, antibody detection methods include assays that can be completed in a few minutes or require a week or more to ascertain.

The utilization of multiple approaches with properly timed specimens assures diagnosis in virtually every case of suspected dengue. This diagnostic rigor is required to conduct research in dengue pathogenesis. Is this type of diagnostic breadth needed for every laboratory to be made available to every clinician? Ideally, every country experiencing or at risk for dengue epidemics should have at least one center of excellence that is proficient to isolate virus, detect genome, and to carefully characterize antibody responses. This would help to better understand the depth and breadth of the dengue pandemic.

The capacity to diagnose clinically similar outbreaks such as leptospirosis is also important. Serotype specific virologic diagnoses over time may someday allow the prediction of the timing and severity of dengue epidemic in order to allow vector control efforts to prevent or abate such epidemics.

For the clinician, a hospital-based laboratory that can perform basic dengue serology can quickly confirm a dengue outbreak. However, since antibody-based assays do not routinely become positive until the danger period for DHF has passed, the diagnosis is of less value to the patient who is recovering. To have an impact on the care of an individual patient, a reliable rapid molecular diagnosis is needed to assist in the differential diagnosis and to reduce morbidity and mortality. For suspected dengue patients, serum specimens should be collected early in the febrile phase and stored at -70°C for virus isolation/detection and at -20°C for acute phase serology to be paired with a specimen drawn at least 7 days later for convalescent serology (WHO, 1997).

The practice in many dengue hyperendemic countries is to draw blood upon admission and at the time of discharge one to two days following defervescence. In this setting, most dengue patients are experiencing secondary infections and the serology will be positive soon after defervescence. For patients with primary infections, a subsequent follow-up specimen is needed to confirm or rule out DENV infection if attempts at virus isolation or identification fail.

For travelers returning to the United States with suspected dengue, acute and convalescent samples can be sent to the state health department with a request to forward them to the CDC for testing.

SEROLOGICAL ASSAYS

Hemagglutination inhibition (HI) assay

The path towards the serologic diagnosis of dengue started in 1950, when Sabin (1950) discovered that arboviruses in general and dengue virus (DENV) in particular, are able to agglutinate certain types of erythrocytes. Normal erythrocytes in a suspension will settle to the bottom of a test tube or well and form a compact, dense button of red blood cells after 30 minutes to one hour. Agglutination of red cells using virus antigen will prevent this normal settling to occur and results in a uniform lattice of cross linked cells covering the lower part of the tube or well (hemagglutination). Casals and Brown (1954) modified the technique using acetone and ether to extract (purify) the hemagglutinating antigens (HA) and developed a hemagglutination inhibition (HI) assay. In this assay, DENV specific antibody binds the HA preventing lattice formation allowing the red blood cells to clump to the bottom of the test well (Fig 2).

Test serum could be tittered to determine when hemagglutination was no longer inhibited. Porterfield (1954, 1957) and Clarke and Casals (1955) further improved this assay by using female goose red blood cells rather than human cells to remove nonspecific inhibitors of hemaglutination. The techniques used for hemagglutination and HI as described by Clarke and Casals in 1958 have
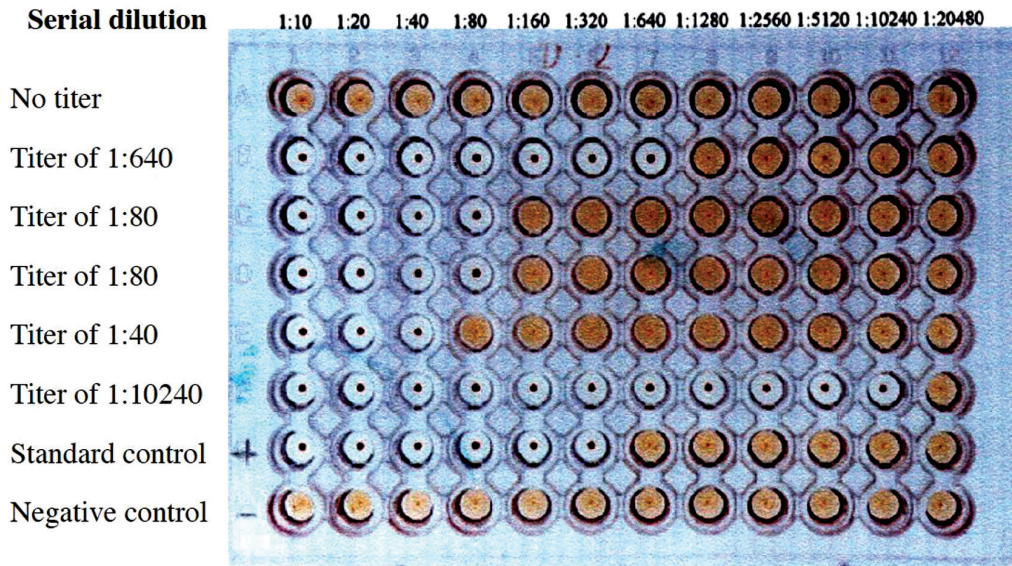
Fig 2–Hemagglutination inhibition assay.

remained largely unchanged today apart from adaptation to microtiter plates in 1980 and remains a fundamental tool to diagnose acute DENV infection and in seroprevalence studies (WHO, 1997).

Agglutination of red cells is dependent on pH and the amount of HA present. HA is quantified as a titer where an HA unit of 1 represents the highest dilution which causes hemagglutination of red cells. An additional 1 HA unit is thus given for each subsequent lower titer of antigen. For example, if the highest titer of a DENV-2 antigen causing hemagglutination was found to be 1:160 and by convention given an HA unit of 1 at this titer, 2 HA units would be a titer of 1:80; 3 HA units for 1:40; 4 HA units for 1:20 and 5 HA units for 1:10. The hemagglutination assay allows quantification and standardization of dengue antigen (HA) produced from a variety of sources with the most common sources being suckling mouse brain and cell culture.

As until recently practiced in the Armed Forces Research Institute of Medical Sciences (AFRIMS) in Bangkok, Thailand, 1-to-2 day old suckling mice (*Mus musculus*) are intracranially inoculated with 0.02 ml of a dengue serotype specific virus suspension. Mice are observed twice a day for

the first signs of encephalitis (failure to eat as evidenced by lack of milk in the stomach, color change, wasting, or tremors), which occurs 3-to-10 days after inoculation. For DENV-1 this usually occurs at day 5, day 4 for DENV-2, day 7 for DENV-3, day 4 for DENV-4, and day 3 for Japanese encephalitis virus. Harvested brain is made into a 20% (weight/volume) suspension in an 8% sucrose solution and homogenized. The homogenate is then acetone extracted, condensed, washed and resuspended in sterile normal saline. All antigen is assayed for the number of HA units.

The hemagglutination assay itself is performed best using goose red blood cells (RBCs) though RBC from other species can be used. Goose RBCs can be obtained from adult female geese (*Anser cinereus*) and should immediately be placed in Alsever's solution (glutaraldehyde-fixed goose RBCs may also be used) (Wolff *et al*, 1977). Goose RBC's are then washed with dextrose-gelatin-veronal solution and are brought up to a final 8% solution just prior to assay use. Buffers of differing pH have been found to be optimal for the agglutination of different flaviviruses and specific to each dengue serotype: pH of 6.2 for DENV-1; 6.4 for DENV-2; 6.6 for DENV-3; and 6.8 for DENV-4. It is essential

that the RBCs be suspended in a buffer set at a specific pH for each dengue serotype prior to use in the assay.

To prepare the test sera, non-specific inhibitors must be removed (that is, some human serum can inhibit agglutination in the absence of measurable dengue antibody). This is done using acetone, ether or kaolin prior to performing the HI assay (Monath *et al,* 1970). Diethylaminoethyl-Sephadex (DEAE-Sephadex) has also been used with success (Altemeier *et al,* 1970). At AFRIMS, acetone extraction is the preferred method and is performed by adding heat-inactivated serum to cold acetone and decanting the mixture. A drop of goose red blood cells is added to the sera, mixed and removed to eliminate non-specific agglutination of erythrocytes.

The HI assay at AFRIMS laboratory is performed in 18 x 10 v-shaped well Lucite plates that are clearly marked for each serum and dilution. Two-fold serial dilutions of the test sera and standard positive and negative controls are placed into each well using a 0.025 ml loop followed by the addition of 0.025 ml of 8 to 16 HA units of specific dengue serotype antigen and the plates are covered and incubated at 4°C overnight. Ideally, all sera from a single patient should be tested in the same assay. As a screening test, two broadly cross-reactive dengue antigens may be used (DENV-2 and DENV-3) with only slight loss of sensitivity. The following morning the test plates are allowed to reach room temperature and 0.05 ml of an 8% goose red cell stock solution diluted 1:24 in the proper pH buffer is added to each well. The plate is allowed to sit undisturbed for one hour at room temperature and the wells that have or have not agglutinated are recorded. The HI titer is taken as the highest serum dilution that causes complete inhibition of agglutination (Fig 2). The interpretation of dengue HI antibody titer is based on WHO criteria (WHO, 1997). Work by Burke and colleagues suggest a good correlation between HI and PRNT50 in paired specimens collected 7 months apart (Burke *et al,* 1988).

The primary sources of inter-assay variation for the HI assay are: 1) the amount and quality of HA (appropriate amount varies by serotype), 2) pH (varies by serotype), and 3) interoperator variation to interpret agglutination in each well. Sources of assay variation can be reduced by: establishing HA units for stock dengue antigen and periodically checking to ensure that antigen HA activity is not lost, strictly following guidelines on the proper pH for each dengue serotype antigen, running both positive and negative serum controls on each plate, testing paired sera on the same plate, and reading the plate on a white background or a low-light fluorescent light box.

Interpretation of results is relatively straightforward (Table 1). For suspected acute dengue, the timing of the collection of paired sera is important. Sera obtained 7 days or more apart should demonstrate a four-fold rise in titer if the patient was acutely infected with DENV: Serum collected a week apart will often require the patient to be seen in follow-up; a problem for many clinics. If serum is collected less than 7 days apart and a 4-fold rise in titer is not seen; the result must be read out as non-diagnostic. If there is a 4-fold increase and the titer rises to 1:2560 or higher, it indicates an acute secondary flavivirus infection. If the end titer is 1:1280 or less, the interpretation is an acute primary DENV infection. For many patients with a secondary DENV infection, a 4-fold increase will be seen well before 7 days. If a single specimen or paired specimens show a titer of 1:2560 or higher without a 4-fold increase, the interpretation is a recent DENV infection, possibly acute or having occurred during the previous couple of months.

Importantly, the HI test fails to discriminate adequately between the closely related flaviviruses such as dengue, JE, and West Nile. This could produce results that are difficult to interpret in countries where these viruses co-circulate. Despite these limitations, the HI assay is a powerful technique that is still a standard assay for seroprevalence studies as well as in the diagnosis of acute primary and secondary DENV infections. The HI assay is a robust assay that can be performed with minimal laboratory equipment, reagents and expense. Equally important is that the reagents can be made locally and are readily sustainable for the developing dengue diagnostic laboratory.

Table 1.   World Health Organization criteria to interpret dengue hemagglutination inhibition assay results.

Change in antibody titer	Sample interval	Antibody titer at time of convalescence	Interpretation
≥ to a 4-fold rise	Paired sera with ≥7 days of separation.	≤1:1280	Acute primary flavivirus infection.
≥ to a 4-fold rise	With any specimen.	≥1:2560	Acute secondary flavivirus infection.
≥ to a 4-fold rise	Paired sera with <7 days of separation.	≤1:1280	Acute flavivirus infection, indeterminate primary/secondary.
No change in titer	With any specimen.	≥1:2560	Recent secondary flavivirus infection.
No change in titer	Paired sera with ≥7 days of separation.	≤1:1280	Not dengue.
No change in titer	Paired sera with <7 days of separation.	≤1:1280	None
Uncertain	One sera specimen.	≤1:1280	None

Source: WHO, 1997.

Plaque reduction neutralization assay (PRNT)

Dulbecco (1952) described a chick embryo fibroblast plaque assay for several viruses including Western equine encephalitis and Newcastle disease viruses. This technique allowed quantification of viruses *in vitro* as plaque forming units (PFU). In 1959, Henderson and Taylor described a method to detect arbovirus viral plaques in an agar overlay stained with neutral red and demonstrated its utility to measure serum antibody neutralization titers. The standard neutralization test prior to the availability of tissue culture was the suckling or weanling mouse neutralization test, usually performed using a constant serum dilution mixed with log dilutions of virus prior to administration to the test animals and monitoring for illness. The ability of the serum to neutralize was calculated as the log neutralization index. The DENV neutralization assay using suckling mice in a challenge virus resistance assay was adapted to BS-C-1, PS and LLC-MK2 cell lines by Halstead *et al* (1964) and Sukhavachana *et al* (1966).

It was not until 1967 however, that a direct *in vitro* assay to measure DENV neutralizing antibody and DENV identification by serology was developed by Russell *et al* (1967). This assay became known as the dengue plaque reduction neutralization test (PRNT) and utilized prototype dengue viruses, monkey sera controls, LLC-MK2 cell lines and an agar overlay media with neutral red staining. A probit analysis was used to measure plaque reduction as the serum titer required to reduce dengue viral plaques by 50% (PRNT$_{50}$). This technique introduced an efficient and reproducible assay to measure dengue serotype specific neutralizing antibody and became the standard assay to measure dengue antibody.

Variations of this technique were introduced thereafter, such as a micrometabolic inhibition test using BHK-21 cells and a microculture plaque-reduction test utilizing the LLC-MK2 cell line (Sukhavachana *et al*, 1969), microplate cultures using BHK-21 cells (Fujita *et al*, 1975), a focus reduction method using peroxidase-anti-peroxidase staining of BHK-21 cells (Okuno *et al*, 1978), a semi-micro method in LLC-MK2 cells using a 70% plaque reduction criteria (Morens *et al*, 1985a), and a simplified PRNT assay using BHK-21 cells (Morens *et al*, 1985b). These assays are being used to determine serological responses to dengue vaccines

(Jacobs and Young, 2003), in seroepidemiologic studies defining outbreaks of DENV (Halstead *et al,* 2001) and in pathogenesis studies of dengue shock syndrome (Green *et al,* 1999).

The microneutralization assay (Vorndam and Beltran, 2002) is based on the same principle as PRNT; however, instead of counting the number of plaques per well, this assay uses a colorimetric measurement of virus-induced cell lysis to determine the end-point dilution.

Enzyme immunosorbent assays (EIA)

Innis and colleagues in 1989 applied the anti-JE IgM antibody capture EIA of Burke and Nisalak (1982) to dengue and developed a serological assay to diagnose acute dengue in countries where JE and dengue viruses co-circulate (Innis *et al,* 1989). This assay allows the rapid (within 48 hours) diagnosis of acute DENV infection. Equally important, this assay was optimized to distinguish primary from secondary dengue on the basis of IgM/IgG ratios and by running a concomitant IgM capture JE ELISA, eliminating the false positives that might occur from acute JE virus infections. This assay has served as a standard to measure newer assays (Vaughn *et al,* 1999).

The ELISA assay is widely used in the diagnosis of viral pathogens due to the relative ease to set up the assay in a 96-well format, its high degree of reproducibility and for the option to use automatic plate washers and scanners. In the Innis assay (Innis *et al,* 1989), anti-dengue isotypecapture enzyme immunoassays measure the proportion of immunoglobulin isotype reactive with dengue or JE antigen. Briefly, the method uses 96 well plates sensitized overnight with either goat anti-human IgM or IgG antibody. Control and test sera are diluted 1:100 and placed in the wells overnight. IgM or IgG is then captured onto the respective plates and followed by tetravalent dengue antigen (16 HA units each of DENV-1, DENV-2, DENV-3, and 8 HA units of DENV-4) for the dengue EIA or JE antigen (50 HA units) for the JE EIA. This is followed by an anti-flavivirus horseradish peroxidase conjugate with substrate producing a quantitative colorimetric result read by an EIA plate

reader. A binding index (81) is calculated using the optical density (0D) of the test sample minus the OD of the negative control all divided by the OD of the weak positive control minus the OD of the negative control. The 81 multiplied by 100 generates EIA units where ≥40 units is considered positive for the IgM capture assay. The sensitivity of diagnosing acute dengue is 78% on admission sera and 97% in paired sera with a specificity of 100%. The value of this assay in addition to diagnosing acute dengue is in distinguishing between acute dengue versus acute JE. A ratio of anti-dengue IgM to anti-JE IgM of ≥1.0 is typical of acute dengue whereas a ratio ≤1.0 is consistent with acute JE virus infection.

The added value of this assay is that it distinguishes primary from secondary DENV infection. Sera defined as consistent with primary or secondary dengue by HI titers established ratio cut-offs for IgM/IgG where a ratio of IgM to IgG units ≥1.8 is consistent with primary DENV infection and a ratio of <1.8 is consistent with secondary dengue. Dengue EIA, when compared to HI using WHO standards, demonstrated a high coefficient of correlation and agreement rate (Chungue *et al,* 1989; WHO, 1997).

As the format of this assay has become widely used in the diagnosis of acute dengue, it is worthwhile to discuss some of the quality control aspects of this assay and the methods employed to decrease inter and intra-assay variation. The primary sources of inter-assay variation for this assay are: 1) the dilution of the anti-flavivirus IgG enzyme conjugate; 2) duration of the chromogen-substrate reaction; and 3) the plate coating sensitization step (amount of anti-isotype antibody bound). Samples and antigens used in this assay are used in quantities that saturate the capture system therefore the key limiting components of the assay are the bound anti-isotype antibody and the IgG-enzyme conjugate. The amount of bound anti-isotype antibody is affected by the quantity used, the duration of sensitization, and the type of plates used.

Reduction of variation in the sensitization step can be accomplished by consistently using plates

that were used to optimize the assay and to pre-sensitize plates in batches and store at -20°C. Plates can be kept at -20°C for up to one month without significant loss of bound anti-isotype antibody. It is also important to determine the assay dilution of the anti-flavivirus IgGHRP conjugate. This is accomplished by determining the assay dilution that yields an OD at 492 nm of 0.4 (established cut-off for a positive test) when a 1:100 dilution of the weak positive control is used with test sera. Intra-assay variation should be 10% or less. Within one complete assay, all four positive standards should be in the OD range of 0.25 to 0.55. Values above this range result in a decrease in the assay's sensitivity and values below this range will result in a decrease of specificity.

EIAs using cell culture derived dengue antigens and those using dengue monoclonal antibodies rather than patient-derived control serum have been found to be as sensitive and specific as the Innis assay (Lam *et al,* 1987; Kuno *et al,* 1991; Cardosa *et al,* 1992). Additionally, commercial alternatives are now available and compare favorably with the original EIA described by Innis and colleagues (Lam *et al,* 1996; Cuzzubbo *et al,* 1997; Sang *et al,* 1998; Vaughn *et al,* 1999; Wu *et al,* 1999; Cuzzubbo *et al,* 2000; Groen *et al,* 2000; Lam *et al,* 2000). The dengue IgM and IgG capture EIA assay (PanBio Dengue Duo) demonstrated a sensitivity of 99% and specificity of 92% compared to the Innis assay (Vaughn *et al,* 1999).

A variation of the traditional serum based form of the EIA has recently been developed using saliva for the diagnosis of acute infection (Cuzzubbo *et al,* 1998; Artimos de Oliveira *et al,* 1999). Saliva contains both dengue-specific IgM and IgG that can be measured in a traditional IgM capture EIA format. One prospective study using the PanBio Dengue Duo ELISA determined a sensitivity of 92% and a specificity of 100% compared to the serumbased Innis EIA (Cuzzubbo *et al,* 1998).

The dengue IgM/IgG EIA allows high throughput and reagents are commercially available or readily available from reference laboratories. The advantages of the anti-dengue IgM and IgG isotype-capture enzyme immunoassay are its ability to detect elevated levels of IgM in samples collected during the acute period, an interval when most DHF patients are still hospitalized; that is, a single specimen positive for dengue IgM indicates acute dengue. However, it may take until 3 days post-defervescence before all dengue patients test-positive by EIA. Serum contains no inhibitor of the EIA, therefore no pre-treatment is needed; and the assay can be optimized to distinguish between different flaviviruses.

A further advantage of the EIA is its ability to detect IgM in the CSF in cases where encephalitis is suspected or saliva to minimize venipuncture in prospective field studies. Commercially available EIA kits have demonstrated high levels of sensitivity and specificity for acute DENV infection making this diagnostic assay a key tool for the dengue diagnostic laboratory.

Indirect fluorescent antibody (IFA) test

This assay to detect dengue specific IgM and IgG using fluorescent antibody is used primarily in research laboratories. Vathanophas and colleagues developed it in 1973 (Vathanophas *et al,* 1973). The assay utilizes a solid phase (usually dengue virus infected cells that are cold acetone fixed onto slides) that binds human dengue antibody, which in turn is detected by fluorescein-conjugated anti-human antibody. Visible fluorescence seen on the slides constitutes a positive antibody test. Serial dilutions of test serum are used to measure the amount of antibody present as an antibody titer. This method is limited due to its time-intensiveness, subjective reading, reliance on infected cells as the antibody capture agent and lack of automation. The major advantage of this assay is its relative low cost to perform with a few samples (Boonpucknavig *et al,* 1975).

Rapid antibody assays

In 1988, Cardosa and colleagues reported a simplified dengue assay that could be used in less developed laboratories with the potential to be used in the field (Cardosa *et al,* 1988). The dot enzyme immunoassay (DEIA) was more sensitive than the HI test and had the advantage of using a

single serum dilution as a cutoff point to diagnose acute DENV infection. This and similar assays are an extension of the western blot where DENV proteins are transferred onto a solid phase paper or dipstick and upon exposure to DENV-specific antibody and an anti-human antibody detection system, whereby a band appears indicating a serological response to DENV.

These dot enzyme immunoassays (DEIA) or dot-blot tests offer a diagnostic tool that is rapid (usually 4-6 hours or less), able to detect both IgM and IgG dengue antibody and hence acute primary or secondary DENV infection, and require minimal expertise or laboratory equipment (just a centrifuge and water bath). In the dot blot assay of Cardosa and colleagues, strips containing DENV antigen were incubated with human sera which allows the binding of dengue-specific antibody to the DENV antigen on the test strip. A biotinylated anti-human IgG, or IgM, depending on the assay, detected the bound dengue antibody and after a signaling reaction, displayed a color band on the test strip. A positive IgM alone signified an acute primary DENV infection and both a positive IgM and IgG indicated an acute secondary DENV infection.

Another example of this approach was the Dengue Rapid Test developed by PanBio, LTD (Lam and Devine, 1998; Vaughn *et al,* 1998). In less than 7 minutes, this immunochromatographic test detected dengue-specific IgM and IgG antibodies. Compared to standard diagnostic techniques and using hospital admission and discharge sera, this test demonstrated 100% sensitivity to diagnose DENV infection and reliably distinguish primary from secondary DENV. This assay has migrated towards the use of recombinant antigens and a dipstick format (Wu *et al,* 1999; Cuzzubbo *et al,* 2001; Parida *et al,* 2001). Additional rapid assays are being developed and coming to the market (Groen *et al,* 2000). The limitations of rapid antibody-based assays are that specificity is decreased due to the cross-reactive nature of antibody to other flaviviruses such as JE virus and its dependence on the appearance of IgM, which can appear late in the course of infection or be blunted during secondary DENV infections.

VIRUS DETECTION

Intracerebral inoculation of suckling mice

DENV was first isolated in August 1942 during an epidemic of dengue that occurred in the Nagasaki-Sasebo region of Japan (Hotta, 1952). Blood taken from patients within 48 hours after the first rise of temperature were inoculated intracerebrally (IC) into suckling white mice. Infected mice developed debility, tremors, and paralysis occurring in the hind legs. Since this first description, IC inoculation of suckling mice has become a standard method to generate dengue serotype-specific antigen (for description of technique see section on HA and HI assays). For routine viral isolation however, the technique is slow and cumbersome and requires a steady supply of suckling mice. For these reasons IC of suckling mice has been supplanted with more reliable and sensitive methods discussed below.

Mosquito inoculation

Mosquito inoculation of patient sera as a technique to isolate DENV was developed in the 1970's taking advantage of the natural vector of DENV as a biological amplification system (Rosen and Gubler, 1974). A variety of mosquito vectors have been used including *Toxorhynchites splendens*, *Aedes albopictus*, or *Aedes aegypti* (Gubler *et al,* 1979; Rosen and Shroyer, 1985; Rosen *et al,* 1985). *Toxorhynchites splendens* mosquitoes have the advantage of being larger and easier to inoculate with human sera (Rosen and Shroyer, 1985). There is no risk of spreading the virus through these mosquitoes, as *Toxorhynchites splendens* are non-blood feeders.

At the AFRIMS laboratory, *Toxorhynchites splendens* is used by injecting 0.02 µl of human sera intrathoracically into the mosquito. After an incubation period of 10-to-14 days, DENV is detected in the mosquito head using anti-DENV immunofluorescence antibody. If positive, the mosquito body is triturated and inoculated onto C6/36 cells for virus expansion and typing. Isolation rates using this method are higher than direct inoculation onto cell lines with isolation rates nearly equivalent to molecular detection of virus

by RT-PCR (Vaughn *et al,* 2000). The requirement for insectaries restricts the use of this approach.

Cell culture inoculation

A variety of cell lines are permissive for DENV infection and can generally be divided into insect or mammalian cell lines. Insect cell lines in common use for propagating DENV are the mosquito derived cell lines: AP-61 from *Ae. pseudoscutellaris*, C6/36 from *Ae. albopictus* and TRA-284 from *Tx. amboinensis* (Varma *et al,* 1974; Igarashi, 1978; Kuno, 1982). Of the three, C6/36 is the most commonly used due to its ease of use, availability, and minimal background when using a dengue typing EIA or direct fluorescent antibody staining.

Mammalian cells in common use are derived from hamsters, BHK-21, or primates such as LLC-MK2 or Vero. Mammalian cells offer the advantage of cytopathic changes and plaque formation when infected (Yuill *et al,* 1968). Therefore, mammalian cells are commonly used in the plaque reduction neutralization assay. For viral isolation, mosquito cell lines are more sensitive than mammalian cells (Rosen and Gubler, 1974). Isolating virus in insect cells and plaque quantifying a second specimen on mammalian cells can combine use of insect and mammalian cell types.

One approach to quantify DENV by plaque formation on monolayers of mammalian cells in culture is to dilute samples containing virus serially ten-fold and then to inoculate 0.2 ml of each dilution onto duplicate wells of cell monolayers in 6-well plates. After a 1-hour virus adsorption, cells are overlaid with agarose in maintenance medium. After an appropriate incubation period, usually five to six days, plaques are detected by staining the monolayers with neutral red, which stains living cells. Therefore, plaques are visualized as clear areas in a red background. The virus titer, reported as PFU per 0.2 ml is calculated as the average number of virus plaques counted at a given dilution (containing about 30-100 PFU) multiplied by the dilution factor (Putnak *et al,* 1996).

Dengue virus serotype identification

Methods to identify the infecting DENV serotype in the serum of a dengue patient based on the antibody response are limited due to the high degree of antibody cross-reactivity among the DENV serotypes (Kuno *et al,* 1993). While molecular approaches using RT-PCR have evolved significantly in recent years, the standard for DENV serotype identification remains the isolation of the virus by animal, cell culture or mosquito inoculation, further expansion in cell culture, and serotype identification using DENV serotype-specific monoclonal antibodies in an immunofluorescence assay or an antigen capture EIA format (Henchal *et al,* 1983). Common monoclonal antibodies used for serotype identification include 1 F1 for DENV-1, 3H5 for DENV-2, 5D4 for DENV-3 and 1 H 10 for DENV-4 (Kuno *et al,* 1987; Malergue and Chungue, 1995).

Antigen capture EIA for the identification of DENV has been demonstrated to be a simple and reliable technique (Kuno *et al,* 1985). At the AFRIMS laboratory, an antigen capture EIA utilizes virus isolated in *Toxorhynchites splendens* mosquitoes after expansion using C6/36 cells. Immulon U plates are sensitized with goat anti-mouse IgG in each well. Dengue serotype specific mouse monoclonal antibodies (4G2 anti-flavivirus, 1 F1 anti-DENV-1, 3H5 anti-DENV-2, 10C10 anti-DENV-3, 1H10 anti-DENV-4, and 2H2 anti-dengue group) are bound onto the plate followed by capture of the unknown DENV serotype. A colorimetric reaction is formed after the addition of anti-flavivirus-horse-radish-peroxidase and its substrate. DENV serotype specific mouse brained derived antigen (DENV-1 Hawaii, DENV-2 NGC, DENV-3 H87, DENV-4 914669) are used in parallel as positive controls. Optical density (OD) is read at a wavelength of 492 nm and the results are interpreted by comparing with positive and negative controls where positive control OD is always ≥0.20 and a negative control OD is <0.20. Matching the highest OD reading to the positive dengue control serotype identifies the DENV serotype (Kuno *et al,* 1985).

IMMUNOHISTOCHEMISTRY

Immunohistochemistry is the staining of tissue specimens for the presence of specific

proteins. Dengue antigen staining is a powerful technique to diagnose dengue in fatal cases when serology is non-diagnostic and virus isolation is not available or not successful. A number of techniques have been used to detect dengue antigen in tissue specimens to include both direct and indirect fluorescent antibody staining, and enzyme conjugates using peroxidase and phosphatase conjugates (Boonpucknavig *et al,* 1975; Boonpucknavig *et al,* 1976; Boonpucknavig *et al,* 1981; Hall *et al,* 1991).

At the AFRIMS laboratory, tissue specimens are fixed in Millonig's formalin for 2 hours, irradiated in a microwave oven and then embedded in paraffin. Viral antigen is detected by performing a modification of the immunoalkaline phosphatase method as described by Hall *et al* (1991). The staining method of the tissue section involves deparaffinization in absolute alcohol and water, immersion in a 0.05% solution of protease VIII, application of HistoMark Blue® (Kirkegaard and Perry Laboratories: Gaithersburg, MD), and blocking with normal horse serum and bovine serum albumin. The specimen is incubated overnight with polyclonal mouse dengue antibody followed by a secondary biotinylated horse anti-mouse IgG then streptavidin-alkaline phosphatase, AS-B1 phosphate, hexazotized new fuchsin and levamisol, as the chromogenic substrate. The tissue specimen is then counterstained with Mayer's hematoxylin.

GENOME BASED ASSAYS

Hybridization probes and polymerase chain reaction (PCR)

In 1987, Henchal and colleagues described molecular techniques to diagnose acute dengue using slot-blot nucleic acid hybridization with a radiolabeled eDNA probe (Henchal *et al,* 1987). In 1991, reverse transcription (RT) of viral RNA followed by polymerase chain reaction (PCR) allowed the rapid (less than 12 hours) detection of DENV in patient sera. A nested technique allowed for the serotype specific diagnosis of dengue (Henchal *et al,* 1991; Morita *et al,* 1991; Lanciotti *et al,* 1992).

Henchal's slot-blot nucleic acid hybridization technique used a radiolabeled eDNA probe to detect as little as 11 plaque forming units of each of the four DENV serotypes (Henchal *et al,* 1987). Unlike antibody based assays that rely on the appearance of dengue-specific IgM or IgG, molecular techniques offer the advantage of detecting the virus directly, early in the course of a DENV infection. Nucleic acid hybridization is not affected by antibody and the appearance of virus-specified RNA coincided with the detection of antigen in infected cells.

A modification of this technique by Ruiz and colleagues utilized a microplate hybridization method (Ruiz *et al,* 1995). DENV RNA was isolated from serum or tissue samples and immobilized onto wells followed by hybridization with a biotin-labeled eDNA-probe with signal detection by peroxidase conjugation. This assay was found to have a sensitivity of 95% and specificity of 100% for all four DENV serotypes.

Henchal and colleagues developed a universal set of sense and anti-sense oligomeric DNA primers that matched all known DENV sequences (Henchal *et al,* 1991). This RT-PCR was found to be 80% sensitive and 100% specific for acute dengue compared to virus isolation in live mosquitoes. Modifications to this assay by Lanciotti *et al* (1992), using nested RT-PCR techniques have increased sensitivity and reduced assay time to less than 6 hours.

The dengue RT-PCR assay provides a rapid, sensitive, diagnostic tool to detect DENV in patient specimens as well as in the mosquito vector (Chan *et al,* 1994). The primary limitation for patient diagnosis is that DENV viremia occurs early in the course of infection and drops to non-diagnostic levels soon after defervescence (Vaughn *et al,* 2000). Other limitations include the need for a laboratory equipped with a ultra-centrifuge, thermocycler, and electrophoresis equipment. The use of positive and negative controls is essential and strict adherence to specified techniques are required to eliminate cross-contamination with RNA and DNA to produce false positive results. Despite these

limitations, dengue RT-PCR is a powerful tool to diagnose dengue serotype-specific viremia. Newer RT-PCR techniques are being developed that may be more practical for the developing dengue diagnostic laboratory including pocket thermocyclers with gel cartridges containing all the essential reagents that can be used in the field and require minimal technical expertise.

Nucleic acid sequence-based amplification (NASBA)

NASBA is an isothermal RNA amplification method that uses electrochemiluminescence to detect mRNA utilizing the NuclisensTM basic kit and the Nuclisens Reader (Organon Teknika). Unlike RTPCR, which relies on the conversion of RNA into eDNA and then amplification, NASBA directly amplifies RNA using primers and capture probes at isothermal temperatures. NASBA has been successfully used in other pathogens such as malaria, cytomegalovirus and human immunodeficiency virus (Berndt *et al,* 2000; Blok *et al,* 2000; Schoone *et al,* 2000; Witt *et al,* 2000). Recently, NASBA has been applied to the diagnosis of dengue (Wu *et al,* 2001). Using spiked sera, NASBA had a detection threshold of 1 to 10 PFU/ml. When tested against clinical samples, a threshold of 25 PFU/ml was observed, a 100% serotype concordance with viral isolation, and a sensitivity of 98.3% and specificity of 100%. NASBA though preliminary in results, may prove to be a useful diagnostic tool in the early viremic phase of acute DENV infection.

Fluorogenic probe-based 5' exonuclease assay (Taqman)

The fluorogenic probe-based 5' exonuclease assay (Taqman) using the PerkinElmer Applied Biosystems automated sequence detection system 7700 has been successfully used to diagnose and quantify a number of human pathogens including many viruses (Morris *et al,* 1996; Hawrami and Breuer, 1999; Jordens *et al,* 2000; Lanciotti *et al,* 2000; Loeb *et al,* 2000; Schutten *et al,* 2000). This technique is based on the use of a fluorescent-tagged probe that hybridizes with the target eDNA sequence (following the RT step). A fluorescent signal is released through the 5'-3' exonuclease

activity of DNA Taq polymerase (Holland *et al,* 1991). This allows real-time monitoring of the targeted PCR product and, with an internal control, a quantitative measurement. Taqman has been successfully used to detect and quantify DENV infection (Laue *et al,* 1999; Callahan *et al,* 2001; Houng *et al,* 2001; Warrilow *et al,* 2002). Taqman may prove to be a useful technique to rapidly diagnosis DENV infection and in particular to rapidly quantify viremia and its correlate in dengue disease severity.

Detection of dengue virus NS1 antigen using enzyme immunoassay

Alcon *et al* (2002) reported that the NS1 antigen was found circulating from the first day after the onset of fever up to day 9: NS1 levels ranged from 0.04 to 2 µg/ml in acute-phase serum samples (from days 0-to-7), and the level for a convalescent phase serum (day 8 and later) was 0.04 µg/ml. In secondary infection, the NS1 level ranged from 0.01 to 2 µg/ml and was not detectable in convalescent-phase sera (Alcon *et al,* 2002). Shu *et al* (2002) reported data from acute-phase sera with either primary or secondary infection that were in agreement with those of Alcon *et al* (2002). Moreover, their data suggested that the NS1 antigen was detectable during days 1-to-8 of illness (Shu *et al,* 2002).

Dengue genotypes studies

Although PCR is the most sensitive and rapid method for the detection of dengue virus in early stage of disease, classical dengue virus culture has important benefits: Longitudinal collections of dengue virus provide material for many studies, such as studies of pathogenesis, phylogenic characterization, as well as antigenic drift. With further sequencing using dengue serotypes seed cultures one is able to tell the genotypes in each serotype including from different years. Studies that attempt to associate virulence with genotypes clearly play an important role in the selection of parent strains for attenuated vaccines.

Dengue1-4 genotypes (Klungthong, Chonticha, Molecular section, Virology Department, AFRIMS, personnel communication) (Figs 3-6):
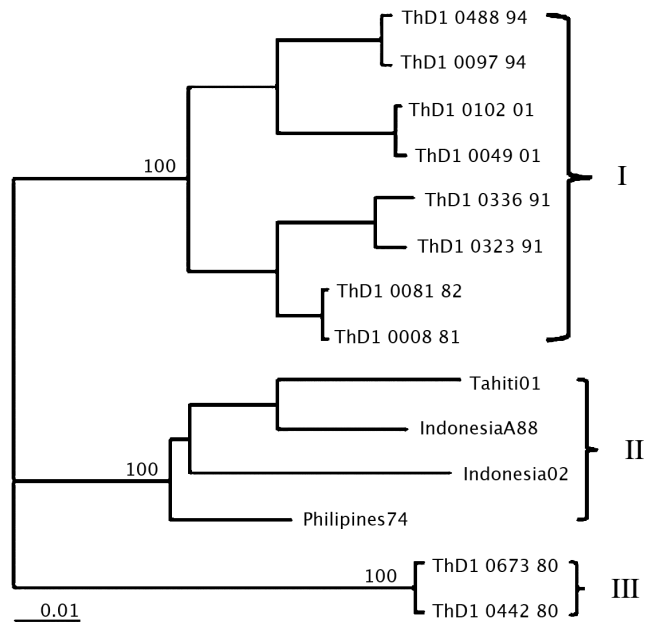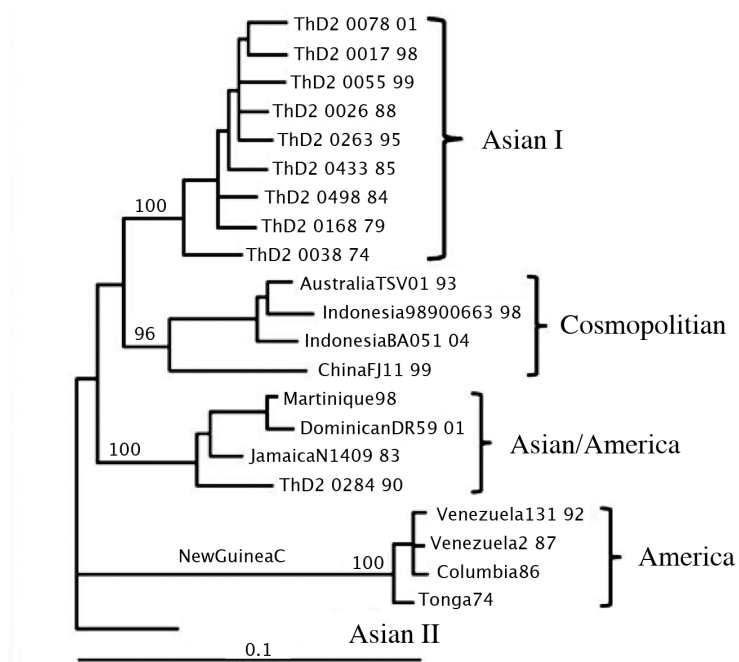
Fig 3–DENV-1 in Thailand.
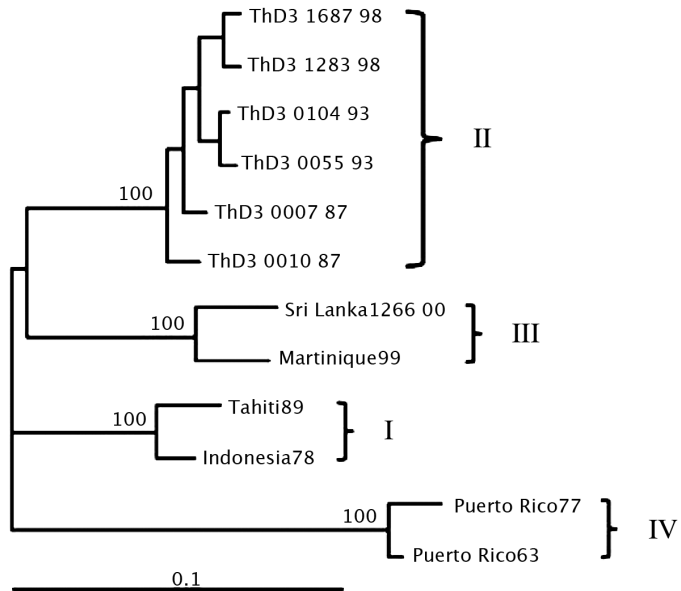

Fig 4–DENV-2 in Thailand.
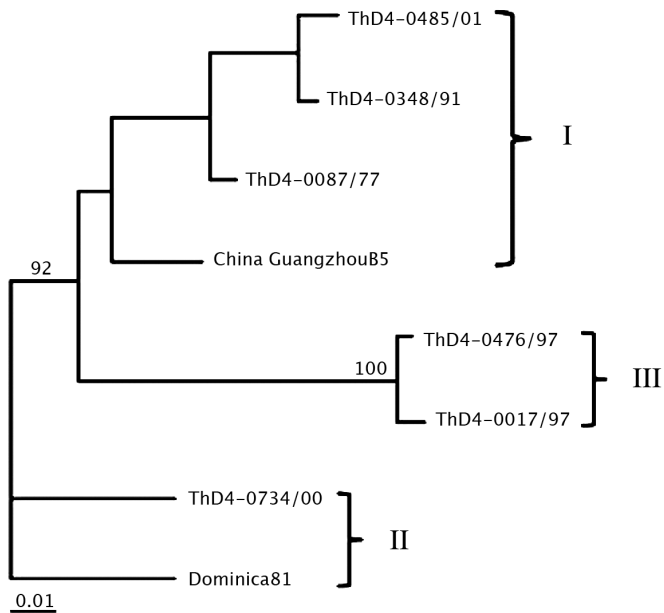
Fig 5–DENV-3 in Thailand.

Fig 6–DENV-4 in Thailand.

DENV-1 in Thailand: Most of the DENV serotype 1 in Thailand is genotype I, circulating since 1980 to the present. A few DENV serotype 1 specimens of genotype III were found during 1980-1983, and then disappeared from circulation.

DENV-2 in Thailand: Most of the DENV serotype 2 in Thailand are Asian I genotype, circulating since 1991 to the present time. A few DENV serotype 2 specimens of Asian/American genotype were found during the 1980 to1990 period and then disappeared from circulation.

DENV-3 in Thailand: Most of the DENV serotype 3 circulating since 1990 to the present are genotype II. Genotype III was also found during 2009 to2010 (This genotype is being investigation until present).

DENV-4 in Thailand: Most of the DENV serotype 4 in Thailand are genotype I, circulating since 1998 to the present. One genotype II was found in the year 2000. A few genotype III specimens were found during the 1997 to2001 timeframe and then disappeared from circulation.

CONCLUSION

Understanding the pattern of immune responses to first or subsequent DENV infections in the context of the clinical illness is essential to identify the appropriate diagnostic tools to diagnose acute DENV infection. Antibody-based assays will not be positive early in the course of disease; patients with suspected dengue should not be sent home believing the fever is not due to dengue. They must be warned about the signs of plasma leakage and appropriately followed. Serotype specific diagnosis is difficult post-defervescence and the limitations of the antibody response in a patient with a previous DENV infection must be taken into account. With these concepts in mind, what would be the ideal assay to diagnose DENV infection? First, the assay must be both sensitive and specific, with a high predictive value despite a low incidence of disease. This will be important in countries where dengue is an emerging disease. It must have low cross-reactivity with other co-circulating flaviviruses such as JE, yellow fever or West Nile virus. The assay must be reproducible with low inter and intra-assay variability and it must be inexpensive so that developing countries with dengue epidemics might be able to utilize the considered diagnostic technique. Likewise the assay must be simple to perform with minimal training and diagnostic equipment. Such an assay would identify serotype-specific dengue antigen during the viremic period and IgM and IgG during the late acute or early convalescent period.

Given these criteria, one can visualize a rapid diagnostic test that will take minutes to perform using a finger-prick of whole blood or saliva placed onto a card with one space for a dengue serotype-specific antigen capture and another for the detection of dengue specific IgM and IgG during the late acute or convalescent period. Future assay will go beyond confirming or refuting dengue as the etiology to distinguish multiple etiologies of fever. Etiologic diagnosis based on gene expression in response to infection needs to be evaluated. These challenges for flavivirologists and commercial companies present an opportunity to aid in the control and treatment of this global health problem.

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Severe Dengue

CHAPTER 4

Clinical features and management of dengue patients

- Childhood dengue diseases: a twenty years prospective study
- Dengue: pitfalls in diagnosis and management
- Role of tourniquet test in dengue patients
- Manifestations of dengue in various age groups of Thai children and adults: a cross-sectional study
- Risk factors for severe dengue
- Dengue in pregnancy
- Maternal and fetal outcomes of dengue infection during pregnancy
- Dengue and the cardiovascular system
- Current management of liver complications in adult dengue infection
- Renal dysfunction in dengue virus infection
- Critical care in dengue management

CHILDHOOD DENGUE DISEASES: A TWENTY YEARS PROSPECTIVE STUDY

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Abstract. Dengue patients admitted to Department of Pediatrics, King Chulalongkorn Memorial Hospital, Thailand during the period of 20 years from 1987-2007 were prospectively studied. All patients were confirmed as having dengue infection by serology and/or virus isolation. The disease was seen all year round with higher incidence during the rainy season. A progressively increase in the number of cases in adolescent age group were seen. For all age groups, fever, anorexia, nausea and vomiting were most common. However seizures, upper respiratory symptoms and diarrhea were more common in children aged less than 1 year. All severity of dengue diseases can be seen in all age groups. Serologically, both primary and secondary dengue infection can be seen in all age groups while primary dengue infection was more common in children aged less than 1 year. The study emphasizes a significant variation of clinical manifestations of dengue diseases in different age groups, suggesting that a successful management must take into account the different age-specific clinical manifestations since early recognition of the disease is one of the key factors for successful treatment.

Keywords: dengue, children, age, clinical manifestations

INTRODUCTION

Dengue, a rapid growing health problem across the globe is the most common arboviral infection of humans transmitted by *Aedes* mosquitoes, principally *Aedes aegypti*. There are four antigenically distinct, closely related serotypes of dengue virus (DEN 1-4), which belong to the genus *Flavivirus* in the family Flaviviridae. A continuum of dengue disease include dengue fever (DF) which causes fever, rash, muscle or joint pain, headache and eye pain; dengue hemorrhagic fever (DHF) causes abnormal hemostasis and increased vascular permeability, with severe cases leading to dengue shock syndrome (DSS). The most common hematological findings include vasculopathy, coagulopathy, and thrombocytopenia. Specific antiviral medications are not available for dengue and successful treat-

ment, which is mainly supportive, depends on early recognition of the disease and careful monitoring for shock (Thisyakorn and Thisyakorn, 2015a). In 2009, the World Health Organization has developed a severity-based revised dengue classification for medical interventions, which has been used in most countries (WHO, 2009).

There is significant variance of clinical manifestations and severity of dengue infection in different age groups such as DF, which have been known in Asia for more than a century are largely age dependent; the disease is mild in children and more severe in adults. Infants and children with DF have symptoms ranging from an undifferentiated fever to a mild febrile illness, sometimes associated with a rash. Older children and adults frequently suffer a more severe form with high fever, pain in various parts of the body, and a maculopapular rash. The infection is only rarely fatal. DSS is more common in children than in adults. Infants with dengue infection present more frequently with convulsions, diarrhea, rash, cyanosis, and splenomegaly while co-morbidities in adults such as peptic ulcers

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disease, preexisting liver disease, which are more likely to be present in adults than in children, can aggravate the disease severity. Thus, proper management of dengue patients must take into account the different age-specific clinical manifestations and severity of dengue disease (Panpitpat *et al*, 2007; Tantawichien, 2015).

Because dengue poses a heavy economic cost to the health system and society, with no specific antiviral medications and successful treatment, which is mainly symptomatic and supportive, depends on early recognition of the disease and careful monitoring for shock. This study aimed at identifying the most prominent clinical manifestations in dengue patients in different age groups in children.

MATERIALS AND METHODS

Clinical data of pediatric dengue patients admitted to King Chulalongkorn Memorial Hospital, Thailand during the period of 20 years from 1987- 2007 were studied. All patients were seen by one or both of the authors and confirmed as having dengue infection by serology and/or virus isolation. The patients were classified into 4 different age groups: 0-1 years, 2-5 years, 6-9 years, 10-15 years. Comparisons of clinical data in different age groups were analyzed. Variables were compared using chi-square test. The level of significant difference was set at a *p*-value <0.05.

RESULTS

A total of 2,090 patients were admitted to King Chulalongkorn Memorial Hospital, a tertiary care hospital in Bangkok, Thailand during 1987 to 2007 with equal number of male and female. One hundred and sixteen cases of which were in the 0-1 year age group (5.6%), 389 cases in the 2-5 years age group (18.6%), 684 cases in the 6-9 years age group (32.7%) and 901 cases in the 10-15 years age group (43.1%) (Fig 1). The disease
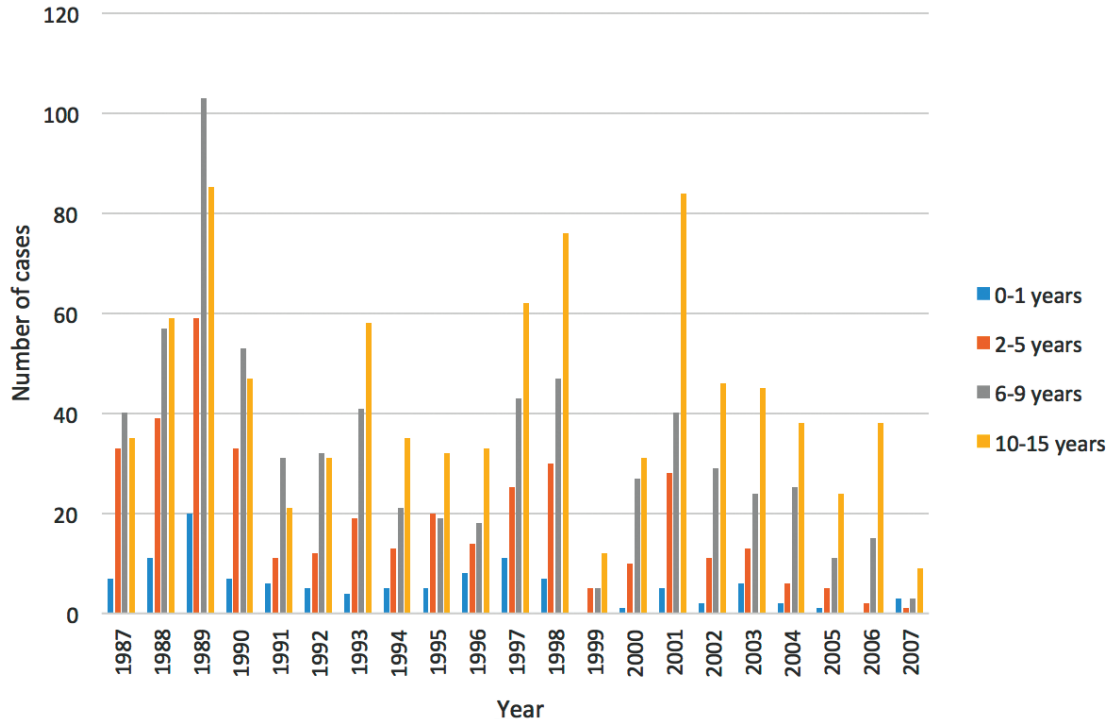
Fig 1–Age distribution of dengue patients in King Chulalongkorn Memorial Hospital between 1987 and 2007.
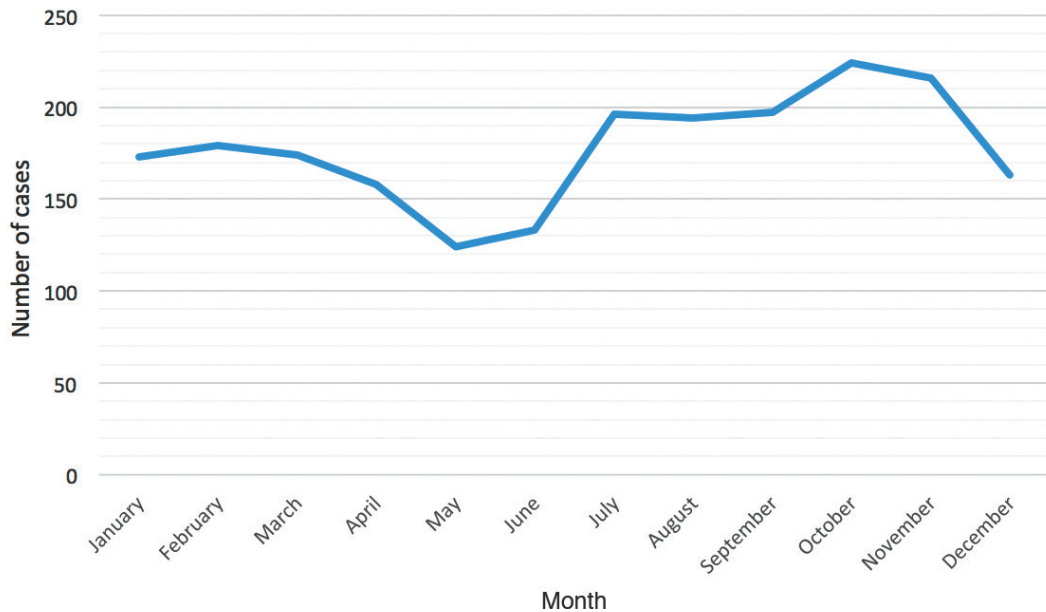
Fig 2–Seasonal distribution of dengue patients in King Chulalongkorn Memorial Hospital between 1987 and 2007.

Table 1. Clinical manifestations of dengue patients in King Chulalongkorn Memorial Hospital between 1987 and 2007.

Clinical manifestation	0-1 years (n=116)	2-5 years (n=389)	6-9 years (n= 684)	10-15 years (n=901)
	n (%)	n (%)	n (%)	n (%)
Fever	116 (100)	389 (100)	684 (100)	901 (100)
Injected conjunctiva	6 (5.17)	35 (9.00)	50 (7.31)	68 (7.55)
Respiratory symptoms	65 (56.03)	186 (47.81)	274 (40.06)	329 (36.51)
Vomiting	51 (43.97)	263 (67.61)	497 (72.66)	635 (70.48)
Abdominal pain	8 (6.90)	167 (42.93)	324 (47.37)	394 (43.73)
Diarrhea	47 (40.52)	68 (17.48)	121 (17.69)	184 (20.42)
Seizures	29 (25.00)	16 (4.11)	9 (1.32)	4 (0.44)
Bleeding	29 (25.00)	96 (24.68)	228 (33.33)	302 (33.52)
Hepatomegaly	98 (84.48)	316 (81.23)	566 (82.75)	619 (68.70)
Splenomegaly	7 (6.03)	5 (1.29)	5 (0.73)	9 (1.00)

was seen all year round with higher incidence during the rainy season from June to October (Fig 2). For all age groups, fever, nausea, anorexia and vomiting were most common. Abdominal pain and vomiting were more common in older children ($p<0.05$). However seizures, upper

respiratory symptoms, diarrhea, hepatomegaly and splenomegaly were more common in children aged less than 1 year ($p<0.05$). Common bleeding sites in all age groups were skin, mucosa, gastrointestinal tract while hypermenorrhea was common in older children (Table 1). All severity of dengue diseases can be seen in all age groups with overall mortality rate of 0.3% (Fig 3). Serologically, both primary and secondary dengue infection can be seen in all age groups while primary dengue infection was more common in children aged less than 1 year (Fig 4). All severity of dengue diseases can be seen in both primary and secondary dengue infection (Fig 5).
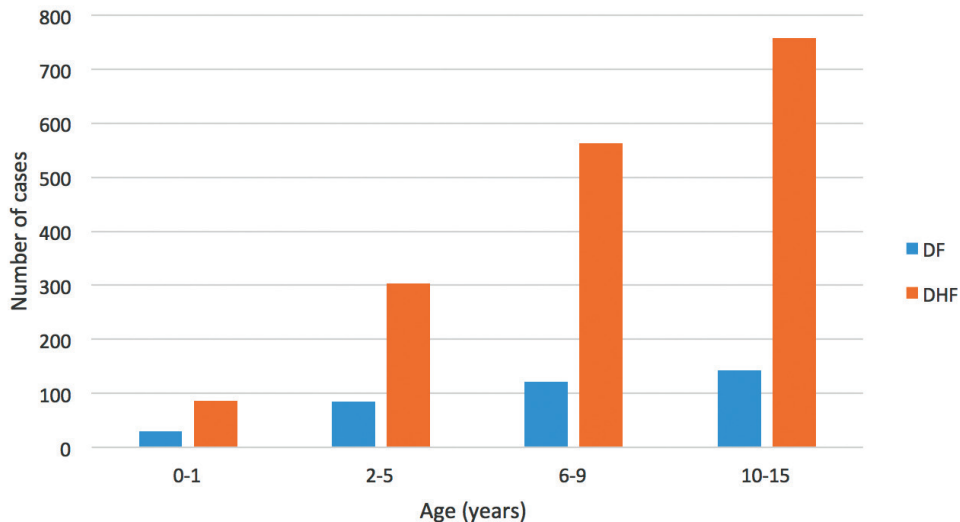

Fig 3–Severity of dengue patients at King Chulalongkorn Memorial Hospital between 1987 and 2007.
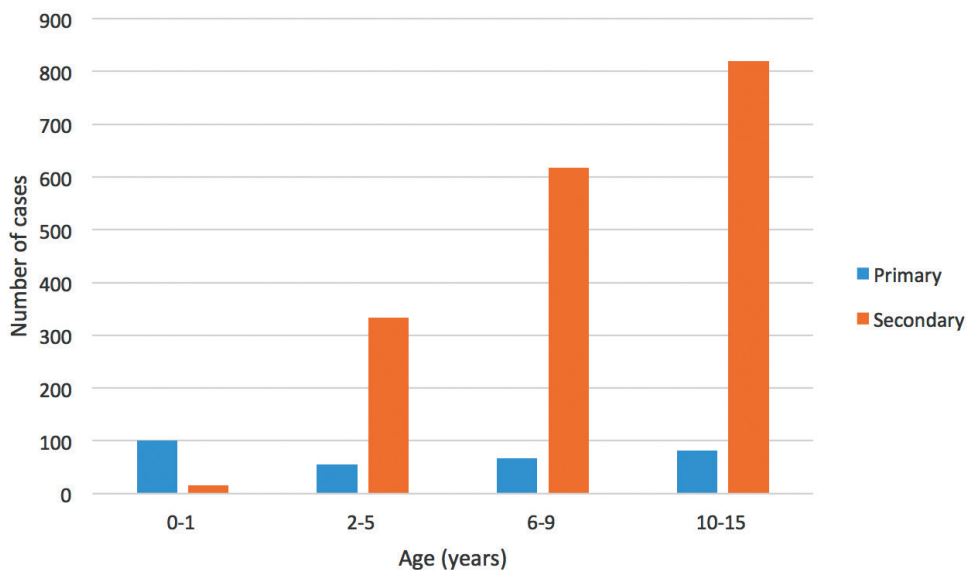

Fig 4–Serological response of dengue patients at King Chulalongkorn Memorial Hospital between 1987 and 2007.
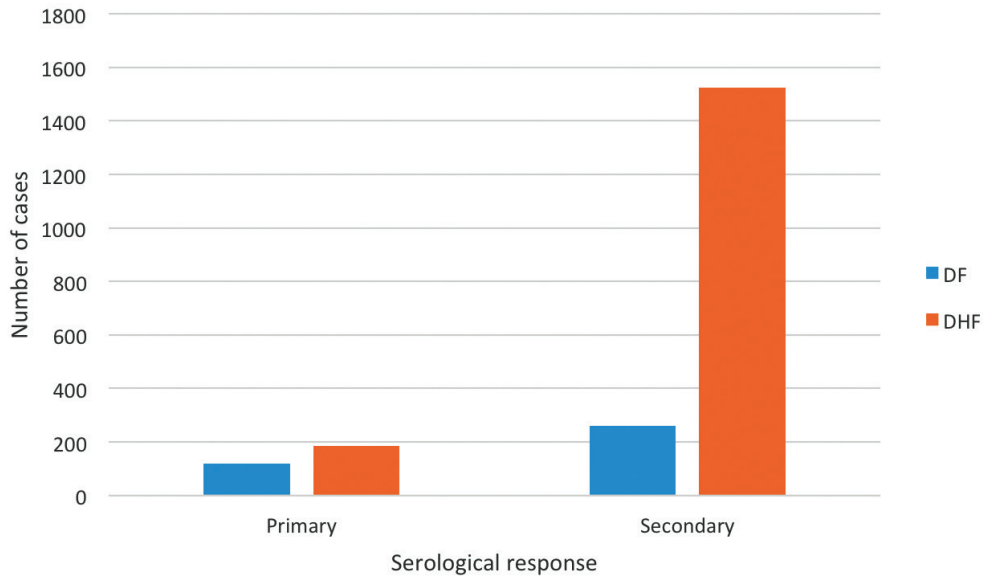
Fig 5–Serological response *vs* dengue severity in dengue patients in King Chulalongkorn Memorial Hospital between 1987 and 2007.

DISCUSSION

Our study shows a progressive shift in age distribution of dengue patients from early childhood to older children during the past decades which is similar to other studies (Tanayapong, 2013; Nunthanid and Tiawilai, 2015). The rising age of dengue patients has been associated with demographic transition, modern housing and commercial development (Kittigul *et al,* 2007). Dengue patients can be seen all year round with a higher incidence in the rainy season, this usually peaks 2-4 weeks after the arrival of the rains which began anytime between June and September. The rainy season in Thailand usually finishes in October, but can last into November (Tanayapong *et al*, 2013). Several clinical characteristics of dengue disease in the early childhood may be different from the older children. Abdominal pain, one of warning signs for severe dengue (WHO, 2009) was more common in older children who are more likely to be able to report their symptoms better than younger children. Seizures, upper respiratory symptoms and diarrhea were more common in children aged less than 1 year. Many infants with dengue infection have severe organ involvement such as central nervous system which may

result in missed or delayed diagnosis. Exhaustive investigations are usually needed in these cases (Thisyakorn and Thisyakorn, 2015b). A higher mortality rate in infants in this study may reflex a severe organopathy which may have happened in this age group. In addition, 25% of infants in this study had seizures which also are more common due to the febrile convulsions in infants. In dengue endemic areas patients with encephalitis and encephalopathy should be investigated for dengue infection whether or not they have other features of dengue disease (Thisyakorn and Thisyakorn, 1994; Thisyakorn *et al*, 1999; Solomon *et al*, 2000).

A severe form of dengue known as DSS was first recognized in outbreaks of dengue in Southeast Asia in the mid-1950s and was initially thought to be previously unknown manifestation of dengue infection. The hypothesis that the disease is more severe if an infection with one dengue serotype follows infection with another serotype was proposed in the mid-1960s to explain an appearance of DSS (Halstead *et al*, 1967). In this study, all severity of dengue diseases can be seen in all age groups no matter they acquired primary or secondary dengue infection. This confirmed the observation that DSS can occur with primary dengue

infection in all age groups and a second dengue infection is not essential for the development of DSS in person of any age. Most infants in this study acquired primary dengue infection and also resulted in all dengue severity. It is now generally agreed that the syndrome was not new but was only more prevalent than in the past (Rosen, 1989).

Specific antiviral medications are not available for dengue and successful treatment, which is mainly supportive, depends on early recognition of the disease and careful monitoring for shock. Laboratory diagnosis includes virus isolation, serology, and detection of dengue ribonucleic acid. Since dengue poses a heavy economic cost to the health system and society, the potential economic benefits are associated with promising dengue prevention interventions such as dengue vaccine and vector control innovations. (Horstick and Ranzinger, 2015; Thisyakorn and Thisyakorn, 2015c).

In summary dengue is one disease entity with different clinical manifestations, often with unpredictable clinical evolutions and outcomes. Clinical profiles show a difference in some aspects across all age groups. Successful treatment which is mainly symptomatic and supportive depends on early recognition of the disease and careful monitoring for the disease severity. Recognition of clinical characteristics in different age groups are essential in early diagnosis of dengue disease.

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DENGUE: PITFALLS IN DIAGNOSIS AND MANAGEMENT

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Abstract. Dengue is a mosquito-borne viral disease, which is currently an important and rapidly growing health problem across the globe. Four closely related dengue serotypes cause the disease, which ranges from asymptomatic infection to undifferentiated fever, dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS). Specific antiviral medications are not available for dengue and successful treatment, which is mainly supportive, depends on early recognition of the disease and careful monitoring for shock. In 2009, the World Health Organization (WHO) developed a severity-based revised dengue classification for medical interventions, which is used in most countries. Laboratory diagnosis includes virus isolation, serology, and detection of dengue ribonucleic acid. Since dengue poses a heavy economic cost to health systems and societies, the potential economic benefits are associated with promising dengue preventive interventions such as dengue vaccines and vector control innovations.

Keywords: dengue, diagnosis, management

INTRODUCTION

Dengue is the most common arboviral infection of humans transmitted by *Aedes* mosquitoes, principally *Aedes aegypt*i. There are four antigenically distinct serotypes of dengue virus (DEN 1–4), which belong to the genus *Flavivirus* in the family Flaviviridae. Global phenomenon such as urbanization and international travel are key factors in facilitating the spread of dengue. The pathogenesis of dengue hemorrhagic fever (DHF) is complex and is the product of host determinants, dengue serotype, and environmental factors.

The continuum of dengue disease includes dengue fever (DF), which causes fever, rash, muscle or joint pain, headache, and eye pain, as well as DHF, which causes abnormal hemostasis and increased vascular permeability with severe cases leading to dengue shock syndrome (DSS). Because the age of dengue patients in several countries has increased from children to adolescents and adults,

the proper diagnosis and management of dengue patients must consider the age-specific variance of clinical manifestations (Thisyakorn and Thisyakorn, 2015a).

CLINICAL FEATURES

There is significant variance in clinical manifestations and severity of dengue infection in different age groups. DF has been known in Asia for more than a century, and its severity is largely age-dependent. This disease is mild in children and more severe in adults. Infants and children with DF have symptoms ranging from an undifferentiated fever to a mild febrile illness, sometimes associated with a rash. Older children and adults frequently suffer a more severe form with high fever, pain in various parts of the body, and a maculopapular rash. However, the infection is only rarely fatal. DSS is more common in children than in adults. Infants with dengue infection present more frequently with convulsions, diarrhea, rash, cyanosis, and splenomegaly while co-morbidities that are more likely to be present in adults than in children such as peptic ulcers and preexisting liver disease can aggravate the disease severity. Thus, proper management of dengue patients must consider the different age-specific clinical manifestations and

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severity of dengue disease (Panpitpat *et al*, 2007; Tantawichien, 2015)

Dengue patients with severe organ involvement such as liver, kidneys, brain, or heart have been increasingly reported. These organopathies may be associated with co-infections, co-morbidities, or complications of prolonged shock. Organopathies can modify clinical presentations of dengue disease and result in missed or delayed diagnosis. Exhaustive investigations are usually needed in these cases. Patients from dengue endemic areas with encephalitis and encephalopathy should be investigated for dengue infection whether or not they have other features of dengue disease.

The clinical spectrum of the infection undermines surveillance activities, particularly because the majority of cases are asymptomatic and remain undetected. A large proportion of infected individuals have a mild form of the disease, which is perceived as insufficiently serious enough to warrant professional care, and this may cause misdiagnosis and underreporting. Over half of the world's population lives in areas at risk of infection. Complex disease presentation and sudden development of hemorrhagic symptoms in a seemingly stable patient can cause fatal outcome even in well-prepared hospitals (Thisyakorn and Thisyakorn, 2015a, b).

An echocardiographic study regarding hemodynamic profiles of DHF patients during the toxic stage shows that the mechanism of decreased cardiac output is complex. Decreased preload is accompanied by decreased left ventricular performance and possibly a subnormal heart rate response in some patients (Khongphatthanayothin *et al*, 2003). Transient myocardial depression is not uncommon in patients with DSS. Cardiac dysfunction in children with DSS may contribute to the clinical severity and the degree of fluid overload in these patients (Khongphatthanayothion *et al*, 2007). Various benign bradyarrhythmias and ectopic beats are detected in dengue patients during the convalescent stage (La-Orkhun *et al*, 2011).

Special consideration for dengue patients during pregnancy are as the following (Royal College of Physicians of Thailand, 2014): Physiologic hemodilution in pregnancy may obscure hemoconcentration in DHF. Differential diagnosis of pregnancy-related conditions should be considered, *eg*, hemolysis, elevated liver enzymes, and thrombocytopenia syndrome (HELLP). There is an increased risk of abortion, premature uterine contraction, intra-partum and post-partum hemorrhage, maternal death, fetal distress, low birth weight, death fetus *in utero*, as well as vertical transmission of dengue virus.

DIAGNOSIS

The incubation period of dengue infection is usually 4-7 days but can range from 3-14 days. Clinical and laboratory criteria for the diagnosis of DHF/DSS as established by the World Health Organization (WHO, 1997) are as follows.

Clinical manifestations:
- Fever: acute onset, high and continuous, lasting 2-to-7 days in most cases.
- Any of the following hemorrhagic manifestations including one positive tourniquet test, petechiae, purpura, ecchymosis, epistaxis, gum bleeding, as well as hematemesis and/or melena.
- Enlargement of the liver is observed at some stage of the illness in 90-98% of children. The frequency varies with time and/or the observer.
- Shock manifested by tachycardia, poor tissue perfusion with weak pulse, as well as narrowed pulse pressure or hypotension with the presence of cold, clammy skin, and/or restlessness.

Laboratory findings:
- Thrombocytopenia (100,000 cells per mm^3 or less).
- Hemoconcentration; a hematocrit increase of more than 20% from the baseline of patient or population of the same age.

A severity-based revised dengue classification for medical interventions has been developed by the WHO and has been adopted in most countries (Fig 1) (WHO, 2009).

Other common laboratory findings are hypoproteinemia, hyponatremia, elevated hepatic enzymes, and elevated blood urea nitrogen level. Metabolic acidosis may be found in patients with prolonged shock. White blood cell count is variable, ranging from leukopenia to mild leukocytosis with an increased percentage of lymphocytes and the presence of atypical forms (Well *et al*, 1980; Thisyakorn *et al*, 1984).

Hematological findings include vasculopathy, reduction of several coagulation factors, reduced platelet count, and platelet dysfunction. Tendency toward bleeding should be monitored in any dengue patient since it may cause severe, uncontrollable hemorrhage. The pathogenesis of bleeding in a dengue patient is not fully understood (Mitrakul and Thisyakorn, 1989). The extent of endothelial cells, coagulation, and fibrinolysis activation in children with dengue infection seems to be correlated with dengue disease severity (Sosothikul *et al*, 2007).

The laboratory diagnosis of dengue infection can be confirmed by serological testing, isolating the virus, and detecting viral RNA by reverse transcriptase polymerase chain reaction. Virologic tests have a high yield in the first few days of illness. However, this yield decreases thereafter, so serological tests should be considered. Commercial kits for rapid dengue diagnostic tests are also available for routine use. However, the sensitivity, specificity, and accuracy vary among these tests. Therefore, these tests are suitable for screening and should be confirmed by the above.

MANAGEMENT

Successful treatment, which is mainly symptomatic and supportive, depends on early recognition of the disease and careful monitoring for shock.
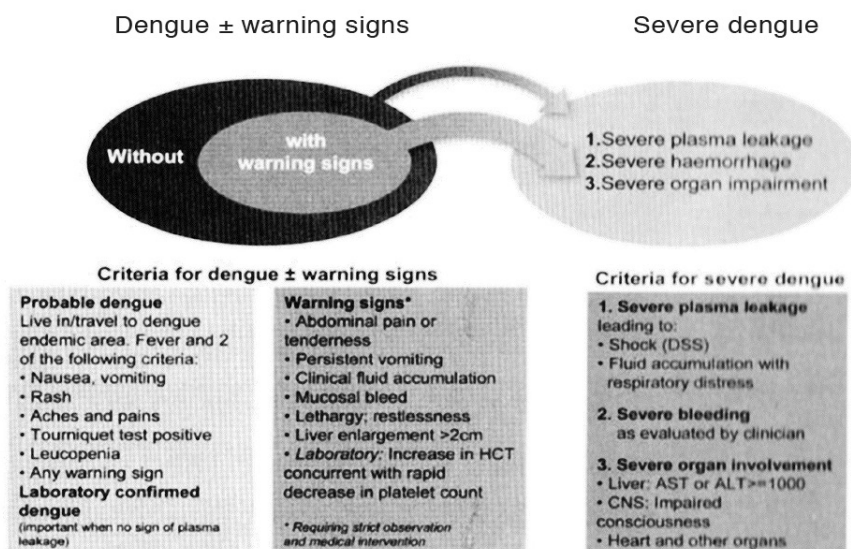

Fig 1–The 2009 WHO dengue case classification (WHO, 2009).

In DHF, early and effective replacement of lost plasma with fluid and electrolyte solutions, plasma, and/or plasma expanders results in a favorable outcome. Blood transfusion is indicated for patients with significant clinical bleeding, mainly from the gastrointestinal tract. Blood components are required when disseminated intravascular coagulation (DIC) causes massive bleeding. Persistent shock despite adequate fluids and a declining hematocrit level suggest significant clinical bleeding requiring prompt treatment. DIC occurs in cases with severe shock and may play an important role in the development of massive bleeding and irreversible shock. Coagulation tests should be monitored in all cases of shock to document the onset and severity of DIC. Blood grouping and matching should be performed as a routine precaution for every shocked patient. The rate of fluid infusion needs to be carefully tailored according to the patient's vital signs, hematocrit, and urine output.

Generally, there is no need for fluid therapy beyond 48 hours after the cessation of shock. Reabsorption of extravasated plasma occurs, which is manifested by a further drop in the hematocrit level. Excessive fluids during the recovery phase may cause hypervolemia, pulmonary edema, or heart failure. An extremely important point is that a drop in the hematocrit level at this stage should not be taken as a sign of internal hemorrhage. A strong pulse and blood pressure with a wide pulse pressure and diuresis indicate good vital signs. They rule out the likelihood of gastrointestinal hemorrhage, which is mostly found during the shock stage (Thisyakorn and Thisyakorn, 1994).

PREVENTION

There is currently no specific antiviral treatment against dengue and successful treatment, which is mainly supportive, depends on early recognition of the disease and careful monitoring for shock. Main public health preventive interventions consist of mosquito control, while a safe and efficacious dengue vaccine is seen as the best hope to fight this disease (Thisyakorn and Thisyakorn, 2015c).

CONCLUSION

Dengue is a disease entity with different clinical manifestations often with unpredictable clinical evolutions and outcomes. Successful treatment of clinical dengue virus infection, which is mainly symptomatic and supportive, depends on early recognition of the disease and careful monitoring of disease severity.

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ROLE OF TOURNIQUET TEST IN DENGUE PATIENTS

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Abstract. The percentage of positive finding in tourniquet test studies in dengue patients has been reported to be between 70-83.9%. However, no data of the positive tourniquet test in relation to days of illness and days before defervescence exist. Thus, our study aimed to evaluate the diagnostic utility of the tourniquet test during the time course of the illness in dengue patients and associations between the tourniquet test result and severity of dengue infection, age, as well as thrombocytopenia. A prospective observational study was conducted in children and adults who were diagnosed as dengue infection hospitalized at King Chulalongkorn Memorial Hospital from December 1997 to December 1998. The tourniquet tests were performed on alternating arms daily until one positive result was obtained. We found the percentage of positive tourniquet tests increased by day of illness. There was no significant difference between tourniquet test result and either shock status or the presence of thrombocytopenia (<100,000/μl). This study reveals that age has no influence on the outcome of the tourniquet test. In conclusion, the tourniquet test is a simple and useful procedure to assist in diagnosis of dengue.

Keywords: dengue infection, tourniquet test

INTRODUCTION

Dengue infection is one of the most important diseases in Thailand and many countries in tropical areas. One of the largest outbreak was in 1987 with the total number of reported cases of 174,285 (Thisyakorn and Thisyakorn, 1994). The clinical features of dengue infection are variable from asymptomatic infection, dengue fever, dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS) (Thisyakorn and Thisyakorn, 1994). Early clinical diagnosis of dengue infection in the first few days of illness can be difficult because the signs and symptoms of dengue patients are similar to many other infectious diseases such as malaria, scrub typhus, influenza, and chikunkunya (Monath, 1995).

The tourniquet test, which is a simple procedure, is one of the most common hemorrhagic phenomenon of DHF and is one of the WHO clinical criteria for DHF diagnosis (WHO, 1997). It was first applied to the dengue diagnosis during the 1928 epidemic in Greece, an outbreak that is now thought to have been an early occurrence of DHF/DSS (Halstead, 1989). A previous study by Nimmannitya, in 1969 showed a percentage of positive tourniquet tests in dengue patient of 83.9% (Nimmannitya, 1997; WHO, 1997), and a study showed the specificity and sensitivity in dengue patients were 90% and 70%, respectively (Tham *et al*, 1996). However, there are no data that show the percentage of positive tourniquet tests by days of illness or days before defervescence.

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We, therefore, designed a prospective cohort study to evaluate the suitable time period for the diagnostic utility of the tourniquet test in dengue patients and to seek associations between positive tourniquet test result and shock status, the presence of thrombocytopenia and age.

MATERIALS AND METHODS

Study subjects

Eligible patients admitted to King Chulalongkorn Memorial Hospital at both adult and child wards. The inclusion criteria were patients who presented with fever equal to or more than 37.5°C or history of fever and having clinical suspicions of dengue infection. All the patients needed to have serologic confirmation of acute dengue infection by either enzymes immunosorbent assay (EIA) or hemagglutination inhibition (HI) test positive (WHO, 1997). Exclusion criteria were patient with fever from other infections, a contraindication to perform the tourniquet test, and intolerance of pain caused by tourniquet test.

Protocol

Tourniquet testing at admission was performed by inflating a blood pressure cuff on the upper arm mid-way between systolic and diastolic blood pressure for 5 minutes, followed by examination of a 2.5 cm^2 area using a standard template. The number of petechiae up to 20 within the template was counted. The tourniquet test was performed by the study investigators on alternating arms daily until the test became positive (WHO, 1997).

Definitions

We defined the first day of illness as the first day of fever and days were counted consecutively afterward. We defined day before defervescence as the number of days before the body temperature was less than 37.5°C. We used standard clinical grading of WHO criteria for the clinical definition of DHF cases (WHO, 1997).

Statistical analysis

The percentage of positive tourniquet test was shown by mean. We used chi-square analysis and Fisher's exact test for statistical analysis to find any associations between tourniquet test and shock status, presence of thrombocytopenia, as well as age. A p-value <0.05 was considered to be significant.

RESULTS

Sixty-six cases were enrolled: 19 adults and 47 children. The overall male to female ratio was 1:1 (males=33, females=33). The mean age (range) was 13.76 years (7 months-66 years). In the first six days of illness, the percentage of positive tourniquet tests increased rapidly. After that, there was only a slight increase. The overall positive tourniquet test percentage was 78.8% (Fig 1).
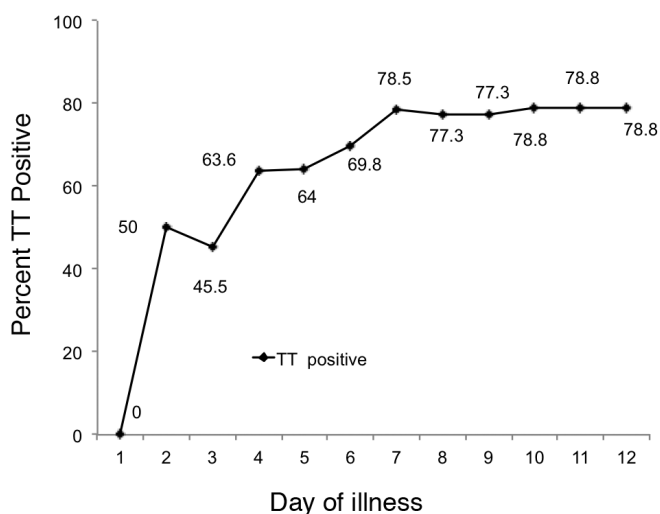
Fig 1–Percentage of positive tourniquet test in dengue patients by days of illness.

Comparing positive tourniquet test between children and adults indicated that the percentage of positive tourniquet tests in children were much higher than in adults in the first 4 days of illness (Fig 2). After that, the percentages were similar.

In addition, there were no significant associations between tourniquet test results and dengue infection severity (p=0.44), age (child or adult) (p=0.55) and thrombocytopenia (platelets <100 x 10^9/l) (p=0.77) (Table 1).

DISCUSSION

Our study was the first study to show the percentages of positive tourniquet test following the day of illness. After day 2 of illness, the percentage of positive tourniquet test markedly increased until the end of first of week. This may imply the suitable time period of diagnostic utility of tourniquet test in dengue infection. We found the percentage of positive tourniquet tests increased by day of illness and day before defervescence. Presence of shock, age, and thrombocytopenia had no significant difference on result of tourniquet test.

The diagnostic role of tourniquet test in dengue infection is still controversial. Recent studies have shown the low sensitivity of tourniquet test in diagnosis dengue infection. A meta-analysis (Grande *et al*, 2016) of 28,739 patients showed the pooled sensitivity and specificity for dengue
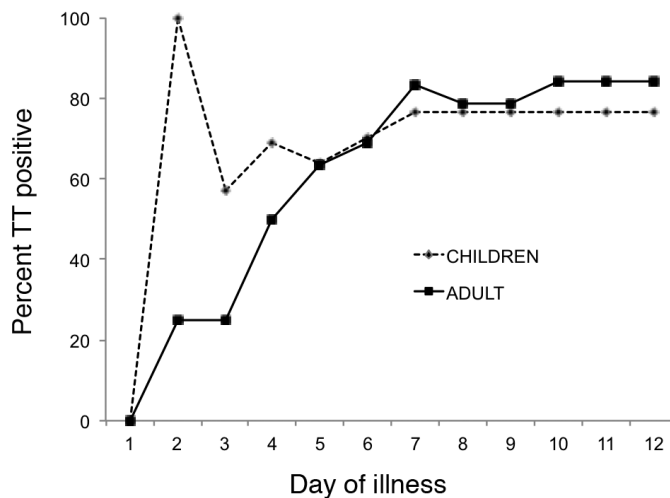
Fig 2- Percentage of positive tourniquet test between adults and children by days of illness.

Table 1. Comparison of percentage of positive tourniquet test between clinical severity, age, and platelet count.

Patient's status	Positive tourniquet test (%)	*p*-value
Shock *vs* non-shock	87.5 *vs* 77.5	NS
Children *vs* adult	76.7 *vs* 84.2	NS
Thrombocytopenia *vs* non-thrombocytopenia	75 *vs* 66	NS

NS, not significant.

diagnosis by tourniquet tests were only 58% and 71%, respectively. Of note, this study was mainly driven by retrospective study from Brazil (Antunes *et al*, 2013), *N* = 9,836, which did not mention time of tourniquet tests were performed.

Another study by Furlan *et al* (2016) of 28,000 dengue patients, found that tourniquet test had a sensitivity of only 11.9%. Again, this study did not mention time of tourniquet test and frequency of test. However, Cavailler *et al* (2016) tested the diagnostic utility of positive tourniquet test in predicting dengue infection and found the odds ratio at 2.17 (95% CI: 1.32-3.59). A study from Puerto Rico has shown the utility benefit of the combination of tourniquet test and white blood of cell count in differentiation of dengue infection from other acute febrile illnesses (Gregory *et al*, 2011). This mean that we should not use only one clinical test but use clinical combination to diagnose dengue infections.

Our study showed the presence of thrombocytopenia had no significant association to the result of the tourniquet test. The positive tourniquet test in dengue infection is not only from the presence of thrombocytopenia but also from other hemostatic factors such as vasculopathy and coagulopathy (Mitrakul, 1987). This means that a tourniquet test can be performed even when the platelet count is higher than 100,000/l.

This study, however, did not collect the daily complete blood count, so we selected the number of platelet counts by in groups when the tourniquet test was positive, we used the maximum number of platelet counts on the first day of positive test. In groups when the tourniquet test was negative, we used the minimum number of platelet counts on the last day of admission.

This study had some strengths. First, we used standard serology (EIA, HI) tests to confirm dengue infection in every case. Second, we have serially performed tourniquet tests until the test was positive, not only single time points. This will provide a better picture of tourniquet test during the time course of dengue infection.

Our study also had some limitations. We did not have the control group and could not demonstrate the sensitivity and specificity of tourniquet test in diagnosing dengue infection. However, as mention earlier, our study aimed to demonstrate the suitable time period to perform a tourniquet test, not the diagnostic accuracy. We also accept that this study was not designed to reveal the potential contributing factors to the result of tourniquet test. Therefore, a better study design to find out those contributing factors is needed.

In summary, we have shown the tourniquet test, a simple bedside procedure, provided a good diagnostic utility during the first week of dengue infection and should be combined with other clinical parameters to help diagnosis dengue suspected cases.

ACKNOWLEDGEMENTS

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MANIFESTATIONS OF DENGUE IN VARIOUS AGE GROUPS OF THAI CHILDREN AND ADULTS: A CROSS SECTIONAL STUDY

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Abstract. Dengue is the most common mosquito-borne virus causing disease in many countries. The disease, caused by dengue serotypes 1 to 4, ranges from asymptomatic infection, undifferentiated fever, dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS). Early recognition of the disease is one of the key factors for successful treatment, and is only possible if the diagnostician knows the patient-specific clinical manifestations. We studied the clinical manifestations in dengue patients to determine the most common manifestations of dengue infection by age group. Clinical data of dengue patients admitted to Photharam Hospital, Ratchaburi, Thailand during 2005-2015 were analyzed. The patients were classified into eight different age groups: 0-1 years, 2-5 years, 6-9 years, 10-14 years, 15-18 years, 19-29 years, 30-59 years, and ≥60 years. For all age groups, fever, anorexia, nausea, and vomiting were most common. However, seizures and diarrhea were more common in children aged <1 year. In regards to dengue severity, DHF was more common in older children and young adults while DF was more common in younger children and ageing adults. Our study emphasizes the significant variance of clinical manifestations in different age groups, suggesting that proper management must consider the different age-specific clinical manifestations.

Keywords: dengue, manifestations, age groups, children, adults

INTRODUCTION

Dengue infection, a mosquito-borne viral disease of humans, is now a significant problem in many countries. It can present itself in many forms, ranging from asymptomatic infection, undifferentiated fever, dengue fever (DF) to severe dengue hemorrhagic fever (DHF) with or without shock. DHF is characterized by fever, bleeding diathesis, and the possibility of developing a potentially fatal shock syndrome. The most common hematological findings include vasculopathy, coagulopathy, and thrombocytopenia (Thisyakorn and Thisyakorn, 2015a). In 2009, the World Health Organization has developed a severity-based revised dengue classification for medical interventions, which has been used in most countries (WHO, 2009).

Laboratory diagnosis includes virus isolation, serology, and detection of dengue ribonucleic acid. Successful treatment depends on early recognition of the disease and careful monitoring for shock. There are currently no licensed antivirals to treat dengue. The use of safe and efficacious dengue vaccine along with vector control innovations will lead to successful prevention and control of dengue (Thisyakorn and Thisyakorn, 2015a).

In the past decades, a trend of increasing age in dengue patients has been evidented. Moreover, clinical presentations and laboratory findings of dengue infections may be different in each age group (Kittigul *et al*, 2007; de Souza *et al*, 2013; Tantawichien, 2015). Thus, this study analyzes the magnitude of clinical manifestation variation among dengue patients of different ages.

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MATERIALS AND METHODS

Study design

This descriptive study was conducted in Photharam Hospital, Ratchaburi, Thailand during 2005- 2015.

Study subjects

The medical charts of all hospitalized patients with dengue infection were reviewed. Dengue patients were divided into eight different age groups: 0-1 years, 2-5 years, 6-9 years, 10-14 years, 15-18 years, 19-29 years, 30-59 years, and ≥60 years. Diagnosis of dengue infection adhered to clinical and laboratory criteria for the diagnosis of DHF as established by the World Health Organization (WHO, 1997) as follows:

Clinical criteria:
1. Acute fever: high, continuous, lasting for 2-7 days.
2. Hemorrhagic manifestations such as a positive tourniquet test, petechiae, purpura, ecchymosis, epistaxis, bleeding gums, hematemesis, or melena.
3. Hepatomegaly.

4. Shock – a rapid, weak pulse with a narrow pulse pressure; hypotension with cold, clammy skin, and restlessness.

Laboratory criteria:
1. Thrombocytopenia (platelet count < 100,000/mm^3).
2. Hemoconcentration (hematocrit increased from baseline by > 20%).

In patients with DHF grade I, a positive tourniquet test is the only hemorrhagic manifestation whereas in DHF grade II spontaneous bleeding occurs. Patients with circulatory failure (a narrowing of the pulse pressure and a rapid and weak pulse) have DHF grade III. Patients in profound shock (no detectable blood pressure and pulse) have DHF Grade IV. DHF III and IV are also referred to as DSS.

Data collection and analysis

Data obtained from the medical records were age, sex, date of hospitalization, severity of disease, clinical manifestations, laboratory findings, complications, and outcome. Descriptive data were analyzed using mean, range, and percentage. Variables were compared using chi-square test.

Fig 1–Seasonal distribution of dengue patients in Photharam Hospital, Ratchaburi, Thailand between 2005 and 2015.

The level of significance was set at *p*-value <0.05.

RESULTS

During the period of study, we analyzed 2,676 cases, of which there were 37 cases in the 0-1 year age group (1.38%), 156 cases in the 2-5 years age group (5.83%), 375 cases in the 6-9 years age group (14.01%), 622 cases in the 10-14 years age group (23.24%), 406 cases in the 15-18 years age group (15.17%), 614 cases in the 19-29 years age group (22.95%), 435 cases in the 30-59 years age group (16.26%), and 31 cases in ≥60 years age group (1.16%).

The gender composition comprised of 1,297 males and 1,379 females. The disease was seen all year round with higher incidence in the rainy season, which begins in June and usually lasts until October (Fig 1). Fig 2 shows the age distribution of dengue patients during the study period.

The most common symptoms across age groups were fever, anorexia, nausea, and vomiting. Respiratory symptoms, diarrhea, and seizures were more common in children aged <1 year (*p*<0.05). However, older children and adults presented more commonly with headache and myalgia. Gastrointestinal bleeding and hypermenorrhea were more common in older children and adults (Table 1).

Complete blood counts in teenagers and adults showed that the maximal hematocrit (Hct) was significantly higher whereas the minimal white blood cell (WBC) count was significantly lower. In contrast, the maximal Hct in infants was significantly lower whereas the WBC count was significantly higher (Table 2).

Patients were diagnosed with either dengue DF, DHF, or DSS. All severities of dengue diseases were seen in all age groups (Fig 3). The overall mortality rate was 0.11%.

DISCUSSION

Our study showed that several manifestations of dengue patients in young children are prominent. They presented significantly more frequently with upper respiratory symptoms and convulsion than the older age groups. Skin bleeding was also more pronounced than bleeding of the mucous membrane or gastrointestinal tract in the infant group.

In the older age groups, we detected five leading manifestations including fever, vomiting, anorexia, abdominal pain, and headache, all
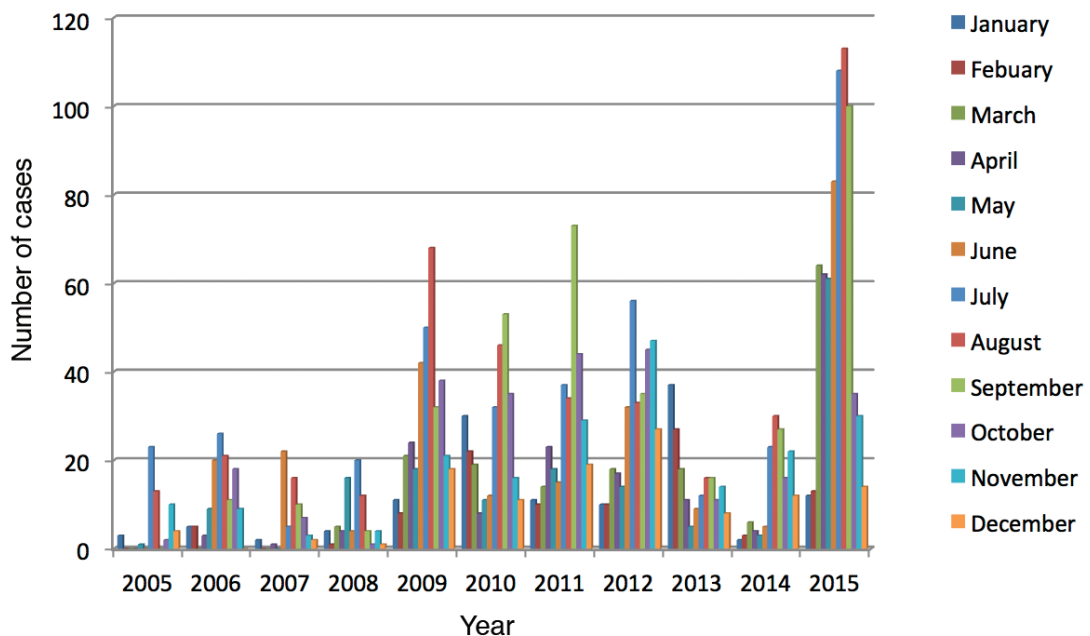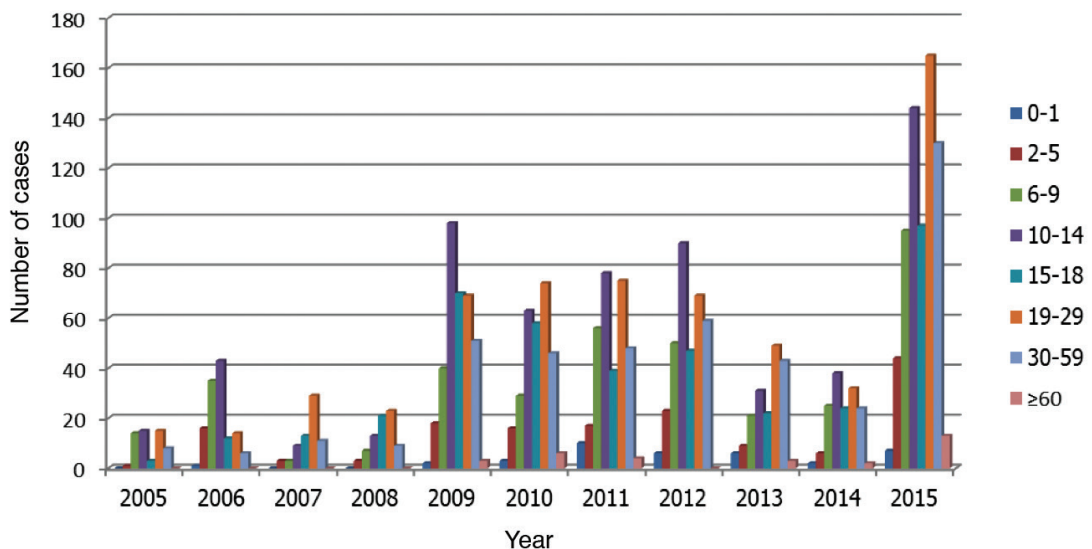

Fig 2–Age distribution of dengue patients in Photharam Hospital, Ratchaburi, Thailand between 2005 and 2015.

Table 1. Clinical manifestations of dengue patients in Photharam Hospital, Ratchaburi, Thailand between 2005 and 2015.

Clinical manifestations	0-1 yrs (n=37) n (%)	2-5 yrs (n=156) n (%)	6-9 yrs (n=375) n (%)	10-14 yrs (n=622) n (%)	15-18 yrs (n=406) n (%)	19-29 yrs (n=614) n (%)	30-59 yrs (n=435) n (%)	≥60 yrs (n=31) n (%)
Headache	1 (2.70)	31 (19.87)	152 (40.53)	316 (50.80)	239 (58.87)	368 (59.93)	258 (59.31)	12 (38.71)
Respiratory symptoms	23 (62.16)	69 (44.23)	160 (42.67)	215 (34.57)	124 (30.54)	156 (25.41)	103 (23.68)	10 (32.26)
Anorexia	14 (37.84)	76 (48.72)	208 (55.47)	325 (52.25)	176 (43.35)	244 (39.74)	168 (38.62)	11 (35.48)
Nausea	6 (16.22)	61 (39.10)	16 (4.27)	291 (46.78)	230 (56.65)	355 (57.82)	262 (60.23)	14 (45.16)
Vomiting	15 (40.54)	80 (51.28)	198 (52.80)	329 (52.89)	203 (50.00)	303 (49.35)	211 (48.51)	11 (35.48)
Abdominal pain	1 (2.70)	33 (21.15)	125 (33.33)	191 (30.71)	113 (27.83)	174 (28.34)	121 (27.82)	7 (22.58)
Diarrhea	14 (37.84)	24 (15.38)	49 (13.07)	95 (15.27)	67 (16.50)	77 (12.54)	45 (10.34)	1 (3.23)
Seizures	6 (16.22)	2 (1.28)	1 (0.27)	6 (0.96)	1 (0.25)	1 (0.16)	1 (0.23)	0 (0.00)
Hepatomegaly	2 (5.41)	9 (5.77)	29 (7.73)	43 (6.91)	8 (1.97)	18 (2.93)	5 (1.15)	0 (0.00)
Splenomegaly	0 (0.00)	1 (0.64)	1 (0.27)	2 (0.32)	2 (0.49)	1 (0.16)	0 (0.00)	0 (0.00)
Site of bleeding								
Skin	2 (5.41)	1 (0.64)	1 (0.27)	2 (0.32)	4 (0.99)	5 (0.81)	17 (3.91)	1 (3.23)
Gastrointestinal tract	0 (0.00)	3 (1.92)	5 (1.33)	25 (4.02)	7 (1.72)	16 (2.61)	12 (2.76)	0 (0.00)
Mucous membrane	0 (0.00)	13 (8.33)	43 (11.47)	70 (11.25)	23 (5.67)	47 (7.65)	27 (6.21)	2 (6.45)
Hypermenorrhea	0 (0.00)	0 (0.00)	0 (0.00)	36 (5.79)	1 (0.25)	59 (9.61)	32 (7.36)	0 (0.00)

Table 2. Mean values of complete blood count of dengue patients in Photharam Hospital, Ratchaburi, Thailand between 2005 and 2015.

Complete blood count	0-1 yrs n=37	2-5 yrs n=156	6-9 yrs n=375	10-14 yrs n=622	15-18 yrs n=406	19-29 yrs n=614	30-59 yrs n=435)	≥60 yrs n=31
Hct max (%)	35.75	37.99	39.39	42.17	43.14	43.09	42.13	39.8
Hct min (%)	31.56	34.14	36.76	37.77	38.23	38.29	37.57	35.78
WBC min (/mm³)	6,828.65	3,896.36	3,237.85	2,717.59	2,795.61	3,392.76	3,595.05	3,180.65
Plt min (/mm³)	61,351.35	88,802.55	85,781.79	75,469.00	64,126.23	57,984.08	60,701.23	67,645.16

Hct, hematocrit; WBC, white blood cell; Plt, platelets.
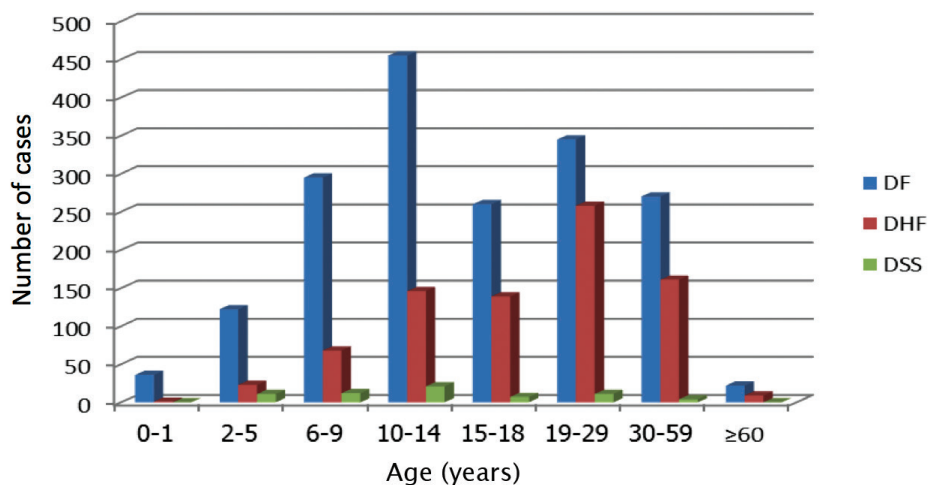
Fig 3–Severity of dengue patients by age group in Photharam Hospital, Ratchaburi, Thailand between 2005 and 2015.

of which are in agreement with those from a previous report that identified the same leading manifestations (Tantawichien, 2015). The older age groups may have more commonly self-reported headache, abdominal pain, and myalgia than children because the older age groups may have been better able to explain their symptoms than young children.

Hypermenorrhea with clinical significance was seen in older girls and young women with dengue infections. The menstrual period was essential in all girls and women who presented with clinical manifestations compatible with dengue infections since hormonal therapy to stop the bleeding may be necessary.

Normal infants tend to have a higher WBC count as their baseline levels whereas older children and adults tend to have higher Hct level and a lower WBC count (Walters and Abelson, 1996). In our study, this may explain why infants with dengue infection had higher profiles on WBC count in comparison to the other age groups, and also why dengue-infected teenagers and adults had significantly higher average values of Hct and lower average values of WBC count. Moreover, the severity of plasma leakage may be higher in older children, resulting in higher Hct levels observed in our study.

A previous study on the dynamics of WBCs in dengue patients demonstrated that the number

and types of WBCs changed dynamically with dates of illnesses (Wells *et al,* 1980). Studying the dynamics of these cells in different age groups of dengue-infected children may help to explain the difference in complete blood counts between different age groups.

Our study showed that all severities of dengue diseases could be seen in all age groups. In infants, the diagnostician should not exclude the possibilities of dengue in the infant who presents with high fever, respiratory problems, and central nervous system manifestations. Those central nervous system manifestations are possibly due to febrile convulsions, encephalopathy, and dengue encephalitis (Thisyakorn and Thisyakorn, 2015b).

Early recognition of dengue patients is essential to arrive at an accurate diagnosis and provide prompt treatment. This study emphasizes the prominently different ways that dengue infections can manifest in different age groups, and also shows that age is probably a major determinant of the clinical manifestations that are most likely in a dengue-infected patient.

In condusion, dengue is one disease entity with different clinical manifestations often with unpredictable clinical outcomes. Successful treatment, which is mainly symptomatic and supportive, depends on the early recognition of the disease and careful monitoring of the disease severity. Proper management must consider the different age-specific clinical manifestations.

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RISK FACTORS FOR SEVERE DENGUE

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Abstract. Dengue viral infection (DVI) is a common cause of morbidity and mortality in both children and adults in tropical countries. Severe dengue is defined as dengue patients with dengue shock syndrome (DSS) and/or organ failure/dysfunction. The mortality rate of severe DVI found in various studies varies from 0.5-13%, depending on the severity of disease in the enrolled patients, the experience of the medical team, age group, and underlying disease(s). In this narrative review, we describe and discuss the risk factors and outcomes of severe DVI in children and adults. In both children and adults, DSS leading to organ failure has a poor outcome with a high mortality rate of 60-70%; severe hepatitis and/or coagulopathy are commonly found in patients with severe bleeding; and patients with non-DSS-caused organ failure have a good prognosis. Obese children are at higher risk of developing severe DVI and subsequent organ failure, notably acute kidney injury (AKI) and acute liver failure (ALF). In adults, severe DVI is caused not only by DSS, but can also result from co-bacteremia and/or underlying diseases. Children rarely have these comorbidities, and adults are thus more vulnerable to organ dysfunction. To reduce the number of DVI patients progressing to severe DVI, early detection of patients at risk of developing severe DVI is important. During the febrile stage, dengue patients should be carefully checked daily. If they have any of the warning signs or risk factors of severe DVI, they should be hospitalized with close monitoring.

Keywords: dengue hemorrhagic fever, dengue, organ failure, risk factors, severe dengue, shock syndrome

DEFINITION OF SEVERE DENGUE VIRAL INFECTION

The definition of severe dengue viral infection (DVI) according to WHO 2009 is a dengue patient who has dengue shock syndrome (DSS), including those with signs of impending shock or dengue hemorrhagic fever grade III (DHF Gr III) and profound shock (DHF Gr IV), fluid accumulation with respiratory distress, severe bleeding and severe organ involvement such as acute respiratory failure (ARF), acute liver failure (ALF) or transaminase enzymes >1,000 units/l, acute kidney injury (AKI), and impaired consciousness (WHO, 2009).

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ARF is defined by severe hypoxemia requiring a mechanical ventilator. Hematologic failure is defined by severe bleeding requiring packed red cells and/or other blood components to control. AKI is defined by a sudden increase in serum creatinine (Cr) level >2 mg/dl or a serum Cr concentration >2 times previous or subsequent values and that is also higher than the upper limit of normal values for the patient's age (Chan *et al*, 2002). ALF is defined as the rapid development of severe acute liver injury with impaired synthetic function (INR ≥1.5 or PT >15 seconds) and encephalopathy or INR ≥2.0 or PT >20 seconds irrespective of hepatic encephalopathy in patients with no history of liver disease (Suchy, 2016).

COMPLICATIONS OF SEVERE DENGUE VIRAL INFECTION

Severe bleeding

Endothelium injury leading to consumptive thrombocytopenia and coagulopathy as well as

increased fibrinolysis, and acute liver injury causing coagulopathy and disseminated intravascular coagulation play a major role in hemorrhage in DVI. Mild mucosal bleeding is common in DVI. However, severe bleeding is not common and can be fatal if uncontrolled. The most common severe bleeding sites leading to death are the upper gastrointestinal tract, the lower respiratory tract, and within the skull (Lee *et al*, 2012; Fariz-Safhan *et al*, 2014; Laoprasopwattana *et al*, 2014).

In an earlier unpublished study from our institution on DVI in children, profound shock, platelets <20,000/mm³ and INR ≥1.5 were the major risk factors of severe bleeding [odds ratio (OR) = 3.4; 95% CI: 1.4-8.6, 2.6; 95%CI: 1.1-6.2], and 10.6; 95%CI: 4.0-28.4, respectively] (Laoprasopwattana *et al*, 2017). Similarly, a study of DVI in adults also found that platelets <20,000/mm³ and/or prothrombin time (PT) prolongation were associated with severe bleeding (Lee *et al*, 2012).

Our study also found that INR had a high correlation with transaminase enzymes (Laoprasopwattana *et al*, 2017). This finding is supported by a recent study in adults in which an increase of aspartate aminotransaminase was associated with an increased risk of severe bleeding (OR = 1.008; 95% CI: 1.005-1.01) (Fariz-Safhan *et al*, 2014). Taken together, these various factors suggest that acute liver injury plays a key role in hemorrhage in DVI patients by causing decreased synthesis and increased consumption of coagulation factors. These subsequently cause prolonged PT and prolonged activated partial thromboplastin time (aPTT).

Acute liver failure

Currently, the most common infectious disease causing ALF in Thailand is DVI (Poovorawan *et al*, 2006). The liver is the target organ of the dengue virus, and mildly elevated transaminase levels are common in patients with DVI with levels of transaminase enzymes usually returning to <200 U/l in 2 weeks (Laoprasopwattana *et al*, 2016). ALF is rare (<1%) in DVI, but severe complications do occur in some patients. These are associated with high fatality rates, especially high-grade hepatic encephalopathy, which has a fatality rate of 50.0-66.7% (Poovorawan *et al*, 2006; Chongsrisawat *et al*, 2009; Laoprasopwattana *et al*, 2016).

Profound shock is a major risk factor of ALF and most DSS-caused ALF patients develop ALF within 48 hours after onset of shock (Laoprasopwattana *et al*, 2016). This suggests that the major cause of ALF in DVI is ischemic hepatic injury. ALF may also result from the immune system through a process in which the dengue virus stimulates Fas ligand formation on hepatocytes, causing cell apoptosis by immune-mediated hepatocytic injury (Pagliari *et al*, 2014).

Acute kidney injury

AKI is rare although mild serum creatinine elevation is common in DHF. The major cause of AKI in both children and adults is DSS (Lee *et al*, 2009; Laoprasopwattana *et al*, 2010). The reported major causes of non-DSS AKI are rhabdomyolysis, acute hemolysis, and direct kidney injury from dengue virus. Immune complex-mediated acute glomerulonephritis, sepsis, or nephrotoxic medications can cause AKI too. Non-DSS-caused AKI has a good prognosis with a very low mortality rate. AKI itself does not increase the risk of fatality in DVI in patients who must have renal replacement therapy, but profound shock subsequently causing respiratory failure and massive bleeding are the major causes of fatal DVI (Laoprasopwattana *et al*, 2010).

AKI was found in 0.9% (25/2,893) of hospitalized children with DVI with a high mortality rate of 64.0%. The main risk factors of AKI were profound shock and obesity (OR 16.9; 95% CI: 4.2-68.5 and OR = 6.3; 95%CI: 1.4-28.8, respectively). Most DSS-caused AKI patients developed AKI within 24 hours after onset of shock. Fatality was more likely in children with profound shock, oliguric AKI, respiratory failure, or prolongation of PT or aPTT of more than twice that of the reference specimen. Among the survivors, none had chronic kidney disease, and serum creatinine levels returned to normal in 1 to 48 days (mean 32 days) (Laoprasopwattana *et al*, 2010).

Lee *et al* (2009) found an AKI rate of 3.3% (10/304) in adult patients hospitalized with DHF with a mortality rate of 60.0%. The higher proportion

of AKI in adults can be explained by the other risk factors of adults that increase their vulnerability to AKI during renal hypoperfusion such as older age, having an underlying disease(s) such as hypertension, diabetes mellitus and chronic kidney disease, as well as/or co-bacterial infection (Table 1).

Fatal outcome

DVI fatality rates generally depend on various factors such as age group or severity of DVI, availability of intensive medical care, and the experience of the medical team. The overall fatality rate of DVI varies from 0.2-2% in tropical countries (WHO, 2009).

Both adult and child fatal DVI patients have a higher proportion of severe DVI at first presentation and higher median leukocyte counts than those who survive the disease (Thein *et al*, 2013; Laoprasopwattana *et al*, 2014). Most fatal cases in children have DSS at presentation while more than half of fatal cases in adults have an underlying disease or diseases, making them prone to unfavorable outcomes (Table 2). The higher leukocyte count in fatal DVI patients could be explained by the high levels of inflammatory cytokines and stress hormones, and concurrent bacterial infection if present. Although documented the numbers of

bacteremia cases in the two studies are low (Thein *et al*, 2013; Laoprasopwattana *et al*, 2014) (Table 2), empirical treatment with antibiotics is common in treating DVI patients with multiple organ failure. This is because these patients are more vulnerable to nosocomial infection.

The DVI children at highest risk of death are those with DHF grade IV who subsequently have multiple organ failure. Our previous study of 238 children with severe DVI found that 30 of these patients subsequently died. Organ failure occurred in nearly one-third of the severe DVI patients. Organ failure, notably ARF, AKI, and ALF, are accompanied by severe bleeding, occurring in 16-18% of severe DVI patients with a high mortality rate of 60-70% (Table 3). We found that patients with both ARF and severe bleeding had an 82% fatality rate, but if they did not have these two risk factors, the chance to survive was 99% (Laoprasopwattana *et al*, 2014).

The causes of fatal DVI in adult patients are not only DSS and subsequent multiple organ failure, but also concurrent or secondary bacteremia and underlying diseases contributing to death (Wang *et al*, 2007; Leo *et al*, 2011; Lee *et al*, 2012; Thein *et al*, 2013). Wang *et al* (2007) found that of 11

Table 1.   Risk factors of acute kidney injury in children and adults hospitalized with DVI.

Risk factor	Laoprasopwattana *et al*, 2010[a]		Lee *et al*, 2009	
	AKI (*n*=25)	No AKI (*n*=50)	AKI (*n*=10)	No AKI (*n*=294)
Age median (range), years	10.0 (0.5-13.1)	10.0 (0.3-13.0)	69.5 (33-78)[b]	55.0 (19-88)
Chronic renal disease, *n* (%)	0.0	0.0	2 (20.0)[b]	9 (5.7)
DSS *n* (%)	19 (76.0)[b]	10 (20.0)	8 (80)[b]	10 (3.4)
Severe bleeding, *n* (%)	21 (84.0)[b]	6 (12.0)	8 (80)[b]	45 (15.3)
Bacteremia, *n* (%)[c]	1 (4.0)	0.0	3/7 (42.8)[b]	3/67 (4.5)

AKI, acute kidney injury; DSS, dengue shock syndrome.
[a]To determine the risk factors of AKI, children who had DHF-caused AKI were matched with patients with DHF by age at a ratio of 1:2.
[b]Significant when comparing children and adults with AKI (*p* <0.05).
[c]Significantly higher in AKI than non-AKI groups (*p* <0.05).

Table 2. Risk factors contributing to fatal outcome in children and adults.

Risk factor	Laoprasopwattana et al, 2014		Leo et al, 2012 Thein et al, 2013	
	Died (n =30)	Survived (n =208)	Died (n =28)	Survived (n =80)
Age range, years	0.5 -13	0.3-15	21-86	21-86
Underlying disease(s)[a]				
Cardiac disorder, n (%)	0	0	10 (47.6)[b]	7 (8.8)
Renal disorder, n (%)	1 (3.3)	0	8 (40)[b]	2 (2.5)
At presentation				
Severe dengue, n (%)	25 (83.3)[b]	121 (59.2)	22 (78.6)[b]	8 (10)
DHF gr. I-II, n (%)	5 (16.7)	87 (41.8)	5 (17.9)	6 (7.5)
DSS[#], n (%)	25 (83.3)[b]	121 (59.2)	2 (7.1)	0
Bacteremia/meningitis, n (%)	1 (3.3)	0	4 (14.3)	NA
Leukocyte count (10^9/l)[c]	18.8[b] (10.4-24.7)	6.6 (4.6-10.3)	8.8[b] (2.8-31.7)	2.5 (1.1-7.6)

DHF, dengue hemorrhagic fever; DSS, dengue shock syndrome; NA, not applicable.
[a]Significant when comparing children and adults with fatal outcome ($p <0.05$).
[b]Significantly higher in non-survival than survival group ($p <0.05$).
[c]In children, the highest leukocyte count; in adults, leukocyte count at presentation.

Table 3. Mortality rate of severe DVI and organ failure in children and adults.

Study	Patient population characteristics	n of fatal outcomes/n of condition (%)	
Laoprasopwattana et al, 2014	238 severe DVI Thai children aged <15 y with DSS 228 (95.8%); referred n=70	Severe bleeding ARF ALF AKI Severe DVI	28/44 (63.6) 30/44 (68.1) 28/41 (63.6) 23/39 (58.9) 30/238 (12.6)
Lam et al, 2013	1,719 DSS Vietnamese children aged <15 years	DSS	8 (0.5)
Lee et al, 2009	304 hospitalized Taiwanese adults >18 years	AKI	6/10 (60)
Wang et al, 2007	661 hospitalized Taiwanese adults >18 years	ARF	8/11 (72.7)

AKI, acute kidney injury; ALF, acute liver failure; ARF, acute respiratory failure; DSS, dengue shock syndrome; DVI, dengue viral infection.

dengue patients with ARF, severe bleeding, AKI, and bacteremia were found in 8, 7, and 6 patients, respectively. The major causes of ARF were sepsis (*n*=6) and upper gastrointestinal tract bleeding (*n*=3), and the mortality rate of ARF was 72.7%.

PREVENTION OF SEVERE DVI

The severity of DVI is associated with the degree of endothelial cell injury, leading to plasma leakage and bleeding disorders. During the febrile period, early differentiation of DVI from other acute febrile illnesses and then monitoring for any warning signs of impending severe DVI are important.

Warning signs of severe DVI include persistent vomiting, abdominal pain/tenderness, hepato-megaly, fluid accumulation, lethargy/ restlessness, mucosal bleeding, and increased hematocrit with rapid decrease in platelets (WHO, 2009). Patients with any warning signs of severe DVI or with any risk factors of unfavorable outcomes such as obesity, having an underlying disease(s) prone to causing organ failure/dysfunction, and old age should be hospitalized for close monitoring and have appropriate treatment readily available to prevent progression to severe DVI and minimize the risk of a fatal outcome.

In summary, DSS is the major cause of severe DVI in children. However, DSS and underlying disease(s) that tend to cause organ failure are the major causes of severe DVI in adults. Profound shock is the major cause of organ failure due to poor tissue perfusion and hypoxia in both children and adults. Obese children are at higher risk to develop AKI or ALF while adults with co-infection with bacteremia and/or an underlying disease(s) that makes them vulnerable to organ dysfunction are at risk of developing severe DVI.

Patients with severe hepatitis/ALF are also prone to develop severe bleeding. Multiple organ failure, especially involving ARF and severe bleeding, is associated with a high risk of death in both children and adults. Early detection of DVI patients who have warning signs of severe DVI is essential. These patients should be closely monitored, so early treatment can be applied to prevent severe

DVI and a fatal outcome.

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DENGUE IN PREGNANCY

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Abstract. Pregnant women living in an endemic area or travel to tropical countries have a risk of dengue infection. When compared to non-pregnant women, pregnant women are at increased risk of dengue hemorrhagic fever (DHF), dengue shock syndrome (DSS), and death. Dengue diseases during pregnancy are found to be associated with a higher risk of cesarean delivery, preeclampsia, preterm birth, and low birthweight infant. Vertical transmission of the virus causing symptomatic diseases in the newborn has been reported. Physiologic changes during pregnancy and some obstetric complications may mask or resemble clinical or laboratory features of dengue leading to misdiagnosis and delayed treatment. Early recognition of dengue during pregnancy and its consequences as well as prompt management would result in a better outcome. In endemic countries, dengue infection should be considered in pregnant women presenting with febrile illness. Hospitalization, close clinical and laboratory monitoring and fluid therapy are recommended in the management of dengue in pregnancy. Vaccination among women in reproductive age in endemic countries prior to pregnancy may be an effective method to prevent dengue disease during pregnancy.

Keywords: dengue, pregnancy, severe dengue

INTRODUCTION

Dengue is a systemic infection caused by dengue virus. It is a major public health problem in more than 100 endemic countries, including Thailand. Globally, this mosquito-borne viral infection was estimated to cause 58.4 million annual symptomatic cases and about 10,000 deaths per year (Stanaway *et al*, 2016). Poorly planned urbanization providing an environment for *Aedes* mosquito proliferation and international travel facilitate the global spread of dengue virus in the recent decades (Messina *et al*, 2014). The virus has four immunologically distinct serotypes, and infection by any of the four serotypes results in a wide range of clinical manifestations from asymptomatic to severe and life-threatening dengue shock syndrome (DSS) (Guzman *et al*, 2016). Disease severity is largely associated with the host immune response. The phenomenon called antibody-dependent enhancement promotes clinically severe dengue, especially in individuals with monotypic immunity during secondary heterotypic infection.

Reports on dengue disease in Thailand between 2000 and 2011 demonstrated a shift in age group predominance of dengue disease from younger towards older individuals over 15 years of age (Limkittikul *et al*, 2014). During 2003-2013, the number of symptomatic dengue infections among the Thai population was reported to be between 20,000 and 80,000 cases each year with a total of 782 deaths (Kaewnorkkao *et al*, 2015). The mortality had the highest rate in cases complicated with DSS and was associated with age more than 15 years old. Unusual bleeding, ageing, co-morbidity, and waning immunity are related to an increased risk of the complications of dengue disease and fatality among adult and elderly patients (Tanta-wichien, 2015).

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Pregnant women living in an endemic area or traveling to tropical countries are also at risk to acquire a dengue infection. Dengue seroprevalence that represents evidence of previous dengue infection among Thai pregnant women has been reported to be between 94.7% and 97.3% (Watanaveeradej *et al*, 2003; Perret *et al*, 2005; Pengsaa *et al*, 2006; Khamim *et al*, 2015). Interestingly, more than 90% of the women had past infection with more than one serotype (Perret *et al*, 2005; Khamim *et al*, 2015). A hospital-based prospective study (Chansamouth *et al*, 2016) conducted in two central hospitals in Vientiane, Lao PDR found that dengue was the most common infection among pregnant women hospitalized due to fever. Severe diseases and poor pregnancy outcomes were reported in these cases.

This narrative review presents updated evidences on the effect of pregnancy on dengue infection as well as on the impact of the disease on pregnancy outcomes. The clinical management of dengue during pregnancy is described.

EFFECT OF PREGNANCY ON DENGUE

The clinical manifestations and outcome of dengue in pregnant women were found to be similar to those of non-pregnant women (Waduge *et al*, 2006). However, a more recent study demonstrated that pregnant women were at increased risk of developing severe dengue [dengue hemorrhagic fever (DHF) and DSS] and mortality when compared with non-pregnant women (Machado *et al*, 2013). Advanced gestational age was associated with severe dengue. Women in the third trimester of pregnancy were four times more likely to have DHF/DSS when compared to those in the first trimester [odds ratios (OR) = 3.98; 95%CI: 1.36-12.65].

The overlapping clinical or laboratory features of dengue with both physiologic change during pregnancy and some more common obstetric complications may cause misdiagnosis, delayed diagnosis, or delayed treatment. For example, an increase in blood volume leading to a lower baseline hematocrit level during the second-half of pregnancy may cause plasma leakage unrecognized by clinicians. When a pregnant woman has thrombocytopenia as well as increased liver enzymes, most obstetricians will consider HELLP (hemolysis, elevated liver enzymes, and low platelets) syndrome, an entity of preeclampsia, rather than DHF. For terminating HELLP syndrome, cesarean section may need to be performed, and this can lead to massive hemorrhage in a misdiagnosed case of DHF. For early recognition of dengue disease in pregnancy, clinicians need awareness of clinical and laboratory manifestations of dengue when dealing with the pregnant woman who presents with fever (Waduge *et al*, 2006; WHO, 2012).

IMPACT OF DENGUE ON PREGNANCY OUTCOMES

A systematic review of 30 published observational studies, mostly case reports or case series, found that the women with dengue infection during pregnancy had high rates of cesarean delivery, preeclampsia, and preterm birth (Pouliot *et al*, 2010). In addition, a higher risk of low birthweight among the infants born to women with dengue infections during pregnancy was demonstrated.

Increasing risks of preterm birth and low birthweight among infants born to women with symptomatic dengue infection during pregnancy were also reported in a retrospective cohort study (Friedman *et al*, 2014). The adjusted OR of preterm birth and low birthweight were 3.34 (95%CI: 1.13-9.89) and 2.23 (95%CI: 1.01-4.90), respectively.

A recent systematic review (Paixao *et al*, 2016) identified and included 16 studies that were case-control, cohort, cross-sectional studies, or unselected case series with a total of 6,071 pregnant women including 292 cases who were exposed to dengue during pregnancy. Similar to the prior reports, the most common adverse pregnancy outcomes for women with dengue infection during pregnancy were preterm birth and low birthweight. The meta-analysis showed that women with clinical dengue symptoms had an increased risk of preterm birth (OR = 2.50; 95%

CI: 1.44-4.34) and low birthweight (OR = 1.84; 95 %CI: 1.04-3.25). Likewise, dengue infection during pregnancy was found to be associated with a higher risk of miscarriage (OR = 3.51; 95% CI: 1.15-10.77).

Vertical transmission of dengue virus during *in utero* or at parturition has been reported in the literature (Tan *et al*, 2008; Pouliot *et al*, 2010). The rate of transmission is inconclusive; however, the risk of vertical transmission is potentially related to the severity and timing of maternal infection (Pouliot *et al*, 2010). Maternal infection with symptoms during the peripartum period was found to increase risk of vertical transmission and symptomatic disease in the newborn (Pouliot *et al*, 2010; Arragain *et al*, 2016). Time interval from maternal symptoms to neonatal onset of fever was reported to be between 5 to 13 days (mean 7 days) (Pouliot *et al*, 2010). Most of the newborns who had vertically-transmitted dengue infections exhibited symptoms. Common clinical manifestations included fever, thrombocytopenia, and hepatomegaly. Documented complications in the infected newborns included pleural effusion, hemorrhage, circulatory failure, and death (Pouliot *et al*, 2010).

MANAGEMENT OF DENGUE DURING PREGNANCY

As previously mentioned, one of the keys to success in the management of dengue during pregnancy is early recognition of the infection and its consequences. A high index of suspicion of dengue is needed when clinicians deal with pregnant women presenting with acute febrile illness who live in or with recent travel to an dengue-endemic area (WHO, 2012). Women are probable to be dengue fever when they have a febrile illness together with two or more of the following manifestations (WHO, 2012; RCPT, 2015):

- nausea, vomiting;
- rash;
- aches and pains: headache, retro-orbital pain, myalgia, arthralgia;
- hemorrhagic manifestations: petechiae, epistaxis, positive tourniquet test; and

- leukopenia, neutropenia, and atypical lymphocyte findings.

Laboratory testing to confirm the diagnosis includes the detection of dengue virus genomic sequences by PCR or demonstration of dengue virus antigen by non-specific protein of dengue virus (dengue NS1) or serological tests. Detection of serum anti-dengue antibody (anti-DEN) IgM or a rise in anti-DEN IgG titer of ≥2 times and convalescent IgG ≥100 units are considered as having recent dengue infection (RCPT, 2015).

After dengue fever is suspected and diagnosed, the pregnant woman should be admitted to the hospital early for close monitoring and supportive management (WHO, 2012). Acetaminophen (10 mg/kg every six hours, maximum 3 g/day) is recommended to reduce the fever and pains, but the patient should be informed that these symptoms might not be alleviated. In the woman using baby aspirin for prevention of preeclampsia, the drug should be withheld. Other NSAIDs and anti-platelet agents should also be avoided. Fluid and electrolyte balance has to be monitored and maintained. If the woman cannot tolerated oral fluid replacement, intravenous isotonic solutions should be started in the same manner as a non-pregnant woman. However, excessive fluid replacement should be avoided (WHO, 2012).

During admission, the woman should be monitored for the warning signs of severe dengue infection that lead to severe plasma leakage, severe hemorrhage, and severe organ impairment (WHO, 2012; Tantawichien, 2015). The warning signs include abdominal pain, persistent vomiting, clinical fluid accumulation (oliguria with respiratory distress), spontaneous mucosal bleeding (epistaxis, bleeding per gum), retinal hemorrhage, alteration of consciousness (either lethargy or restlessness), liver enlargement and tenderness, as well as hemoconcentration with rapid decrease in platelet count. Judicious volume replacement by intravenous fluid therapy in the patient with these warning signs or signs of dehydration may modify the course and the severity of disease (WHO, 2012). Parameters

that should be monitored include vital signs (1-4 hourly), urine output (4-6 hourly), hematocrit (6-12 hourly), blood glucose, renal function tests, liver function tests, and coagulation profile. Fetal monitoring is needed in the late second or third trimester of pregnancy. Maternal monitoring for detection of DHF/DSS should be performed until the woman is out of the critical phase. In the convalescent phase, the patient appetite returns, and their general condition improves. Laboratory findings show the platelet count and hematocrit both returning to baseline level.

There is no indication for termination of pregnancy in a pregnant woman with dengue infection. In some instances, administration of tocolytic agents may be considered in the pregnant woman who is in labor during the critical phase of dengue disease (WHO, 2012). If delivery is inevitable, blood and blood products should be prepared, and the delivery should take place in a hospital where skilled obstetricians and neonatologists are available. Platelet transfusion is indicated when the platelet count is <50,000/mm^3 during labor (RCPT, 2015) and should be initiated during or at delivery (WHO, 2012). Transfusion of packed red cells should be administered if indicated. Interventions to prevent postpartum hemorrhage should be commenced immediately after delivery. The newborn whose mothers had dengue just before or at delivery should be closely observed to determine whether vertically transmitted dengue infection is apparent (Pouliot *et al*, 2010; Arragain *et al*, 2016).

PREVENTIVE MEASURES

Vector control has been used for prevention and control dengue in most endemic countries for decades. However, the global dengue burden and spreading of both the virus and the mosquito have demonstrated the failure of this preventive measure (Guzman *et al*, 2016). Development of safe and effective dengue vaccines might be the most effective means to control dengue disease (Thisyakorn and Thisyakorn, 2015). To date, a live attenuated (recombinant) tetravalent vaccine (CYD-TDV) has been registered in several countries (WHO, 2016). To maximize the public health impact

and cost-effectiveness, the populations targeted for vaccination should have a seroprevalence of dengue of approximately 70% or greater (WHO, 2016). Because the registered dengue vaccine is a live attenuated vaccine, use in pregnant women is not recommended. Vaccination among women of reproductive age in endemic countries prior to pregnancy may be an effective measure to prevent dengue disease during pregnancy.

ACKNOWLEDGEMENTS

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MATERNAL AND FETAL OUTCOMES OF DENGUE INFECTION DURING PREGNANCY

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Abstract. Thirteen pregnant women with dengue infection were admitted to Ban Pong Hospital, Ratchaburi and Photharam Hospitals, Ratchaburi, Thailand during 2007-2015. The diagnosis of dengue disease in these pregnant women adhered to clinical and laboratory criteria for the diagnosis of dengue disease as established by the World Health Organization (WHO) 1997. Our study showed dengue infection can happened in any pregnancy trimester with all severities of dengue disease, namely dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS). All were symptomatic, received supportive treatment, and had uneventful recovery. Eleven of 13 cases proceeded to normal delivery, but two cases proceeded to abortion. All 11 cases proceeding to labor delivered normal newborn remaining healthy up to the time of this report.

Keywords: dengue, pregnancy, fetus

INTRODUCTION

The World Health Organization (WHO) has declared dengue the fastest-spreading mosquito-borne viral disease in the world (WHO, 2012). Four closely related dengue serotypes cause the disease, which ranges from asymptomatic infection to undifferentiated fever dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). DF causes fever, rash, muscle or joint pain, headache, and eye pain, and it is rarely fatal. DHF is characterized by increased vascular permeability leading to leakage of plasma and DSS (Thisyakorn and Thisyakorn, 2015).

A shift in age group of the dengue patients toward adulthood has been widely seen in Asia (Tantawichien, 2015). It also affects child bearing age and pregnant women (Khamim *et al*, 2015). In dengue endemic areas, many cases of dengue disease among pregnant women have been reported. Dengue infected pregnant patients have higher risk of severe disease than non-pregnant patients. Dengue infection during pregnancy can increase risks of abortion, premature uterine contraction, intra-partum and post-partum hemorrhage, maternal death, fetal distress, low birth weight, or death of the fetus *in utero* that is associated with disease severity and gestational age (RCPT, 2015).

Obstetricians must be aware that dengue infection in pregnant women may occur and surgical procedures performed on patients with dengue disease may unmask dengue induced hemostatic defects resulting in unexpected hemorrhage that is difficult to control. It also has been reported that dengue infection was vertically transmitted to the fetus and led to dengue illness in the neonate (Thaithumyanon *et al*, 1994).

This study aimed to describe maternal and fetal outcomes of dengue infection during pregnancy.

MATERIALS AND METHODS

A retrospective analysis of 13 pregnant women with dengue diseases admitted at Ban Pong Hospital, Ratchaburi and Photharam Hospital, Ratchaburi, Thailand during 2007-2015 was

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done. The diagnosis of dengue patients adhered to clinical and laboratory criteria for the diagnosis of dengue patients as established by the WHO (WHO, 1997). The analysis also included maternal and fetal outcomes at delivery and during various durations of follow-up.

RESULTS

Thirteen pregnant women with dengue disease admitted to Ban Pong Hospital, Ratchaburi and Photharam Hospital, Ratchaburi, Thailand during 2007-2015.

There were five severe dengue cases with four DHF and one DSS. One of the DHF patients was in the first trimester of pregnancy and had spontaneous abortion while the other four were in the second trimester of pregnancy and proceeded to normal delivery.

Eight cases had DF, of which two were in the first trimester with one of those two in the first trimester having threatened abortion. Both of those first trimester DF cases proceeded to normal delivery. Two DF cases were in the second trimester with one spontaneous abortion and the other proceeding to normal delivery. Other 4 DF cases were in the third trimester of pregnancy and all proceeded to normal delivery (Table 1).

All 11 cases proceeding to normal labor delivered normal newborn babies and all of them remained healthy thereafter up to the time of this report (Table 2).

DISCUSSION

Our study showed that dengue infection presented in all pregnancy trimesters. In an endemic area of dengue, dengue infection should be considered in a pregnant woman with fever. The clinical characteristics of dengue-infected pregnant women are the same as other dengue infected patients who usually present with fever, myalgia, nausea, and vomiting. Leukopenia and thrombocytopenia are also common findings. Those with DHF show a rising of hematocrit as seen in our study.

Table 1. Characteristics of dengue-infected pregnant women.

Case number	1	2	3	4	5	6	7	8	9	10	11	12	13
Age (years)	15	33	33	16	37	27	19	22	20	19	22	27	27
Diagnosis	DHF	DF	DF	DF	DF	DF	DF	DF	DHF	DSS	DHF	DF	DHF
Gestational age (weeks)	12	14	33	38	26	5	10	14	14	16	16	36	20
Duration of fever (days)	5	3	3	3	3	3	5	6	4	3	4	6	5
Nausea/Vomiting	Yes	No	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Myalgia	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No
Petechiae	No	No	No	Yes	Yes	Yes	No	Yes	No	No	No	Yes	No
Epistaxis	No	No	Yes	No	Yes	No	No	Yes	No	No	No	No	No
White blood cell	2,100	4,900	3,110	6,700	4,980	1,680	3,500	3,150	3,300	3,500	4,500	4,890	4,230
Platelet count	74,100	84,400	95,800	73,200	26,100	83,200	39,000	59,900	42,100	11,400	24,800	84,200	34,000

DF, dengue fever; DHF, dengue hemorrhagic fever; DSS, dengue shock syndrome.

Table 2. Fetal outcomes of dengue-infected pregnant women.

Case number	1	2	3	4	5	6	7	8	9	10	11	12	13
Gestational age at delivery, (weeks)	21	17	40	38	37	40	40	38	39	40	38	40	38
Fetal status at birth	Abortion	Abortion	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	LBW	Normal	Normal
Birth weight, (g)	-	-	3,560	2,750	2,650	2,710	2,950	3,240	2,740	3,090	2,340	2,700	3,400
Sex	-	-	Male	Female	Female	Female	Male	Female	Female	Male	Male	Female	Female
Period of follow up, (years)	-	-	7	5	4	4	3	6	9	7	4	1	5

LBW, low birth weight.

Special considerations for diagnosis of DHF in pregnant women include physiologic hemodilution in pregnancy, which may obscure hemoconcentration in DHF and a differential diagnosis of pregnancy-related conditions, especially HELLP (hemolysis, elevated liver enzymes, and thrombocytopenia) syndrome (RCPT, 2015). All thirteen pregnant women with dengue disease in this study had uneventful recovery from dengue disease.

There is no specific dengue therapy and successful treatment, which is mainly supportive, depends on early recognition of the disease and careful monitoring for shock. (Thisyakorn and Thisyakorn, 2015). The 2009 WHO case classification which is a severity-based revised dengue classification for medical interventions has been implemented in many countries (WHO, 2009). The systematic literature review indicated that the 2009 WHO case classification has clear advantages for clinical use (Horstick and Ranzinger, 2015).

Dengue infection during pregnancy can increase risk of abortion, premature uterine contraction, intra-partum and post-partum hemorrhage, maternal death, fetal distress, low birth weight, or death of the fetus *in utero* that is associated with disease severity and gestational age (RCPT, 2015). As seen in our study, two cases had abortion: one in the first trimester of pregnancy and the other in the early second trimester of pregnancy. One of 11 dengue infected pregnant woman in this study who proceeded to labor delivered a low birth weight newborn.

In conclusion, this report emphasizes a shift in age group of the dengue patients toward adulthood which also affected child bearing age and pregnant women. Favorable outcomes can be obtained by early recognition of the disease and careful monitoring of shock. Dengue infection during pregnancy can increase risks of abortion, premature uterine contraction, intra-partum and post-partum hemorrhage, maternal death, fetal distress, low birth weight, or death of the fetus *in utero* that is associated with disease severity and gestational age.

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DENGUE AND THE CARDIOVASCULAR SYSTEM

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Abstract. Dengue is a mosquito-borne viral disease which is currently an important and rapid growing health problem across the globe. It is one disease entity with different clinical manifestations, often with unpredictable clinical evolutions and outcomes. Hypovolemia plays an important role in hemodynamic changes of dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) as seen by a favorable response to appropriate volume replacement in majority of dengue patients with DHF and DSS. Cardiac involvement secondary to dengue virus infection is not uncommon and is often transient. It may vary from functional myocardial impairment, both self-limiting and arrhythmias that need treatment to severe and even fatal myocarditis. Successful treatment, which is mainly symptomatic and supportive, depends on early recognition of the disease and careful monitoring for the disease severity. For patients with severe myocarditis, in addition to intensive care and careful vasopressor and catecholamine therapy, mechanical circulatory support such as intra-aortic balloon pump (IABP), extracorporeal membrane oxygenation (ECMO) or ventricular assist device can be beneficial.

Keywords: dengue, cardiac, arrhythmias, myocarditis

INTRODUCTION

The World Health Organization (WHO) has declared dengue the most rapidly spreading mosquito-borne viral disease with a 30-fold increase in global incidence over the past 50 years (WHO, 2012). Infection with any one of the 4 antigenically-related serotypes: DEN-1, DEN-2, DEN-3, and DEN-4 can produce a broad spectrum of effects from mild to severe dengue which includes asymptomatic infection, undifferentiated febrile illness, dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). (WHO, 1997, 2009). The pathogenesis of dengue disease is still not clearly understood (Thisyakorn and Thisyakorn, 2015a).

Despite being traditionally considered a disease of children, dengue is now known to affect individuals of any age and cardiac involvement is not uncommon especially in adult who can present with fulminant myocarditis, which sometimes can masquerade as acute myocardial infarction (Lee *et al*, 2009). Clinical presentation in adult older than 65 years can be even more complicated because of higher frequency of comorbidities such as hypertension, ischemic heart disease, and diabetes mellitus.

The clinical course of dengue is divided into 3 phases: febrile, critical, and recovery. Febrile phase: the patient develops high-grade fever suddenly. This phase usually lasts 2-7 days. The patient has anorexia, nausea and vomiting, facial flushing, skin erythema, generalized body ache, myalgia, arthralgia, and headache. Critical phase: around the time of defervescence, usually on days 3-7 of illness, an increase in capillary permeability in parallel with increasing hematocrit level may occur. Progressive leukopenia followed by a rapid decrease in platelet count usually precedes plasma leakage. Those patients who deteriorate will manifest with warning signs such as abdominal pain and tenderness, persistent vomiting, clinical

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fluid accumulation, mucosal bleed, lethargy, restlessness, liver enlargement, increase in hematocrit concurrent with rapid decrease in platelet count. Recovery phase: if the patient survives the 24-48 hour critical phase, a gradual reabsorption of extravascular compartment fluid takes place in the following 48-72 hours. General well-being improves, appetite returns, hemodynamic status stabilizes and diuresis ensues. An extremely important point is that a drop in the hematocrit level at this stage not be taken as a sign of internal hemorrhage. A strong pulse and blood pressure, with a wide pulse pressure and diuresis, indicate good vital signs. They rule out the likelihood of gastrointestinal hemorrhage, which is mostly found during the critical or shock stage (Thisyakorn and Thisyakorn, 1994). Bradycardia and electrocardiogram (ECG) changes are common during this stage (WHO, 2009).

Severe dengue.

Although majority of dengue virus infections are either asymptomatic or mild, an estimated 1-5% of patients admitting to hospital develop complications including organ impairment, bleeding and plasma leakage that can result in potential cardiovascular collapse if severe (Yacoub et al, 2014).

Severe dengue is defined by one or more of the following (WHO, 2009):
1. Plasma leakage that may lead to shock if fluid replacement therapy is not enough for maintaining adequate circulating blood volume. Shock can be intractable culminating death.
2. Severe bleeding usually does not happen in children unless they have pathologic bleeding site or have disseminated intravascular coagulation (DIC) leading to death.
3. Severe organ impairment is not a usual finding in dengue infection, it can be a result of prolonged shock of any origin or can be severe disease from direct involvement of that organ by dengue, eg, fulminant myocarditis that can be fatal.

CARDIAC INVOLVEMENT

Dengue virus affects the heart functionally and structurally. It is difficult to define cardiac involvement in dengue virus infection due to lack of clear criteria. Cardiac involvement of dengue include functional myocardial impairment, both self-limiting and arrhythmias that need treatment (Mahmod et al, 2009) to fulminant myocarditis leading to hypotension, cardiac failure, pulmonary edema, cardiogenic shock, and death (Miranda et al, 2013b; Guadalajara-Boo et al, 2014; Bich et al, 2015; Shivanthan et al, 2015).

FUNCTIONAL MYOCARDIAL IMPAIRMENT

Functional myocardial impairment usually results in a benign and self-limiting disease (Wali et al, 1998; Khongphatthanayothin et al, 2007; Kirawittaya et al, 2015). Myocardial dysfunction has been documented in dengue virus infection by using a variety of methods including 12-lead ECG, 24-hour Holter, echocardiography, radionucleotide ventriculography, Tc pyrophosphate imaging, cardiac magnetic resonance (CMR) imaging (Kabra et al, 1998; Wali et al, 1998; Khongphatthanayothin et al, 2003; Khongphatthanayothin et al, 2007; Kularatne et al, 2007; Lateef et al, 2007; Satarasinghe et al, 2007; Salgado et al, 2009; Salgado et al, 2010; La-Orkhun et al, 2011; Yacoub et al, 2012; Miranda et al, 2013a; Sengupta et al, 2013; Yadav et al, 2013).

The dysfunction can be found in different severity of dengue infection but is more prevalent in disease with more severity (Khongphatthanayothin et al, 2007). Cardiac functional abnormalities were found related to the severity of plasma leakage (Kirawittaya et al, 2015). In most patients the dysfunction is asymptomatic, requires no treatment and usually the abnormal findings revert to normal during the follow-up (Wali et al, 1998) or even as rapid as 24-48 hours after the toxic or critical stage (Khongphatthanayothin et al, 2007). Except in the rare case of fulminant myocarditis, the patient is usually symptomatic, needs treatment, and abnormal findings may last longer.

There was statistically significant correlation between cardiac manifestations and all the

warning signs except persistent vomiting (Sheetal and Jacob, 2016). Myocardial functions need to be accessed in patients with this disease especially those who have persistent hypotension in spite of adequate intravenous fluid resuscitation since more severe concurrent involvement such as myocarditis should be considered, it can progress to cardiogenic shock and death.

The postulated mechanisms for myocardial dysfunction include direct viral invasion, immune mechanisms, electrolyte imbalance (Shivanthan et al, 2015), myocardial edema from capillary leakage, presence of myocardial depressant factor, coronary hypoperfusion, altered calcium homeostasis (Salgado et al, 2010), lactic acidosis, or a combination of these factors. Another possibility is non-fulminant myocarditis, which can be the sole factor or a contributory factor of myocardial dysfunction.

ELECTROCARDIOGRAPHIC ABNORMALITIES

Just like the functional myocardial impairment, ECG abnormalities are quite common in dengue virus infection. Most of the ECG abnormalities are benign, asymptomatic and self-limiting but some may cause symptoms and need treatment (Mahmod et al, 2009). The reported ECG abnormalities are diverse and include rate and rhythm abnormalities, sinus bradycardia is the commonest (Sheetal and Jacob, 2016), waveform and voltage abnormalities. These ECG abnormalities can be detected during both the critical and the recovery phases. The possibility that these ECG abnormalities can be caused by concealed dengue myocarditis cannot be ruled out since myocarditis can be asymptomatic and undiagnosed.

Although ECG is an easy and useful test to screen for cardiac abnormalities, the use of ECG changes alone to denote cardiac involvement is inaccurate. ECG abnormalities were found in 5-of-17 patients of DHF/DSS studied with horizontal ST elevation and T inversion and the changes reverted back to normal within 3 weeks (Wali et al, 1998). Reported rhythm abnormalities include relative bradycardia (Lateef et al, 2007),

sinoatrial block (Kaushik et al, 2010), disorders of atrioventricular conduction (Junctional rhythm) (Donegani and Briceno, 1986; Promphan et al, 2004; Kaushik et al, 2010), first degree (Naresh et al, 2008), second degree (Khongphatthanayothin et al, 2000), and complete heart block (Virk et al, 2016), monomorphic premature ventricular contractions on a background of heart block (Khongphatthanayothin et al, 2000), transient (Pahadiya et al, 2015) and non-self-limiting (Mahmod et al, 2009), atrial fibrillation, self-limiting tachy-brady arrhythmia (Lee et al, 2010), sinoatrial block and uniform ventricular ectopics progressing to ventricular bigemini (Chuah, 1987), ventricular trigeminy (Matthias et al, 2014).

Electrocardiographic features mimicking acute myocardial infarction have also been reported (Lee et al, 2009). In a prospective study in Thailand, overnight 18-24 hour Holter monitoring was performed in 35 clinically diagnosed dengue virus infection children (mean age 11.7 years) at least 24 hours after defervescence, Cardiac rhythm abnormalities were found in ten patients (29%), including sinus pause (1), first degree (2) and Mobitz type I second-degree AV block (Wenckebach) (3), and atrial (4) and ventricular ectopic beats (5).

There was no relationship between the clinical severity of dengue virus infection (DF, DHF without shock, and DSS) and the incidence of cardiac arrhythmia. All patients were asymptomatic during episodes of cardiac arrhythmia (La-Orkhun et al, 2011). In another case series of 120 patients with dengue, 62.5% showed ECG abnormalities: T inversions, ST depression, bundle branch blocks (Kularatne et al, 2007).

Arrhythmia management

The reported ECG abnormalities may be asymptomatic or go undetected, most of the patients only need conservative follow-up of the ECG abnormalities that are usually self-limiting and revert to normal without any treatment (Wali et al, 1998). However, some types of arrhythmia should be treated aggressively. Supraventricular tachyarrhythmias may respond to digitalis or other

medications. Ventricular arrhythmias have been known to respond to lidocaine or intravenous amiodarone. Despite aggressive treatment of these arrhythmias, rapid deterioration to ventricular fibrillation may occur and should be treated immediately by direct current defibrillation. Complete atrioventricular block requires a temporary transvenous pacemaker (Vashist *et al*, 2009).

MYOCARDITIS

Cardiac involvement in dengue patients can range from benign myocardial impairment and arrhythmias to fulminant myocarditis (Miranda *et al*, 2013a; Yacoub *et al*, 2014). Dengue myocarditis can present at any time during the illness, unlike other severe manifestations that present during the critical phase around defervescence (Simmons *et al*, 2012). The incidence of dengue myocarditis is unknown, but the prevalence of myocarditis in hospitalized dengue patients is 11.28% (Li *et al*, 2016) according to the European Society of Cardiology myocarditis criteria (Caforio *et al*, 2013) using ultrasound cardiogram as the imaging diagnosis method of myocarditis rather than cardiac magnetic resonance (CMR) imaging and no endomyocardium biopsy (EMB) was performed.

Dengue myocarditis can be found both in primary and secondary dengue infection and cardiac involvement was not more prevalent in dengue patients with secondary infection (Miranda *et al*, 2013a; Sane *et al*, 2015). All four serotypes of dengue virus can cause myocarditis (Weerakoon *et al*, 2011; Marques *et al*, 2013; Bich *et al*, 2015; Sane *et al*, 2015). Myocarditis presents in many different ways. It can be completely asymptomatic, as shown by a study in Sri Lanka, revealing 24% of dengue patients with echocardiographic evidence of myocarditis without any cardiac complaints and with complete resolution during convalescence (Satarasinghe *et al*, 2007).

The main mechanism of dengue myocarditis is still unknown though both direct viral infection and immune mediated damage have been suggested to be the cause of myocardial damage (Hober *et al*, 1993; Hober *et al*, 1996). The histopathological findings of dengue myocarditis include: areas of myocytolytic necrosis of myocardial fibers with inflammatory cell infiltration and marked interstitial edema causing fiber separation (Weerakoon *et al*, 2011; Guadalajara-Boo *et al*, 2014). One report also included electron microscopic findings showing clusters of dengue-like virus particles inside the cells and in the interstitial space, providing evidence of a possible direct action of dengue virus on myocardium (Miranda *et al*, 2013b).

Diagnosis of myocarditis

Non-invasive imaging techniques such as cardiac magnetic resonance (CMR) imaging can be useful in making the diagnosis of myocarditis and for monitoring disease progression, but endomyocardial biopsy (EMB) should be the gold standard for the diagnosis of definite myocarditis (Caforio *et al*, 2013).

Standard 12-lead ECG should be performed in all patients with clinically suspect myocarditis. ECG is usually abnormal in myocarditis though ECG signs are neither specific nor sensitive. Echocardiography helps to rule out non-inflammatory cardiac diseases such as valve disease. Global ventricular dysfunction, regional wall motion abnormalities, and diastolic dysfunction with preserved ejection fraction may occur in myocarditis. Fulminant myocarditis often presents with a non-dilated, thickened, and hypocontractile left ventricle as the intense inflammatory response results in interstitial edema and loss of ventricular contractility (Pinamonti *et al*, 1988; Felker *et al*, 2000).

Cardiac magnetic resonance (CMR) imaging provides non-invasive tissue characterization of the myocardium and can support the diagnosis of myocarditis. One study has demonstrated good correlation between CMR and EMB in troponin-positive patients without coronary artery disease (Baccouche *et al*, 2009).

Troponin and Brain Natriuretc Peptides (BNP) levels: while cardiac troponins are more sensitive of myocyte injury in patients with clinically suspect myocarditis than creatine kinase level (Lauer *et al*, 1997), they are nonspecific and when normal do

not exclude myocarditis (Heymans, 2007). This also applies to cardiac hormones such as brain natriuretic peptides.

Endomyocardial biopsy (EMB) should be the gold standard for the diagnosis of definite myocarditis. Besides confirming the diagnosis of myocarditis, it identifies the underlying etiology and the type of inflammation, which implies different treatment and prognosis. EMB should be performed early in the course of the disease and multiple specimens should be taken. At least three samples, each 1-2 mm in size, should be taken from the right or from the left ventricle for light microscopy; additional samples should be snap frozen in liquid nitrogen and stored at -80ºC or store in RNA later tubes at room temperature for viral PCR (Leone *et al*, 2012). EMB has been rarely reported in dengue myocarditis (Guadalajara-Boo *et al*, 2014).

TREATMENT OF MYOCARDITIS

Most patients with acute myocarditis do not require therapy. For dengue patients with myocarditis and hemodynamically stable heart failure should be treated with diuretics, angiotensin converting enzyme inhibitor, or angiotensin receptor blockage and beta-adrenergic blockage with or without aldosterone antagonists. For dengue patients with myocarditis and hemodynamically unstable, heart failure should be treated aggressively and supportive care is indicated for fulminant myocarditis. In cases with cardiogenic shock and severe ventricular dysfunction, norepinephrine should be the first choice as a vasopressor because treatment with dopamine in comparison with norepinephrine was associated with significant more arrhythmic events (De Backer *et al*, 2010).

Because myocardial oxygen consumption increases under catecholamine therapy and vasoconstrictors may impair the microcirculation and tissue perfusion, their use should be restricted to the shortest duration and the lowest possible dose. Inotropic dobutamine may be administered in addition to norepinephrine in an attempt to improve cardiac contractility, which is often

performed in clinical practice. In fulminant cases with cardiogenic shock and severe ventricular dysfunction, mechanical circulatory support such as intra-aortic balloon pump (IABP), ventricular assist device or extracorporeal membrane oxygenation (ECMO) may be needed to provide a bridge to heart transplantation or to recovery (Japanese Circulation Society Joint Working Group, 2011).

Immunomodulatory therapy

Therapy to enhance immune function may benefit patients with myocarditis, especially during the viral replication phase (Liu and Mason, 2001).

Interferon

An uncontrolled trial of interferon in humans with biopsy-positive enteroviral or adenoviral myocarditis demonstrated clearance from the myocardium with a decrease in left ventricular size and improvement in left ventricle ejection fraction (Kuhl *et al*, 2003).

Intravenous immunoglobulin

High dose intravenous immunoglobulin (IVIG) modulates the immune and inflammatory response by a variety of mechanisms and is used in a number of systemic autoimmune diseases (Orange *et al*, 2006). Its use has been associated with improved left ventricular ejection fraction in chronic symptomatic heart failure of various causes (Gullestad *et al*, 2001). A Cochrane review analyzing the use of IVIG for presumed viral myocarditis in children and adults concluded that evidence from one trial does not support the use of IVIG for the treatment of adults with presumed viral myocarditis.

The only pediatric trial had high risk of bias but suggested that benefit may be seen in the select group of children beyond the neonatal period who have viral encephalitis with myocarditis. Until higher-quality studies have demonstrated benefit in a particular group of patients, IVIG for presumed viral myocarditis should not be provided as routine practice in any situation (Robinson *et al*, 2015).

Immunosuppressive therapy

The recognition that the pathogenesis of myocardial dysfunction in myocarditis is at least

partially the result of an immune activation or autoimmune process (Liu *et al*, 2001) led to attempts to ameliorate this process with a variety of immunosuppressive regimens using prednisone or various combinations of steroids, azathioprine, and cyclosporine (Maisch *et al*, 1995). A single-center controlled trial suggested a beneficial effect of combined steroid and azathioprine therapy in virus-negative myocarditis (Frustaci *et al*, 2009)

Heart transplantation

Despite advances in therapy and the high rate of improvement in patients with myocarditis, some patients with myocarditis will require cardiac transplantation.

PREVENTION

There is currently no specific antiviral treatment against dengue and successful treatment, which is mainly supportive, depends on early recognition of the disease and careful monitoring for shock and treatment of complications. Main public health preventive interventions consist of mosquito control while safe and efficacious dengue vaccine is seen as the best hope to fight this disease (Thisyakorn and Thisyakorn, 2015b).

CONCLUSION

Dengue virus is the causative agent of a very wide spectrum of clinical manifestations, ranging from asymptomatic illness, to undifferentiated febrile illness, DF, DHF, and DSS. Cardiac involvement in dengue virus infection is also very difficult to define since cardiac manifestations can be either caused by direct pathology of the heart or indirectly from the pathophysiologic changes of the cardiovascular system secondary to the dengue virus infection.

Although severe cardiac impairment in dengue patients is not commonly seen, it can cause significant morbidity and even mortality. Careful interpretation of clinical parameters will help avoid unnecessary invasive interventions. Successful treatment that is mainly symptomatic and supportive depends on early recognition of the disease and careful monitoring for the disease severity.

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CURRENT MANAGEMENT OF LIVER COMPLICATIONS IN ADULT DENGUE INFECTION

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Abstract. Adult dengue patients have a high prevalence of abnormal liver function tests in about 75-80% for aspartate aminotransferase (AST), followed by 52-54% for alanine aminotransferase (ALT) whereas the incidence of patients with acute liver failure (ALF) caused by dengue infection was 0.31%. The average duration from onset of fever to clinical symptoms of ALF was 7.5 days with a mortality rate of 67%. Our article aims to detail the clinical findings of liver involvement, especially those presenting with ALF, and the current management including N-acetylcysteine (NAC) and artificial liver dialysis.

Keywords: liver complications, adults, dengue

INTRODUCTION

Dengue infection is a major mosquito-borne viral disease of the tropical countries and is becoming a global problem (Stanaway *et al*, 2016; Subedi and Taylor-Robinson, 2016; Zambrano *et al,* 2016) with an estimated number of 60 million symptomatic dengue infections annually as well as 10,000 deaths per year. Clinical presentations in adult patients are different from child patients (Martinez Vega *et al*, 2016). Severe infection, namely DHF or dengue shock syndrome (DSS), were more prevalent in adults than in children (Wichmann *et al*, 2004).

Liver involvement is common in dengue infection, and acute liver failure (ALF) is one of the serious and emergent complications of dengue (Souza *et al,* 2004). This review article aims to describe the prevalence and severity of abnormalities of liver function test in adult patients with dengue infection. In addition, we aim to describe the current management of the severe dengue-infected patients with liver involvements, especially those presenting with ALF.

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MATERIALS AND METHODS

We retrospectively reviewed data from a PubMed search, which includes Medline, that was performed to identify relevant literature using search terms "**liver** and **dengue infection** and **burden**" during a 20-year period from 1996 to 2016. All relevant literatures of adult dengue patients with liver involvement were reviewed. Specific terms were defined: 1) adult was defined as age greater than or equal to 15 years; 2) abnormal AST and/or alanine aminotransferase (ALT) were defined as their level above the normal value with blood tests taken within 7 days after the onset of fever; 3) dengue infection was defined by World Health Organization (WHO) criteria with serological confirmation by enzyme-linked immunosorbent assay test or rapid immunochromatographic test; and 4) the 2009 WHO classifications were used to categorize dengue patients as dengue (with or without warning signs) and severe dengue.

RESULTS

Prevalence and severity of abnormalities of liver function test in adult patients with dengue infection

Recently, the data from 15 articles related to liver tests abnormalities in dengue-infected patients during 1997-2013 showed a high prevalence of 75-80% for the presence of an abnormal AST, followed by 52-54% for the presence of an abnormal ALT (Martinez Vega *et al,* 2016; Wang *et al,* 2016). Additionally, liver impairment in adult dengue patients was more frequently found compared with child dengue patients [liver impairment defined as ALT > 2 × upper limit of normal (ULN])], 47.1 *vs.* 25.5%; or severe hepatitis defined as AST/ALT >10 × ULN, 16.5 versus 4.3%) (Martinez Vega *et al,* 2016; Wang *et al,* 2016).

Our previous report showed that the mean age of adult dengue patients ±SD was 26.4±11.5 years, two-thirds of them had a severe form of dengue infection, and most of them had no underlying liver disease (Kittitrakul *et al,* 2015). Recently, data from Thailand showed that the incidence of patients with ALF caused by dengue infection was 0.31% (Kye Mon *et al,* 2016).

The dengue-infected patients with high levels of transaminitis usually presented with longer duration of fever of at least 1 week (Kittitrakul *et al,* 2015). The average duration from onset of fever to clinical symptoms of ALF was 7.5 days with a mortality rate of 67% (Kye Mon *et al,* 2016). Previous studies have shown that the dengue-infected patients presenting with severe acute hepatitis were usually infected with dengue serotypes 3 or 4 (Gasperino *et al,* 2007; Soundravally *et al,* 2010). The characteristic histological change to the liver parenchyma in these patients was midzonal (zone 2) hepatic necrosis, which is the classic pathological finding (Gasperino *et al,* 2007). However, there was an evidence of multi-serotype dengue viral infections from Indonesia showing the concurrent presence of dengue serotype 2 and type 3 infections, both of which may contribute to the severity of disease (Lardo *et al,* 2016). Recent studies have shown the pattern of abnormal liver tests, in which the highest AST levels were seen on day 6 of the illness, and AST were significantly higher in patients with severe dengue than those with non-severe dengue (Fernando *et al,* 2016).

The clinical findings of liver complications in adult patients with dengue infection

The common clinical findings of liver complications in patients with dengue infection are hepatomegaly (28-72%) (Wichmann *et al,* 2004; Wichmann and Jelinek, 2004; Wang *et al,* 2009), abnormal liver tests (52-80%) (Wang *et al,* 2016), and severe hepatitis defined as AST/ALT >10 × ULN (16.5%) (Wang *et al,* 2016). The clinical presentation of severe hepatitis in dengue-infected patients had a significantly greater proportion of hypotension than those with low level ALT (25% *vs* 5%, respectively) (Kittitrakul *et al,* 2015). These findings are consistent with a previous report that the transaminase levels were associated with severity of vascular leakage and increased severity of bleeding (Trung *et al,* 2010).

In clinical practice, the primary physicians should be aware of and work up for the common causes of abnormal liver tests in critical patients, including ischemic hepatitis, liver test abnormality related to bacteremia, and drug-induced liver injury (Thomson *et al,* 2009). If the clinical course of a dengue patient is worsening despite full supportive treatments, the clinician must exclude co-infection with other tropical diseases or complications, for example, malaria (Assir *et al,* 2014), bacterial sepsis (Ahmed *et al,* 2014), acute acalculous cholecystitis (Tan *et al,* 2005), leptospirosis, and acute hepatitis E (Parkash *et al,* 2010; Behera *et al,* 2010).

The current principles of management of ALT in adult patients with dengue infection
A. The current principles of management for hospitalized critically ill patients with acute hepatitis are the following:

1. Identify those patients with underlying chronic liver disease (Thomson *et al,* 2010). There is an evidence from a retrospective study showing that the cirrhotic patients presenting with dengue infection had poor clinical outcomes from acute liver decompensation with a mortality rate of 25%, which was higher than that reported in patients without chronic liver diseases (1.4%) (Table 1) (Kulkarni *et al,* 2016).

2. Exclude treatable and/or emergency hepato-

Table 1. Outcomes of DHF infection in cirrhosis patients: acute hepatic decompensation – retrospective study; 7/2015-4/2016 – India.

Outcome	Gr 1 No liver disease (*n*=71)	Gr 2 Chronic hepatitis (*n*=12)	Gr 3 Cirrhosis (*n*=12)	*p* value
Age, year (SD)	39.2 (1.6)	40.8 (16.3)	50.9 (7.9)	0.02
Jaundice, *n* (%)	6 (8.5)	1 (8.3)	7 (58)	<0.001
Ascites, *n* (%)	5 (7%)	0	7 (58)	<0.001
Death, *n* (%)	1 (1.4)	0	3 (25)	0.001
Survived, *n* (%)	70 (98.6)	12 (100)	9 (75)	

Modified from Kulkarni, *et al*, 2016.

biliary diseases, for example, gallstone cholangitis or ALF. The high-risk group for developing ALF from dengue infection is those patients with a comorbidity, especially diabetes mellitus (Sam *et al,* 2013).

3. Identify the common causes of abnormal liver tests in critically ill patients, especially the causes from ischemic hepatitis and sepsis (Thomson *et al,* 2009). In addition, the specific host characteristics may influence the etiologies of abnormal liver tests, for example, pregnancy (Malhotra *et al,* 2006), AIDS, or elderly patients. There have been few studies focusing on co-infection of dengue virus and HIV infection. However, the seroprevalence of dengue virus infection in HIV-infected children was not different from that in healthy children (Thisyakorn *et al,* 2016; Torrentes-Carvalho *et al,* 2016; Delgado-Enciso *et al,* 2017). Finally, the physician should look for the possibility of abnormal liver tests caused by drug-induced liver injury. Recently, two reports have found evidences that acetaminophen overdose may play an important role in dengue-infected patients presenting with ALF (Ranganathan *et al,* 2006; Gan *et al,* 2013). Supra-therapeutic doses of acetaminophen to control fever in children (the average dose was 145 mg/kg/d) were reported in all children with fulminant hepatitis compared with none in the control group (Ranganathan *et al,* 2006).

B. The current principles of management of ALF in adult patients with dengue infection are the following:

1. N-acetylcysteine (NAC)

2. Provide temporary liver support as a bridge to liver transplantation: artificial liver support.

Both treatment modalities are mainly reported in case series that have limitations to inform guidelines because of the lack of good study design, small sample size, and the different definitions of ALF used in each study.

1. N-acetylcysteine (NAC)
The rationale for NAC use as an adjunctive therapy is its ability to restore hepatocellular glutathione and its action as a free radical scavenger. In addition, NAC may improve antioxidant defense (Senanayake *et al,* 2013; Habaragamuwa and Dissanayaka, 2014). In non-acetaminophen-related ALF, the following NAC dosage regimen has been used: an intravenous (iv) loading dose of 150 mg/kg/d in 5% dextrose in water for up to 72 hours or 7 days (Lee *et al,* 2009; Squires *et al,* 2013). The prescribed dosage in children was 100 mg/kg/24h until an INR of < 1.4 was achieved.

Previous studies have shown that dengue patients with ALF who were prescribed NAC had favorable outcomes as shown in Table 2 (Sklar and Subramaniam, 2004; Senanayake *et al,* 2013;

Table 2. The outcomes of treatment with NAC in patients with dengue infection and severe hepatitis.

Studies	Case report	LFT - presentations	Complications	Outcomes
Habaragamuwa et al, 2014	54-year female	Acute liver failure: AST 16,261 U/l, ALT 4,545 U/l, INR 1.7, TB 6 mg/dl	GCS=11	Survived with normal liver tests in 2 weeks
Manoj et al, 2014	37-year male	FHF-AST 220 U/l, ALT 157 U/l, TO Day 7; AST/ALT 12,500/ 2,700 U/l, INR 1.7, TB 10 mg/dl	Gr III HE, Resp fail, UGIB-massive	Survived with discharge – Day 18
Lim et al, 2012	6-year boy	Acute liver failure	No details	Survived
Abeysekera et al, 2012	52-year; HT male	Acute liver failure	Gr II HE	Survived
Kumarasena et al, 2010	N =7 cases; Age: 6 mo-12 y	Acute liver failure	Low GCS, prolonged shock	Survived

GCS; Glasgow Coma Scale provides a score in the range 3-15; patients with scores of 3-8 are usually in a coma.

Habaragamuwa and Dissanayaka, 2014). The standard dosage and duration for NAC regimens remain controversial, but have been suggested as follows:

1. IV NAC 100 mg/kg/d infusion for 5 days (Habaragamuwa and Dissanayaka, 2014).

2. IV NAC with 150 mg/kg loading dose, followed by iv administration over 15 minutes, then followed by 12.5 mg/kg/h for 4 hours, and finally iv drip administration 6.25 mg/kg/h for up to 72 hours (Kumarasena et al, 2010).

The second regimen was reported in a retrospective study of eight consecutive dengue-infected patients with ALF who showed complete recovery without adverse events from NAC treatment (Kumarasena et al, 2010).

2. Artificial liver support

Artificial liver support aims to provide temporary support of liver function while maintaining the treatment of specific causes of liver failure. It can provide detoxification through different dialysis procedures. It is different from bioartificial liver support because there is no addition of the viable porcine cellular component into the system, the addition of which may have a safety concern with xenotransplantation of porcine cells (Banares et al, 2013; Wang et al, 2013). The current use of non-biological systems including the albumin dialysis and plasma exchange are available worldwide as follows (Carpentier et al, 2009):

2.1 Molecular Adsorbent Recirculating System (MARS, Gambro, Sweden). This was developed by Stange and Mitzner in 1993 (Fig 1). The key principle of this system is albumin dialysis, in which albumin plays an important role in scavenging function, and it can remove toxins as well as reduce hyperbilirubinemia in ALF patients (Sen et al, 2005). There was a case report that described the use of MARS in a critically ill dengue-infected patient who had a rapid improvement of biochemical tests and encephalopathy (Penafiel et al, 2006). MARS has some limitations including high cost and some technical difficulty in its usage (Penafiel et al, 2006). Recently, a meta-analytic study reported
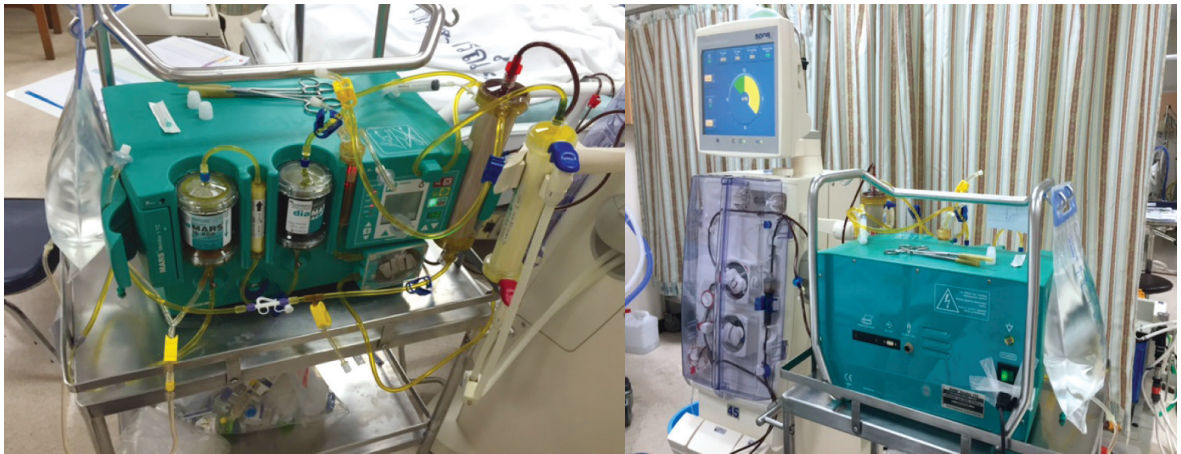
Fig 1–The Molecular Adsorbent Recirculating System (MARS) in an intensive care unit.

the outcomes of MARS treatment in patients with ALF from 10 randomized control trials. It showed that MARS improved survival, but MARS did not show a survival benefit in cirrhotic patients with acute decompensation (He *et al,* 2015).

2.2Prometheus (Fresenius, Germany). This was developed by Falkenhagen, *et al* in 1999. It uses the principle of fractionated plasma separation, adsorption, and hemodialysis (Tsipotis *et al,* 2015). Currently, there are no reports about the use of Prometheus in dengue-infected patients with ALF.

2.3SPAD (Single pass albumin dialysis). These non-biological systems have been used as a treatment for different types of liver failure. The overall outcome of using these devices is safe. In addition, they have shown several clinical benefits including improvement of jaundice, improvement of hemodynamic instability, reduction of portal pressure, reduction of intracranial pressure, and improvement of hepatic encephalopathy (Nevens and Laleman, 2012). Recently, there was a non-inferiority crossover study design comparing MARS with SPAD system procedures for total bilirubin reduction and clinical outcomes (Sponholz *et al,* 2016). The major findings showed that both systems were safe and had similar efficacy in plasma bilirubin reduction. However, MARS had better efficacy in reduction of serum bile acids, albumin-binding capacity than that by SPAD (Sponholz *et al,* 2016). At King Chulalongkorn

Memorial Hospital in Thailand, we have used albumin dialysis more often than other modalities due to the availability of equipment, lower cost, and ease of usage (Boonsrirat *et al,* 2009). Our previous study of using SPAD in patients with ALF showed favorable outcomes and had no serious complications (Boonsrirat *et al,* 2009). We used 2% human serum albumin dialysate for 6 hours, and SPAD reduced the level of total bilirubin by an average of 23% without serious complications (Boonsrirat *et al,* 2009).

Recently, a meta-analysis (7 trials of MARS and 3 trials of Prometheus), showed that albumin dialysis was superior in reducing serum total bilirubin level and improving hepatic encephalopathy compared with the standard medical therapy of 8.0 mg/dl; however, it did not show superior efficacy in reducing serum ammonia or bile acids (Tsipotis *et al,* 2015).

In summary, at least two-thirds of adult dengue patients have shown abnormal liver function tests. Acute severe hepatitis with an elevation of transaminase levels of at least 10 times has occurred in 4-15% of adult dengue-infected patients, and this should be a concern for the physician. Transaminases gradually decrease to normal levels within 2 weeks. The clinical findings of acute severe hepatitis or jaundice can be used as associated factors for dengue severity with an odds ratio of 1.9. The evidence of acetaminophen overdose, co-infection, or underlying chronic liver diseases

may play important roles in causing ALF in dengue-infected patients. NAC and artificial liver support is currently used as a bridge to liver transplantation. However, studies of both treatments have had some limitations including lack of randomization, small sample size, and the nature of multiple organ failure in severe forms of dengue infection. These new treatment modalities should be considered for use on a case-by-case basis, and more data are needed to support their usage.

ACKNOWLEDGEMENTS

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RENAL DYSFUNCTION IN DENGUE VIRUS INFECTION

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Abstract. Dengue virus infection can exhibit a wide spectrum of renal dysfunction including tubular dysfunction (dyskalemia, dysnatremia, and acute tubular necrosis), and less commonly glomerular injury (*ie* microscopic hematuria or sub-nephrotic to nephrotic range proteinuria). Data from previous reports have shown that the incidence could be up to 80%. The pathogenesis of dengue-associated renal dysfunction is still unclear. Proposed mechanisms include two major effects: direct effect and indirect effect such as hemodynamic factor and immunologic factor. Until the present time, supportive treatment is still the only key treatment in dengue-associated renal dysfunction. This narrative review aims to discuss current evidences regarding the epidemiology, pathogenesis, and various renal manifestations in dengue virus infection.

Keywords: denque infection, renal dysfunction, glomerular injury

INTRODUCTION

Dengue virus infection is one of the most significant human viral mosquito-borne infections. The main pathogen is dengue virus, an RNA *Flavivirus,* which comprises of four serotypes, DEN-1 to DEN-4. The female *Aedes aegypti* is the main vector. Once infection occurs with one serotype, there will be lifelong protection to that serotype but only a few-month protection for the remaining serotypes (Gibbons and Vaughn, 2002; Guzman and Kuri, 2002).

Dengue has an incubation period of 3-14 days and will replicate in reticuloendothelial system during this phase. The clinical spectrum ranges from asymptomatic (as many as 50% of individual infections), dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS), which is the most severe form of dengue infection.

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Renal involvement is one of the most significant target organ involvements in dengue infection. Acute kidney injury (AKI) has been increasingly recognized in dengue virus infection. The incidence of AKI was once as low as 1.6% among 617 Colombian children with dengue virus infection (Mendez and Gonzalez, 2003), but in the recent report, the incidence of AKI had risen to as high as 35.7% in adults infected by dengue virus admitted to the tertiary hospital in India (Basu *et al,* 2011). Most cases of patients who developed AKI were related to hypotension (42.9%), rhabdomyolysis (21.4%), and hemolysis (7.1%), carrying an increased risk of death (Lima *et al,* 2007). Microscopic hematuria and proteinuria could be detected in up to 12.5% and 80% of patients with DHF, respectively (Futrakul *et al,* 1973). Lastly, evidence from a few reports with available renal biopsy demonstrated the glomerular involvement and hemolytic uremic syndrome in patients with dengue virus infection (Wiersinga *et al,* 2006).

EPIDEMIOLOGY AND RISK FACTORS

The prevalence of AKI in previous studies has varied widely due to heterogeneous criteria of AKI used, differing populations, and differing severities of dengue virus infection in those studies. Current available data were mainly derived from

case reports and retrospective studies. Two studies used an elevation of serum creatinine of at least 2 mg/dl for the definition of AKI, and these studies may have underestimated the prevalence of AKI (Laoprasopwattana *et al,* 2010; Lee *et al,* 2009). However, later studies have applied the Acute Kidney Injury Network (AKIN) criteria and the risk, injury, failure, loss of kidney function, and end-stage acute kidney disease (RIFLE) criteria, both of which have allowed for more diagnoses of AKI (Kuo *et al,* 2008; Basu *et al,* 2011; Khalil *et al,* 2012; Mehra *et al,* 2012; Khan *et al,* 2014; Mallhi *et al,* 2015). Table 1 shows the prevalence of AKI, ranging from 0.2% to 35.7%. There is higher proportion of AKI in earlier stages. Mallhi *et al* (2016) used the conventional definition (serum creatinine ≥ 2 mg/dl), RIFLE, and AKIN criteria for diagnosis of AKI in dengue, and showed the incidence of AKI to be 4.2%, 12.6%, and 14.6%, respectively. The AKIN and RIFLE criteria were comparable while the conventional definition was the least sensitive criteria and might miss earlier stage of AKI (Mallhi *et al,* 2016).

Risk factors for dengue-associated AKI have been previously reported in the literatures. In a retrospective study of 304 hospitalized patients with DHF in Taiwan where AKI developed in 10 patients (3.3%) (Lee *et al,* 2009), the multivariable logistic regression was performed using various clinical and laboratory factors including age, gender, history of stroke, chronic kidney disease, concurrent gastrointestinal bleeding, concurrent bacterial infection, hemoglobin, activated partial thromboplastin time, and development of DSS, all of which showed significant difference between DHF patients with and without AKI. The result revealed that only DSS was the independent risk factor for development of AKI [odds ratio (OR) = 220; 95% CI: 19.8-2443.9; $p<0.001$]. However, the study had major limitation due to small number of AKI patients for the multivariate analysis. In the adjusted model there was almost 10 variables using in the model. This limitation made the disputable results. Another retrospective study in Pakistan reviewed 532 adult patients with dengue virus infection, 71 of which developed AKI

(13.3%) (Khalil *et al,* 2012). After multivariable logistic regression, the independent predictors of AKI were male gender (OR=4.43; 95%CI: 1.92-10.23; $p<0.001$), development of DHF or DSS (OR=2.14; 95%CI: 1.06-4.32; $p=0.03$), neurological involvement (OR=12.08; 95%CI: 2.82-51.77; $p=0.001$), and prolonged aPTT (OR= 1.81; 95%CI: 1.003-3.26; $p=0.04$). Recently, in a study of 667 dengue patients in Malaysia in which AKI were diagnosed in 95 patients (14.2%) by AKIN criteria, multivariable logistic regression disclosed that presence of DHF (OR=8.0; 95% CI:3.64-17.59; $p<0.001$), rhabdomyolysis (OR=7.9; 95%CI: 3.04-20.49; $p<0.001$), multiple organ dysfunction (OR=17.19; 95%CI: 9.14-35.12; $p<0.001$), diabetes (OR=4.7; 95% CI: 1.12-19.86; $p=0.034$), late hospitalization (OR=2.1; 95% CI: 1.06-4.13; $p=0.033$), and use of nephrotoxic drugs (OR=2.9; 95% CI: 1.34-6.11; $p=0.006$) were independent risk factors for AKI (Mallhi *et al,* 2015).

CLINICAL MANIFESTATION AND PATHOGENESIS

Dengue-associated AKI

Clinical and pathological data concerning the histopathology of AKI in dengue virus-infected patients is limited. From these limited data, the histopathology of AKI in dengue virus infection patients comprises of acute tubular necrosis (ATN), glomerulopathy, and rarely thrombotic microangiopathy. Hemodynamic instability, which results from plasma leakage syndrome, plays a major role in the pathogenesis of AKI. Other mechanisms have also been proposed including rhabdomyolysis or hemolysis leading to ATN and acute glomerular injury (Oliveira and Burdmann, 2015) (Fig 1 and Fig 2).

Hemodynamic factors. Patients with severe dengue virus infection, mostly in secondary infection, develop plasma leakage syndrome resulting in clinical signs of hemodynamic instability including hemoconcentration, tachycardia, narrow pulse pressure, and eventually hypotension. This process is believed to result from inflammatory cytokines particularly tumor necrosis factor

Table 1. Summary prevalence of acute kidney injury (AKI) and mortality.

Author	Year	Country	No.	Age (yrs)	Severity of dengue	Definition of AKI	AKI (%)	Mortality of AKI vs non-AKI (%)
Mendez and Gonzalez	2003	Columbia	617	<13	DHF	NR	1.6	NR
Khan et al	2008	Saudi Arabia	91	6-94	DHF	NR	2.2	NR
Kuo et al	2008	Taiwan	519	>18	DF/DHF/DSS	RIFLE	9.3 I 79.7 II 10.8 III 9.5	28.6 vs 1.2
Lee et al	2009	Taiwan	304	>18	DHF/DSS	Cr > 2 mg/dl	3.3	60 vs 0
Laoprasopwattana et al	2010	Thailand	2,893	<15	DF/DHF/DSS	Cr > 2 mg/dl	0.9	64 vs 0
Bhaskar et al	2010	India	128	>18	DHF	NR	12	NR[a]
Bunnag et al	2011	Thailand	50	<15	DSS	NR	10	NR
Basu et al	2011	India	28	>18	NR	RIFLE	35.7	60 vs 5.6
Mehra et al	2012	India	223	>18	DF/DHF	AKIN	10.8 I 50 II 29.2 III 20.8	NR[b]
Lumbiganon et al	2012	Thailand	99	<18	Fatal DF	NR	25.0	NR
Khalil et al	2012	Pakistan	532	>18	Fatal DHF DF/DHF/DSS	AKIN	14.3 13.3 I 64.8 II 18.3 III 16.9	11.3 vs 0
Khan et al	2014	Malaysia	124	>18	DHF	AKIN	7.2 I 22 II 78	NR
Vachvanichsanong et al	2015	Thailand	2,221	<15	DF/DHF/DSS	NR	0.2	NR
Mallhi et al	2015	Malaysia	667	>18	DF/DHF/DSS	AKIN	14.2 I 76.8 II 16.8 III 6.4	8.4 vs 0

DF, dengue fever; DHF, dengue hemorrhagic fever; DSS, dengue shock syndrome; AKI, acute kidney injury; NR, not reported; Cr, creatinine; I, AKIN stage 1; II, AKIN stage 2; III, AKIN stage 3.
[a]Overall mortality was 14%, but comparison between patients with and without AKI was not reported.
[b]Mortality was significantly higher in patients with AKI ($p < 0.01$) but the exact proportion was not described in the literature.
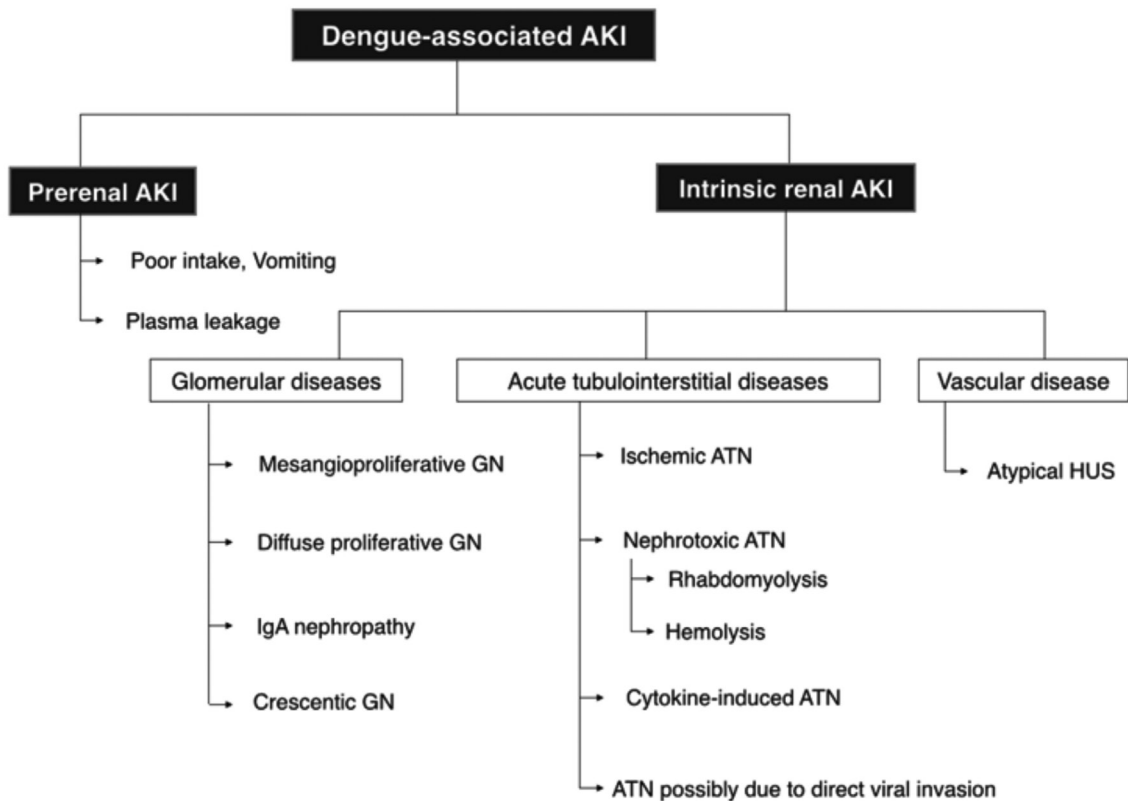
Fig 1- Clinical approach to dengue associated AKI. DVI, dengue virus infection; AKI, acute kidney injury; ATN, acute tubular necrosis; GN, glomerulonephritis; HUS, hemolytic uremic syndrome.

alpha (TNF-α), interleukin (IL)-6, IL-17, and IL-18, all of which are produced by dengue-infected monocytes (Anderson *et al,* 1997; Pagliari *et al,* 2016) and mast cells (Brown *et al,* 2011). Such changes lead to renal hypoperfusion and AKI. A retrospective study of 532 patients with dengue virus infection, in which AKI developed in 71 patients (13.3%), found that the presence of DHF or DSS was an independent predictor of AKI (OR=2.14; 95%CI: 1.06-4.32, *p* = 0.03), thus confirming the significance of hemodynamic factors in the development of AKI (Khalil *et al,* 2012). The pattern of AKI in this regard is likely ATN although kidney biopsy has not been performed generally.

Rhabdomyolysis and hemolysis. Rhabdomyolysis rarely occurs in dengue virus infection and the underlying pathogenesis of dengue-induced

muscle injury has not been established (Huang *et al,* 2015). Direct invasion by the virus is a possibility suggested by identification of the viral particle by electron microscopy in the thigh muscles of dengue-infected mice (Nath *et al,* 1982). On the other hand, inflammatory cytokines can be myotoxic, especially TNF-α, causing injury to the muscles (Gandini *et al,* 2011). Rhabdomyolysis causes AKI by multiple mechanisms. Firstly, muscle injury leads to fluid sequestration into the damaged muscles, followed by intravascular volume depletion. The subsequent reduction in renal blood flow results in activation of a neuroendocrine homeostatic response as well as the release of vasoactive mediators, including endothelin-1, thromboxane A2, and TNF-α, which eventually promotes intrarenal vasoconstriction and AKI. Secondly, direct tubular damage can be caused

by myoglobin, which is excessively produced and freely filtered through the glomerular filtration slits. Lastly, myoglobinuria can also cause intratubular obstruction by forming casts in the tubular lumen (Bosch *et al,* 2009).

Reported cases of dengue virus infection, rhabdomyolysis, and AKI are available. Serum creatine phosphate kinase level ranged from 4,063 to 156,900 IU/l, and most patients were oliguric at diagnosis (Repizo *et al,* 2014). Repizo and colleagues also performed renal biopsy in a 28-year-old Brazilian man presented with DF, rhabdomyolysis, and non-recovery AKI, showing acute tubular necrosis. Immunohistochemistry for myoglobin was positive in the renal tubules suggesting that ATN might be contributed, in part, by deposition of toxic myoglobin (Repizo *et al,* 2014).

Direct viral invasion. Data from autopsies of fatal dengue virus infection cases demonstrated the presence of dengue virus in the skin, liver, spleen, lymph node, bone marrow, lung, brain, and kidney, which indicates the possibility of direct cytopathic effects of dengue virus on renal tissues (Martina *et al,* 2009). Data from animal studies provide insight in this issue. Kidneys obtained from mice transfected with human DEN-2 for 48 hours revealed diffuse increase in glomerular volume, mesangial and endocapillary hypercellularity, and immunoglobulin (Ig) M deposition in the glomeruli (Barreto *et al,* 2004). The viruses were also detected after inoculation of mouse renal tissue into mosquito cell cultures using an electron microscopy and immunofluorescence technique, thus confirming the presence of viral particle in affected organs. Unfortunately, detailed localization of dengue virus in the tissue could not be attained.

Similar findings were also reported in humans. Renal biopsies in 12 of 20 dengue patients with renal disease detected dense spherical particles of 40-50 nm in diameter in the monocyte-like cells infiltrating the glomeruli (Boonpucknavig *et al,* 1976). Further localization of the dengue antigen was conducted on renal tissues from dengue patients by using immunohistochemistry (IHC) demonstrating viral antigens only within the tubular cells (Jessie *et al,* 2004). However, *in situ* hybridization (ISH) was also performed in the same study to detect positive-strand dengue virus RNA. In contrast to the IHC result, ISH did not detect dengue virus RNA in the renal tubular cells, but only observed dengue virus RNA in the spleen and blood-clot leukocytes. These findings imply a lack of viral replication in the renal tissues, which is against the pathogenic hypothesis of direct viral invasion.

A rare model of possible direct viral invasion and AKI is dengue-induced hemolytic uremic syndrome. This condition has only been reported in three patients to date (Wiersinga *et al,* 2006; Boyer and Niaudet, 2011). The first reported case presented with DF, malignant hypertension, and microangiopathic hemolytic anemia. Renal biopsy showed glomerular and arteriolar microthrombi consistent with acute thrombotic microangiopathy. Electron microscopy also revealed microtubulo-reticular structures in vascular endothelial cells, suggesting a process of viral infection (Wiersinga *et al,* 2006).

Immune response. Host immune response may be involved in the pathogenesis of dengue associated AKI. The concept of secondary infection as the key pathogenic factor for developing DHF/DSS has been well established for decades. Once infected with dengue virus, a human develops life-long antibodies to that particular serotype while antibodies to other serotypes only last for a few months. When the patient gets infected by another strain of dengue virus, a severe inflammatory response ensues, causing DHF or DSS (Whitehorn and Simmons, 2011). This phenomenon is explained by antibody-dependent enhancement (ADE). A previous infection by one serotype of dengue virus results in development of subneutralizing antibodies. These antibodies possess high viral attachment efficiency, enhancing internalization of virus into cells through FCγ receptor (FCγR)-dependent or FCγR-independent mechanisms. The FCγR-dependent mechanism is also proposed to suppress type I interferon-

mediated antiviral responses and promotes the T-helper-2 response, whose antiviral effect is less than the T-helper-1. Eventually, ADE enhances viral replication and cytokine or chemokine production, leading to cytokine-mediated endothelial activation mainly by TNF-α and plasma leakage syndrome (Wan *et al,* 2013).

Support for the role of the immunologic mechanism in the pathogenesis of dengue-associated AKI is based on a report of DHF patients who developed AKI in the absence of hypotension, hemolysis, and rhabdomyolysis (Lima *et al,* 2007). These observations raise the possibility of direct viral invasion as well as immune-mediated mechanisms. As previously mentioned, one series of DHF cases from Thailand reported albuminuria, hematuria, and low complement factor 3 (C_3) in 71%, 12.5%, and 82%, respectively, together with azotemia, which suggested immune-complex-mediated acute glomerular disease (Futrakul *et al,* 1973). There were reports of DHF and AKI associated with proteinuria, hematuria, and reduced serum C_3 level without the presence of hypotension, rhabdomyolysis, hemolysis, or use of nephrotoxic agents, making acute glomerulonephritis very likely, albeit in the absence of confirmatory histopathology (Ghosh *et al,* 2011; Bhagat *et al,* 2012).

These interesting cases highlight the possible immunopathogenic mechanism of glomerular and tubular damage either by immune complex-mediated mechanism or cytokine-mediated tubular injury.

Autoimmunity induced by molecular mimicry is another possible mechanism for dengue-associated renal disease since this phenomenon has also been reported in other viral infections, namely coxsackie virus and Epstein-Barr virus (Lin *et al,* 2011). Autoantibodies against platelets, endothelial cells, and coagulatory molecules have been demonstrated in patients with dengue virus infection and are believed to result from cross-reactivity to dengue virus antigen; that is, NS1, prM, and E proteins, respectively. These antibodies can cause platelet dysfunction,

endothelial injury, and coagulopathy upon binding to their corresponding antigens. An example of autoimmunity and AKI came from a report of an elderly woman presenting with DHF and AKI in Honduras with serologically and pathologically confirmed anti-glomerular basement membrane (GBM) disease in association with positive P-ANCA, and anti-myeloperoxidase antibody on serologic studies (Lizarraga *et al,* 2015). Development of auto-antibodies to GBM or neutrophil antigen is believed to be induced by environmental factors including infection (Tarzi *et al,* 2011), which was possibly dengue virus infection in this Honduran patient.

Hemolytic-uremic syndrome

Hemolytic-uremic syndrome is another rare manifestation of dengue infection. There have been three reported cases (Wiersinga *et al,* 2006; Hadianto and Mellyama, 2011; Aroor *et al,* 2014). Patients presented with a triad of thrombocytopenia, hemolytic anemia, and AKI. One patient had renal biopsy, and it found microthrombi in the glomeruli. Electron microscopy showed microtubuloreticular structure, suggesting viral infection (Wiersinga *et al,* 2006).

Abnormal urinalysis

There is an abnormal urinalysis in up to 90%, increasing with dengue severity, mostly self-limiting subnephrotic range proteinuria in up to 71% and microscopic hematuria in up to 80% (Futrakul *et al,* 1973; Kuo *et al,* 2008; Lumpaopong *et al,* 2010; Vachvanichsanong *et al,* 2010).

There are four cases of reported nephrotic-range proteinuria without nephrotic syndrome that disappeared on resolution of dengue virus infection suggesting association of proteinuria with the disease process (Vasanwala *et al,* 2009; Hutsopardol *et al,* 2011; Hebbal *et al,* 2016). Moreover, patients who developed significant proteinuria defined by spot urine protein creatinine ratio of 0.2 g/g or more had association with higher degree of thrombocytopenia compared to those without significant proteinuria and higher peak proteinuria (0.56 *vs* 0.08 g/day; *p*<0.001) was associated with the development of DHF/DSS. As

a result, monitoring of urine protein to identify peak proteinuria has been proposed to predict development of DHF/DSS (Vasanwala *et al,* 2011).

Microscopic hematuria must be differentiated between glomerular and non-glomerular hematuria due to bleeding disorder or catheterization. Glucosuria, ketonuria, and abnormal tubular casts were also found, suggesting tubular injury after dengue viral infection (Futrakul *et al,* 1973).

Glomerulopathy

Multiple evidences from animal and human studies supported the causal relationship between dengue virus infection and various types of glomerulopathy. Barreto *et al* (2004) demonstrated in mice infected by dengue virus type 2 that 48 hours after the onset of infection, there were glomerular enlargement, endocapillary and mesangial hypercellularity together with glomerular IgM deposition. Similarly, Boonpucknavig *et al* (1981) studied renal histopathology in dengue virus type 2-infected mice at the end of the third week of infection and observed immune-complex deposition as well as proliferative lesions in the glomeruli.

Another study demonstrated IgG, IgM, and C_3 deposition in 50% of patients with dengue virus infection and renal abnormalities (Boonpucknavig *et al,* 1976). Ultrastructural examination under transmission electron microscope revealed glomerular immune-complex-type deposits associated with mesangial cell hypertrophy and the presence of dense spherical particles ranging from 40-50 nm in diameter, possibly dengue virus particles, in 60% of patients.

Other pathological patterns have also been reported. IgA-dominant immune complex deposition with mesangial proliferation presenting with hematuria and proteinuria was documented with resolution of mesangial proliferation and IgA deposition six weeks later (Upadhaya *et al,* 2010). One patient presented with rapidly progressive glomerulonephritis with serum positive for anti-myeloperoxidase and anti-glomerular basement membrane (GBM) antibodies. Renal biopsy revealed diffuse crescentic glomerulonephritis.

Immunofluorescence examination demonstrated strong linear IgG deposition along capillary walls. A diagnosis of anti-GBM with anti-neurtophilic cytoplasmic antibody (ANCA) was made (Lizarraga *et al,* 2015). The proliferative pattern with hypocomplementemia has also been reported (Bhagat *et al,* 2012). Another young female presented with nephritonephrotic features with hypocomplementemia. Renal biopsy showed diffuse proliferative glomerulonephritis consistent with lupus nephritis (Rajadhyaksha and Mehra, 2012). Table 2 summarizes the pathological patterns of dengue virus infection in each structure of the kidney.

Electrolyte abnormalities

Electrolyte disturbances are common in dengue infection, yet might be frequently unreported. Higher electrolyte imbalances were associated with dengue severity: 25.1 % with DF, 33.5 % with DHF, and 39.8 % with DSS (Vachvanichsanong *et al,* 2015). The most common electrolyte disturbances are hyponatremia and hypokalemia (Futrakul *et al,* 1973; Kuo *et al,* 2008; Lumpaopong *et al,* 2010; Bunnag and Kalayanarooj, 2011). Hyponatremia may be from plasma leakage, hypotonic therapy, or renal loss. Hyponatremia was reported in 66% of 150 children. Fifty percent of the cases had mild hyponatremia (serum Na 130-134 mEq/l), 14.7% had moderate hyponatremia (serum Na 125-129 mEq/l), and 1.3% had profound hyponatremia (serum Na <125 mEq/l) (Lumpaopong *et al,* 2010). Hyperkalemia is possibly due to rhabdomyolysis or AKI. Interestingly, a case series by Bunnag and Kalayanarooj (2001) reported a high prevalence of hypocalcemia in patients with DSS. Metabolic acidosis is another frequent complication found in 8.6-14% possibly due to hypoperfusion or hyperchloremia after saline infusion (Lumpaopong *et al,* 2010; Vachvanichsanong *et al,* 2015).

Management

In the vast majority of cases, dengue-associated renal disorders are self-limiting. Intensive monitoring for early diagnosis of complications and supportive care according to dengue staging is the mainstay of patient care. Blood urea nitrogen, creatinine, and creatinine-kinase phosphate

Table 2. Renal pathology in dengue infection.

Tubules

- Red blood cells in tubular lumens
- Acute tubular necrosis

Glomeruli

- Immune complex of IgG, IgM and C_3 deposited in the glomeruli also with focal thickening of the glomerular basement membrane and mesangial hypertrophy
- Glomerular enlargement as well as increased endocapillary and mesangial cellularity
- Proliferative lesions
- IgA, lupus nephritis, and anti-GBM (rare)

Interstitium

- Acute interstitial nephritis

Vessels

- Thrombotic microangiopathy

monitoring is advisable. Frequent volume status assessment and judicious fluid administration is mandatory. Based on the three randomized controlled trials in children, colloid has no clear advantage over crystalloid regarding the overall outcomes. Therefore, crystalloid is still the fluid of choice in severe dengue infection. Colloid, however, may restore blood pressure rapidly in patients with refractory shock with a pulse pressure less than 10 mmHg (Dung *et al*, 1999; Ngo *et al*, 2001; Wills *et al*, 2005). The amount of fluid should be minimized to maintain hemodynamic stability but prevent plasma leakage. Corticosteroid is not recommended for severe dengue (Zhang and Kramer, 2014). Renal replacement therapy (RRT) should be started in patients with persistent volume overload, refractory/severe hyperkalemia, refractory acidosis, or uremia despite a maximally conservative strategy. There are no data regarding time-to-initiate, dosing, or modes of RRT in severe AKI, which suggests further studies as to whether continuous RRT for gradual volume removal can improve clinical outcomes are needed. Hemodialysis maybe preferred to peritoneal dialysis due to bleeding disorder accounted by dengue virus infection, which may prohibit Tenchkoff catheter insertion.

CONCLUSION

AKI following dengue viral infection is one of the most significant complications of dengue. The incidence of AKI increases with more severe dengue. Apart from ATN, rhabdomyolysis and hemolytic-uremic syndrome are rare but deadly adverse events. Non-specific proteinuria and hematuria can occur in dengue infection although some patients experience acute glomerulonephritis. Electrolytes must be monitored because dysnatremia, dyskalemia, and acid-base disturbances are common. RRT should be initiated as per conventional indications. Finally, further studies should focus on clarifying the pathogenesis of AKI in dengue and using novel biomarkers to assist in improving clinical outcomes.

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CRITICAL CARE IN DENGUE MANAGEMENT

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Abstract. Dengue is one of the re-emerging infections in the Tropics. There is no specific drug to treat this condition. Supportive treatment including hemodynamic optimization, fever control, and prevention end organ injury is the only available treatment. Therefore, every suspected/confirmed dengue patients should be assessed for fluid status. Lactate and bedside ultrasound has been applied to detect plasma leakage early in severe dengue infection. Currently, dynamic parameters (stroke volume variation, pulse pressure variation, inferior vena cava (IVC) collapsibility index, passive leg raising test, and end expiratory occlusion test) predict the fluid responsiveness better than static parameters (central venous pressure, pulmonary capillary wedge pressure). If the patient shows signs of dehydration, the fluid of choice is still crystalloid rather than colloid. Norepinephrine is still the vasopressor of choice. Finally, the target mean arterial pressure (MAP) should be at least 65 mmHg except in chronic hypertension patients who required a MAP of at least 80 mmHg.

Keywords: critical care, dengue, adult

INTRODUCTION

Dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) are among the most common causes of hospital admission, death, and disability in children in the Tropics. Recently, the age group of dengue infection has shifted to adolescents and adults. Data from Southeast Asia have shown that the mean age of reported dengue cases has increased from 5-9 years to older children and adults. In Thailand, affected adults over 15 years of age comprise 30-40% of dengue cases (Chareonsook *et al*, 1999; Tantawichien, 2000; Pongsumpan *et al*, 2002; Kularatne *et al*, 2005).

Plasma leakage is the hallmark of severe dengue infection and leads to DSS. Until the present time, there has been no specific treatment for this condition. Hemodynamic optimization is the only mainstay treatment as supportive treatment during

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this critical period. The aim of this article was to review the most up-to-date knowledge of critical care management focusing on fluid management, choice of vasopressor, and target blood pressure in severe adult dengue infection.

FLUID ASSESSMENT IN DENGUE PATIENTS

Every patient who is a suspected/confirmed dengue infection should be assessed for volume status as part of hemodynamic optimization. Volume depletion is associated with poor clinical outcome. On the other hand, some suspected/confirmed dengue patients can present with clinical of volume overload. In practice, we define fluid overload condition as a difference between cumulative fluid intake and cumulative fluid output, divided by initial body weight (Bouchard *et al*, 2009). A combination of history taking including medications, physical examination, laboratory testing, and hemodynamic parameters both static and dynamic should still be performed to obtain the best information for fluid assessment.

Clinical variables used for fluid assessment include baseline body weight, history of recent fluid loss, cumulative fluid balance, vital signs, urine

output, capillary refill, and skin turgor. Several trials have shown the limitation of static hemodynamic parameters such as central venous pressure (CVP) and pulmonary capillary wedge pressure in guiding fluid responsiveness (Osman *et al*, 2007). One of the explanations of the limitation of static hemodynamic parameters is heart contractility. At the same CVP, a patient who has normal heart contraction might still be at the steep part of the Frank-Starling curve and still respond to fluid loading. However, a patient who has impaired heart contractility might stay at the plateau phase of Frank-Startling curve and not respond to fluid loading. Therefore, the interpretation of these parameters should be cautious. Dynamic hemodynamic variables including stroke volume or pulse pressure variation, change in vena cava diameter, and passive leg raising test have been introduced as part of clinical decision-making during fluid assessment, and these variables have shown superior results compared with static hemodynamic variables (Feissel *et al,* 2007; Gruenewal *et al,* 2011). However, no study has shown the superiority in major clinical outcome of any particular method. Therefore, a combination of all data of these variables should be used to make a decision on fluid administration.

Recently, blood lactate, which represents global tissue oxygenation, has been introduced into the dengue research field. Thanachartwet *et al* (2016) has studied the role of lactate to predict dengue progression. Plasma lactate was tested on the first day of admission and revealed an area under the curve of 0.84 for identifying severe dengue. At the optimal cutoff value (plasma lactate 2.5 mmol/l), the sensitivity and specificity were 65.0% (95% CI: 40.8-84.6%) and 96.2% (95% CI: 90.5-99.0%), respectively.

Clinical reassessment is one the key concepts of fluid administration. It is becoming apparent that the concept of "one size fits all" cannot apply to fluid therapy in suspected/confirmed dengue patients. The amount of fluid should be based on requirements of the individual.

Careful fluid assessment can be performed many ways depending on the site of care and stage of the disease. We propose the minimum parameters/treatment for fluid administration in Table 1. In the primary care setting, initial fluid management combined with simple bedside physical examination should be applied. In the ICU setting, complex testing such as dynamic hemodynamic parameters should be used. In any setting, we recommend that the clinician/ health care personnel should reassess the clinical response as soon as possible (within a few hours) without leaving the bedside. If the patients do not respond within a few hours (possibly within a 6-hour period), we recommend escalate care or transfer out to a tertiary care hospital.

Role of ultrasound for fluid assessment in dengue patients

Ultrasound has recently been introduced into the field of dengue. We can apply ultrasound as a tool for early detection of fluid leakage and for guiding fluid treatment. By lung ultrasound, fluid leakage may be evidenced by the B line sign (sign of interstitial edema) (Fig 1), sign of pleural effusion, or sign of pericardial effusion (Fig 2). By abdominal ultrasound, fluid leakage may be evidenced by gallbladder wall thickness as well as fluid at the hepatorenal or splenorenal pouches. These parameters might be incorporated into WHO warning signs in the future. Parameters obtained from ultrasound such as inferior vena cava (IVC) collapsibility index, IVC distensibility index, IVC variability index, and stroke volume variation can be applied to guide fluid therapy. This avoids unnecessary or overuse of fluid administration.

FLUID OF CHOICE IN DENGUE INFECTION

This section will compare the evidence base of colloid *vs* crystalloid fluid and of balanced crystalloid fluid *vs* non-balanced crystalloid fluid for fluid administration in dengue infection. Unfortunately, there have been few studies that have directly compared fluid type in dengue patients.

Crystalloid *vs* colloid
Colloid has been widely used for fluid therapy in the critical care setting during the past few years

Table 1. Minimum treatment and parameter requirements in dengue patients based on site of care.

Treatments/Parameters	Community setting	Hospital setting	ICU setting
Fluid challenge	✓		
Mental status	✓		
Hemodynamic parameters: HR, BP	✓		
Capillary refill	✓		
Urinalysis	✓		
UOP, fluid balance	✓		
Lactate		✓	✓
Dynamic pressure parameters such as PPV, SVV, IVC collapsibility, PLR testing			✓
Static pressure parameters such as CVP			✓
Echocardiogram			✓
Cardiac output monitoring			✓
ScvO2			✓

BP, blood pressure; CVP, central venous pressure; HR, heart rate; IVC, inferior vena cava; PLR, passive leg raising test; PPV, pulse pressure variation; SVV, stroke volume variation.
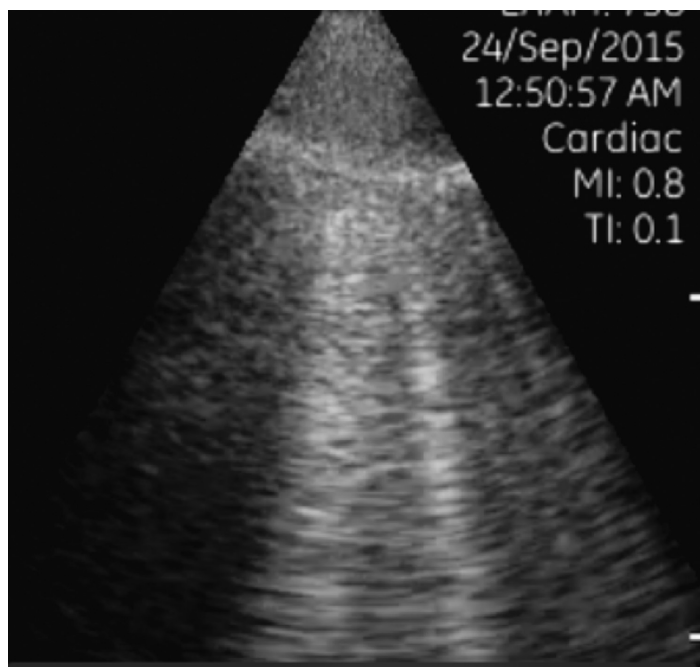

Fig 1–B-line in dengue patients, which is a kind of comet-tailed artifact indicating subpleural interstitial edema.
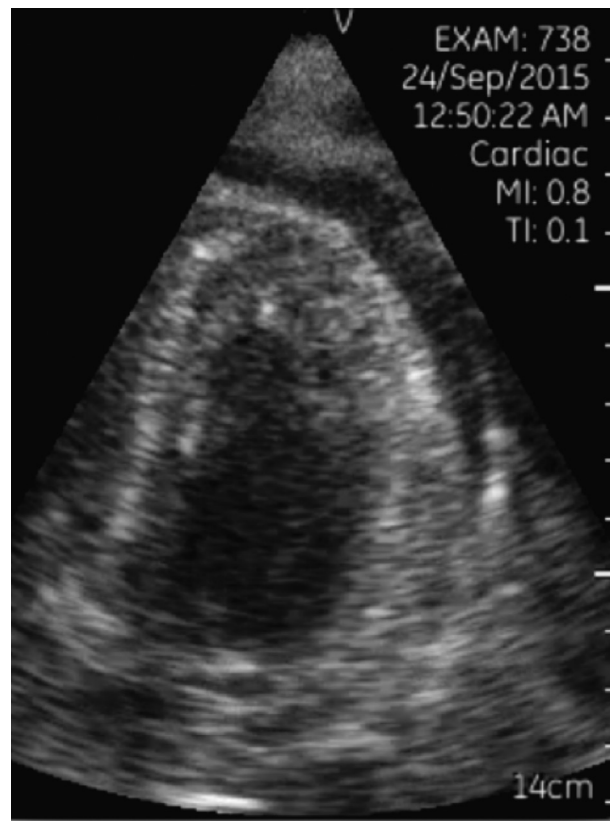
Fig 2–Pericardial effusion due to pericarditis in dengue patients.

(Finfer and Vincent, 2013). One of the main reasons is based on physiologic models. Starling's model assumes colloid can maintain intravascular volume better than crystalloid. The amount of colloid used for fluid resuscitation was expected to be around three times less than the amount of crystalloid (Starling, 1896). However, the assumptions of the Starling's model have been challenged by the endothelial glycocalyx (EG) model. In this model, the vascular integrity is maintained by EG located on the luminal side of vascular endothelium. EG will be directly damaged by process of systemic inflammation and lead to vascular leakage and finally tissue edema. Based on this model, there should be no difference in efficacy among fluid types in holding intravascular fluid when EG has been damaged during process of sepsis. Therefore, the amount of fluid may be more important than the type of fluid to prevent interstitial edema (Varadhan and Lobo, 2010).

Since the publication of two large randomized controlled trials (RCTs), namely, the 6S and CHEST trials 4 years previous, the use of hydroxyethyl starch (HES) has been restricted by regulatory authorities because of its potential for worsening kidney function (Myburgh et al, 2012; Perner et al, 2012). The 6S trial studied fluid optimization in severe sepsis/septic shock patient. Third generation HES, 6% HES 130/0.4 increased the primary composite end point (dead or dialysis dependent rate on day 90) more than Ringer's acetate (51 vs 43%, respectively; p=0.03). Also, the HES group had a higher incidence rate of renal replacement therapy (RRT) than the Ringer's acetate group (22 vs 16%, respectively; p=0.04). The CHEST trial studied fluid optimization in ICU patients. There was no difference of mortality rate between HES and saline, but the HES group had a higher incidence rate of RRT than saline group (7 vs 5.8%, respectively; p=0.04).

In theory, human albumin is the main protein for maintaining plasma colloid oncotic pressure. It also works as a carrier for several endogenous and exogenous compounds with antioxidant and anti-inflammatory properties. Also, albumin can act as a buffer molecule for controlling acid-base homeostasis (King, 1961; Sudlow *et al*, 1975; Weil *et al*, 1979; Quinlan *et al*, 1998; 2005). The results from large RCTs such as the SAFE study in the ICU setting and the latest ALBIOS study of severe sepsis/septic shock have not shown the benefit of human albumin over crystalloid. In addition, there was no difference of renal outcome between human albumin and crystalloid in both studies (Finfer *et al*, 2004; Caironi *et al*, 2014). It appears to be safe for the kidney to use albumin in the high-risk setting. With high cost and no obvious advantage over crystalloid, human albumin should not be used as the first line therapy.

There are few studies that have compared crystalloids to colloids use in dengue infection. Wills *et al* (2005) conducted a double-blinded RCT of three fluids, Ringer's lactate, 6% dextran 70, and 6% HES, for initial resuscitation in Vietnamese children with DSS. There was no difference in the primary outcome that was rescue colloid administration at any time during the study.

Balanced crystalloid solution *vs* non-balanced crystalloid solution

There are several studies that have addressed the adverse effect of non-balanced crystalloid solution (isotonic saline) on the kidney (Hadimioglu *et al*, 2008; Khajavi *et al*, 2008; Hasman *et al*, 2012). Isotonic saline contains 154 mmol/l of chloride, so its administration with a large volume can result in hyperchloremic metabolic acidosis. This condition can lead to renal vasoconstriction, decreased renal artery flow velocity, decreased renal artery blood flow, afferent arteriole vasoconstriction, and finally decreased glomerular filtration rate (Wilcox *et al*, 1983). Current evidence from three large observational studies has also suggested that the high chloride content of isotonic saline may cause harm, especially to the kidney. A study of 30,994 adult patients undergoing major abdominal surgery found that patients receiving isotonic saline had significantly greater blood transfusion requirements, more infectious complications, and more renal support requirements than those receiving balanced crystalloids (Shaw *et al*, 2012). However, there was no difference in mortality rate between the two groups.

Yunos *et al* (2012) conducted an open-labeled, prospective sequential study comparing between traditional chloride-rich solutions (isotonic sodium chloride, 4% succinylated gelatin solution, or 4% albumin solution) and chloride-restricted fluids (Hartmann's solution, Plasma-Lyte 148 or chloride-poor 20% albumin). After adjusting for confounding variables, the chloride-restricted group had a decreased incidence of acute kidney injury [AKI] [odds ratio (OR)=0.52, $p<0.001$] and reduced use of RRT (OR = 0.52, $p=0.004$). Again, there were no differences in hospital mortality as well as hospital or ICU length of stay. Also, a study by McCluskey *et al* (2013) on postoperative patients showed that the incidence of acute postoperative hyperchloremia was 22%.

Patients with hyperchloremia were found to be at increased risk of 30-day postoperative mortality (3.0 *vs* 1.9%; OR=1.58), have a longer length of hospital stay, and were more likely to have postoperative renal dysfunction (McCluskey *et al*, 2013). These large observational studies suggest that it may be time to consider the use of balanced crystalloid solution as the fluid of choice, especially in metabolic acidosis. However, the SPLIT trial, the largest RCT aiming to compare the effect of balanced crystalloid and non-balanced crystalloid on kidney injury, did not show the difference of AKI incidence within 90 days between Plasma-Lyte 148 solution and isotonic saline (9.6 *vs* 0.2%, $p=0.77$). Moreover, no differences of RRT incidence rate and hospital mortality rate between two groups were found. However, it is noteworthy that the incidence of AKI in this study was quite low, and this low incidence rate might have caused difficulty in demonstrating the effect of isotonic saline on AKI outcome (Young *et al*, 2015). Therefore, it is too early to conclude that isotonic saline has no harmful effect of on kidney function based on only this RCT.

In resource-limited settings such as middle to low income countries, isotonic saline could still be the crystalloid of choice for fluid resuscitation in dengue infection.

Amount of fluid, choice of vasopressor, and target blood pressure in dengue patients

Hemodynamic alteration in severe dengue infection is not the same as in other severe sepsis/septic shock cases. Ranjit *et al* (2007) compared hemodynamic parameters between DSS and severe sepsis/septic shock in 32 patients (16 DSS and 16 severe sepsis patients). The DSS patients presented with narrower pulse pressure (25±8 *vs* 43±8 mmHg; *p*<0.01), less presence of systemic inflammatory response syndrome, (9/16 *vs* 15/16; *p*<0.05), and less requirement of fluid administration (28.5 *vs* 57.5 ml/kg; *p*=0.03).

Since the publication of Early Goal-Directed Therapy (EGDT) study by Rivers *et al* (2001), the concept of protocolized strategy that comprises of fluids, vasopressor, and blood transfusion targeting hemodynamic parameters has been widely adopted (Dellinger *et al,* 2008). The average fluid administration during the first 72 hours in this single-centered study was 13 liters. However, during the past few years, there have been studies that have shown the adverse effect of fluid overload to patient outcome (Bouchard *et al,* 2009). Three large RCTs studies have supported the concept of restricted fluid therapy, namely the PROCESS study (Yealy *et al,* 2014), ARISE study (Peake *et al,* 2014), and ProMISe study (Mouncey *et al,* 2015). These studies compared the mortality between protocolized care and usual care in sepsis patients and showed only 3 to 4 liters of fluid intake during the first 72 hours. All of these three major RCTs also suggested that protocolized therapy and the usual care provided a comparable outcome. This emphasizes the concept that *the amount of fluid to be given should be individualized based on the initial assessment of volume status and clinical background/associated co-morbidities.*

Concerns have been raised about the use of fluid bolus following the Fluid Expansion As Supportive Therapy (FEAST) study by Maitland *et al* (2011). African children who suffered from severe sepsis (mainly malaria) were randomized to receive no fluid bolus, or to receive fluid bolus with either isotonic saline or albumin. At 48 hours, patients who received fluid bolus had higher mortality compared with control patients (relative risk 1.45, *p*=0.003). However, this trial was conducted in resource-limited setting with no access to ventilation to optimize the management of sepsis.

The role of oral fluid administration should be considered in the community setting (Harris *et al,* 2003). This strategy, accompanied by thorough clinical assessment, could decrease the rate of hospitalization. In mild dengue infection, ingestion of fluid in the 24 hours before visiting the clinician was found to be protective against hospitalization after adjusting for distance from health facility, date of symptom onset, and thrombocytopenia (OR=0.74 per each additional glass consumed, *p*<0.01). The most common liquid ingested was water (70%), followed by fruit juice (42%), lemonade (27%), milk (25%), coffee (14%), oral dehydration serum (6%), and tea (2%).

Vasopressor is the essential treatment to achieve the hemodynamic goal after the intravascular volume restoration. Persistent hypotension after initial fluid administration places the patients at risk for organ injuries such as kidney injury, bowel ischemia, and shocked liver. There has been no clinical study to show which vasopressor agents (norepinephrine, dopamine, and vasopressin/terlipressin) is the most effective for prevention or treatment of AKI patients.

A study comparing the efficacy of norepinephrine and dopamine did not show the difference in mortality and AKI incidence between the groups (De Backer *et al,* 2010). However, the use of dopamine in a subgroup of patients with cardiogenic shock from this study was associated with more adverse events such as cardiac arrhythmia.

Vasopressin is another potent vasopressor agent that works at vasopressin receptor of smooth muscle cell. This vasopressor has become more popular in treating shock that is refractory to

norepinephrine (Delmas *et al*, 2005). Compared to norepinephrine, vasopressin increases blood pressure, enhances diuresis, and may lower the rate of AKI progression, but it has neither been proven to enhance survival nor to reduce the need for RRT (Russel *et al*, 2008; Gordon *et al*, 2010).

The Surviving Sepsis Campaign: International Guidelines for Management of Severe Sepsis and Septic Shock: 2012 recommends initial resuscitation with vasopressors to reverse hypotension with a mean arterial pressure (MAP) target of at least 65 mm Hg (Dellinger *et al*, 2013). This recommendation is based on previous studies that have shown no significant difference in lactate level or regional blood flow if the MAP was elevated to more than 65 mmHg in patients with septic shock (LeDoux *et al*, 2000). The kidney is one of the organs prone to compromised blood supply when decreasing MAP. Recently, a large retrospective study shows that a MAP of more than 75 mmHg may be required to maintain kidney function (Dünser *et al*, 2009).

The SEPSISPAM investigator group has conducted a multicentered, open-labeled RCT in patients with septic shock undergoing resuscitation with a MAP of either 80-to-85 mmHg or 65-to-70 mmHg. There was no difference in mortality rate between the two targets MAPs. However, patients in the high-target MAP group with chronic hypertension required less renal-replacement therapy and less doubling of serum creatinine than those in the low-target group (Asfar *et al*, 2014).

ACKNOWLEDGEMENTS

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Severe Dengue

CHAPTER 5

Dengue prevention and control

- Dengue vector control: assessing what works?
- Prospects for the development of a dengue vaccine
- The estimated impact and cost-effectiveness of dengue vaccination

DENGUE VECTOR CONTROL: ASSESSING WHAT WORKS?

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Abstract. Primary prevention of dengue remains difficult, and continues to be difficult, relying mostly on vector control, with historical success, but lately there is also a partially effective vaccine. Vector control may continue to play a role, with the most efficacious and effective vector control methods. To establish this, high level evidence such as systematic reviews have been developed for applied vector cotrol methods, but also on service delivery. The systematic reviews followed the PRISMA statement. For single vector control interventions work has been undertaken on peridomestic space spraying, Temephos, *Bacillus thuringiensis israelensis*, Copepods and larvivorous fish. Further work is currently published on pyriproxifen and indoor residual house spraying (IRS). For a particular service delivery, there is existing work on outbreak response and on vector control service delivery. Nearly all vector control methods showed excellent results in at least one study, either on larvae, or adults, or even perhaps on dengue transmission: 1) Vector control can be effective, implementation remains an issue, including delivery structures, 2) Single interventions are probably not useful, efficacy varies, with little sustainability, 3) Combinations of interventions have mixed results, 4) Interventions are often applied in outbreaks (compared to routine vector control), effectiveness is also questionable, 5) Key elements for more effective vector control measures may be timely alerts of outbreaks, followed by immediate vector control measures, including health promotional campaigns, 6) Careful implementation may be most important.

Keywords: dengue, vector, control methods

INTRODUCTION

In light of the ongoing global Chikungunya, dengue, yellow fever and Zika outbreaks, vector control of *Aedes aegypti* and *Aedes albopictus* mosquitos has received more attention. This review summarizes existing high-level evidence, such as systematic reviews, for dengue vector control, and updates a previous review on the topic (Horstick and Runge-Ranzinger, 2015).

Whereas secondary and tertiary prevention strategies for dengue are improving, with low case fatality rates in most countries (WHO/TDR, 2009), primary prevention strategies have yet to demonstrate significant progress, with an estimated 390 million infections each year (Bhatt *et al*, 2013). The first dengue vaccine is now commercially available, but it is only partially effective, with an estimated efficacy of 47-83% against the four dengue serotypes (Hadinegoro *et al*, 2015). Prior to vaccine introduction, vector control was the only available method for primary prevention of dengue. Vector control strategies have shown some success to control dengue (Gubler, 2011), most notably in the past in Cuba and Singapore, but for most countries, vector control strategies have produced mixed results. Even with the introduction of the first vaccine, vector control will likely continue to play a role in dengue prevention. Further studies, utilizing the most efficacious and effective vector control methods (Reiner *et al*, 2016) should be conducted to test for possible synergies between these two approaches.

Dengue vector control comprises chemical, biological and environmental methods (WHO,

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TDR, 2009) targeting adult or larval stages of mosquitoes. Chemical methods can generally be classified into 1) the use of insecticides for residual sprayings, both intra-domiciliary (including IRS) or peri-domestic, 2) the use of long-lasting insecticide treated materials (ITM), including insecticide treated nets (ITN) or curtains (ITC) and 3) control of larval breeding to include the application of Temephos or pyriproxyfen in breeding sites. Chemical control of dengue vectors, however, has limitations, including environmental contamination, bioaccumulation of toxins, concerns regarding human toxicity, and the potential development of resistance in the vector. Biological methods to control larval stages include *Bacillus thuringiensis israelensis* (Bti), or the introduction of larvivorous fish and copedods. Environmental management strategies attempt to eliminate productive breeding habitats, *eg*, emptying of water containers, waste disposal, provision of piped water or employ physical barriers against mosquito vectors, such as window screens and water container covers. However, the latter approaches are often combined with the use of insecticides. There are other methods that are not currently used in large scale control programs, such as the introduction of the bacteria *Wolbachia* and/or genetically modified mosquitoes with the intent of replacing and/or reducing the naturally occurring vector with vectors that have a limited capacity to reproduce and/or to transmit the dengue virus. Integrated control measures have also been developed in the context of Integrated Vector Management (IVM) (WHO, 2004), with possible synergies between chemical, biological, and environmental approaches (Horstick and Runge-Ranzinger, 2017).

Summary evidence–systematic reviews and meta-analyses–helps to assess the efficacy and community effectiveness of interventions and should provide clear policy recommendations for or against the use of such interventions (Moher *et al*, 2009). However, very little summary evidence exists for neglected tropical diseases such as dengue (Nagpal, 2013). A previous meta-analysis examining dengue vector control methods highlighted the efficacies of each approach (Erlanger *et al*, 2008). The constraint of meta-analyses is that they are limited to studies of comparable design and outcome measures, and thus exclude many published studies. We hypothesized that further analyses of vector control methods for the control of dengue vectors with systematic reviews (SR), rather than meta-analysis, may contribute to a better understanding of the value of vector control for primary prevention of dengue.

This review summarizes the findings of our efforts to use systemic reviews of published, high-quality scientific literature in order to determine:

1) The efficacy and/or community effectiveness of each vector control method,
2) the efficacy and/or community effectiveness of combinations of vector control methods,
3) existing research gaps, and
4) practical recommendations concerning the implementation of vector control strategies to reduce dengue transmission.

MATERIALS AND METHODS

Following up with individual SRs on the existing meta-analysis of dengue vector control methods (Erlanger *et al*, 2008), the author designed a framework to describe dengue vector control methods in the 2009 WHO dengue guidelines (WHO/TDR, 2009). The framework has been adapted towards three levels of on-going research: 1) vector control methods, including biological, chemical or environmental, 2) vector control of a particular service function, *eg*, outbreak detection and response, and 3) organization of vector control services.

The full methods for each reported SR are presented in the original articles, however, the methods followed the PRISMA statement (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) (Moher *et al*, 2009), with preformulated study objectives, searches on all relevant databases, combinations of categories of search terms, documentation of data searches to obtain the PRISMA flowchart, screening by title of potential hits, screening by abstract when relevant to the topic, removal of duplicates, retrieval of full articles to apply full inclusion and exclusion

criteria, searches for further references from the bibliographies of included articles and searches of grey literature. Two data extractors independently conducted the searches, and extracted relevant information into predefined data extraction forms, which became the evidence tables for each of the respective studies.

Study quality was assessed using the validated tools appropriate to the study type. Given the limited number of published studies in most areas, study quality was rarely a reason for exclusion, but was addressed in the reporting and subsequent discussion sections. If study quality was used to exclude a study, the quality assessment was summarized in a table, scored and taken into consideration for analysis.

Studies were often classified into efficacy studies - those that were performed under laboratory conditions - and community effectiveness studies-those that were conducted under program-like conditions. The descriptive part of the analysis was performed plotting the included studies against the geographical background and describing clustering over time. The study types and outcome measures used were described. For the analytical components, study results were summarized for vector and human disease outcome measures. For the former, results were compared to estimates needed for a potential reduction of transmission. The discussion sections followed content analysis methods, using categories that emerged from the analysis (Pope *et al*, 2000).

When using mixed methods, the results of stakeholder interviews and questionnaires were added to the data abstraction form. Methodology and analysis of the interviews followed the relevant standards (Pope *et al*, 2000). For questionnaires, these followed the same broad heading and topic areas as the interviews. Finally, information from all parts of the study was compared (Mays and Pore, 2000).

For the analysis of vector control and vector control methods in this study, studies have been included from the entire framework of published studies if relevant to the topic (dengue vector control) and were summarized according to the above-mentioned, predefined categories. Furthermore, implementation aspects derived from individual SRs were analyzed, with a view towards practical public health recommendations.

RESULTS

Descriptive analysis

According to the analytical framework, SRs have been published by the author on the following topics: 1) single vector control methods including peridomestic space spraying (Esu *et al*, 2010), Bti (Boyce *et al*, 2013), Temephos (George *et al*, 2015), copepods (Lazaro *et al*, 2015) and larvivorous fish (Han *et al*, 2015). Further work is in press on pyriproxifen (Maoz *et al*, 2017) and IRS (Samuel *et al*, 2017), 2) service orientated purposes: Outbreak control, including clinical and vector control responses (Pilger *et al*, 2010) and 3) organizational context of vector control: Vector control service organization (Horstick *et al*, 2010). These nine SRs on vector control are summarized below (see evidence tables of the SRs).

A total of 31,836 articles have been screened by title and abstract for inclusion. PRISMA flowcharts are included with the original articles. The authors assessed 430 full text articles and a total of 167 articles were included in the nine SRs. For the seven SRs describing single interventions for dengue vector control, there were 15 articles on peridomestic space spraying (Esu *et al*, 2010), 14 for Bti (Boyce *et al*, 2013), 27 for Temephos (George *et al*, 2015), 11 for copepods (Lazaro *et al*, 2015), 13 for larvivorous fish (Han *et al*, 2015), 17 for pyriproxifen (Maoz *et al*, 2017) and 7 for IRS (Samuel *et al*, 2017). Additionally, there were 24 articles included in the SR assessing outbreak control (Pilger *et al*, 2010) and 32 for vector control service organization (Horstick *et al*, 2010). For each SRs, there was ample evidence for meaningful analyses.

The SRs analyzed community effectiveness and/ or efficacy, as defined by the individual author of each review. Community effectiveness was the preferred outcome of interest, since the intent of the SRs was to provide valid, yet practical public

health recommendations. Of the nine SRs, eight included only community effectiveness studies, while one study (larvivorous fish) incorporated both outcomes in order to achieve a higher volume of studies (Han *et al*, 2015). However, this study did stratify results by efficacy and community effectiveness in both the reporting and discussion. We did observe a general trend across interventions that when efficacy has been tested and validated under laboratory conditions, studies focus more on community effectiveness.

Databases searched were fairly standardized across SRs with the majority including PubMed, EMBASE and WHOLIS, but often LILACS and Web of Science. More than 90% of all articles included were available on PubMed. Additional articles were more often identified from searches of the reference section of included articles, but seldom from the grey literature.

Inclusion and exclusion criteria were also fairly standardized, focusing on the respective research question, but also on study quality. For SRs focusing on vector control methods, an attempt was made to include only controlled studies, however, this was not always possible, depending on the number of studies identified during the search process. There is also a variation depending on the method tested. For example, the SR on *Bti* (Boyce *et al*, 2013) specified study duration in order to assess long-term effects of this method. The two service oriented SRs (Horstick *et al*, 2010; Pilger *et al*, 2010), required inclusion and exclusion criteria more specifically tailored around the research question.

Selected outcome measures of interest varied considerably for the SRs on vector control methods, and largely depended on the included studies. However, standard entomological indices including Breteau Index (BI), Container Index (CI), House Index (HI) and pupal indices are reported in most studies. It is also important to note that for most SRs there are studies measuring human transmission, although with very different measures of effect.

As for study types encountered for the SRs on vector control methods, these were mostly non-randomized controlled trials (NRCTs). However,

there was at least one randomized controlled trial (RCT) or cluster randomized controlled trial (cRCT) available for most methods, with the SRs on larvivorous fish and copepods (Han *et al*, 2015; Lazaro *et al*, 2015) being the exceptions. This is likely related to publication date, since most the cRCTs are more recent studies.

Analysis of vector control with SRs

SRs of single vector control methods. Among the chemical methods, peridomestic space spraying using various insecticides is commonly used to control dengue vectors, the popularity of which may be related to its high visibility (Esu *et al*, 2010). For the purpose of this SR, peridomestic space spraying was defined as the "application of small droplets of insecticide into the air in an attempt to kill adult mosquitoes in and around houses". Of the 15 included studies, 13 reported a reduction in entomological indices, typically around 90% for adult mosquitos post-spraying. This effect was not sustained and mosquito populations general returned to baseline levels within a few days to weeks. Two studies showed no reduction of entomological indices. The analysis also demonstrated that study designs and outcome parameters are heterogeneous, while measures of disease incidence are rarely reported. Even when incidence was measured, the study authors concluded that the observed reduction of cases could not be linked to the intervention. The SR showed that there is a short-term effect on adult mosquito populations, however there is no conclusive evidence for or against the use of peridomestic space spraying to control dengue.

In regard to IRS (Samuel *et al*, 2017), the SR author considered the use of all types of insecticides, although most studies utilised synthetic pyrethroids, with one study applying deltacide, a mixture of Deltamethrin 0.5%, S-Bioallethrin 0.75% and Piperonyl Butoxide 10%. The results of the seven included studies demonstrated that both adult and immature mosquito stages were suppressed, often by more than 90% and over sustained periods of time. The effect on immature mosquitoes is less strong on all studies measuring larval indices. For human dengue infection parame-

ters, there are only two IRS studies, but with good results. The SR concluded "…*evidence obtained from this systematic review showed that the use of IRS either solely or in combination with other control measures can produce significant reductions of Aedes populations (mature and immature forms). IRS can also produce reductions in human dengue cases.*"

Temephos to control larval breeding is one of the most commonly applied substances in larval habitats. The SR included single interventions with Temephos as well as combinations with other interventions (George *et al*, 2015). Of the 27 included studies, the interventions were as follows: 11 single intervention studies (Group 1) and 16 combinations (Group 2). No outcome measures to assess for changes in the incidence of human cases were incorporated in any of the studies. Group 1 showed that all 11 studies reported a post-intervention reduction in the immature stages with a prolonged effect of 4-8 weeks in the dry season and 6-12 weeks in the wet season, if regular re-application has been pursued. Combination interventions in Group 2 included Temephos with health education and information, environmental management and the use of malathion, *Bti*, or larvivorous fish. Ten studies reported a reduction of immature mosquito stages, while three failed to show an effect and three had only a very small effect. This was very surprising, given that the single intervention studies of Temephos showed clear evidence of community effectiveness. Operational issues may have been important, including surveillance and coverage, regular application, mode of application, acceptability and limited residuality of Temephos. The SR concluded "…*while there is little doubt concerning the effectiveness of Temephos in controlling Aedes breeding sites, the same level of effectiveness was not clear from the studies using Temephos combined with other interventions. This could be due to operational issues, delivering several interventions.*"

The final SRs for chemical methods reviewed the use of pyriproxifen (Maoz *et al*, 2017), and was unique in that it described the auto-dissemination effect of the intervention. Of 17 included studies,

two studies included human disease parameters including serological surveys (IgM) and dengue incidence. Studies were categorized by mechanism of application as follows: 1) container treatment studies: six studies showed a reduction above 80% of larval indices. However two RCTs showed a limited effect; 2) two fumigation studies in combination with Permethrin showed a good inhibitory effect; 3) studies measuring autodissemination showed good results of reduction of adult emergence between 20% and 85%, and 4) combination with adulticides seemed to increase overall effectiveness. Human transmission data were weak and could not demonstrate a significant effect. With these results, the evidence presented suggests that pyriproxifen can effectively control adult emergence of immature stages of dengue vector mosquitoes in a variety of breeding sites in a community setting and there is a clear consensus that pyriproxyfen effectively inhibits *Aedes* adult emergence at concentrations of <1 ppb. However, the SR concluded that "*more and larger studies with appropriate study designs and relevant, standardized outcome measures are needed; also, tolerance/resistance of vectors to pyriproxyfen has been reported* (,… and needs to be investigated)."

Bti is often classified with the chemical control options, although - being a bacterium - it is a biological substance. The SR on *Bti* (Boyce *et al*, 2013) analyzed 14 studies with *Bti* eliminating all larvae from treated containers within 24 hours, and for most containers there was a prolonged effect of 14 days. One study that measured an effect on human transmission showed only one case in the intervention area, compared to 15 in the control. No single formulations demonstrated superiority in the four studies testing these products. Higher doses of *Bti* showed a longer duration of effect in one study. Study design and quality need to be improved in future studies. The study concluded that "*there is evidence that Bti is effective in reducing the density of immature dengue vectors when it is applied to targeted containers as demonstrated by the efficacy studies. However, the evidence to suggest that Bti is effective as a single agent, when used in a community setting, is limited.*"

Other biological methods include the use of copepods and larvivorous fish. These methods carry the advantage that there are limited environmental effects. Furthermore, both Copepods and larvivorous fish are part of the natural food chain and re-application of the intervention is also necessary. The SR for Copepods (Lazaro *et al*, 2015) analyses 11 studies, The Copepods used were mostly *Mesocyclops spp*. Copepods controlled larval *Aedes* populations up to 100%. At the household level, reductions of households' positive for *Aedes* larvae between 30-97% were observed. When looking at adult mosquito landing rates and oviposition, reductions to zero were reported. Adult *Aedes* per household measurements showed reductions between 30 - 100%. Adult mosquito indices reductions from 0.12-1.16 to 0-0.01 per community after a period of three years were shown. Additionally, in three studies dengue transmission data were measured with results that ranged from zero reported cases in both the intervention and control communities to a 76.7 % reduction of dengue incidence, as determined by a reduction of serological parameters. However, the study also noted that there was a large geographical discrepancy in the results, with the positive studies having been conducted in one country only (Vietnam), by the same research team, while the success could not be replicated elsewhere. Also, study design and quality were again mentioned as issues. The study concluded "*the use of copepods as a single intervention may be a community effective and sustainable dengue vector control method to control dengue vectors and dengue transmission. However, this is perhaps only possible provided several specific criteria are met: as clearly shown in the fives studies conducted in Vietnam, these would include rigid delivery of intervention; development of community management committees and collaborators; efficient mobilization and sustained interest of the community residents.*"

Finally, the SR on larvivorous fish (Han *et al*, 2015) analyzed 13 studies. Eight of nine intervention studies showed a reduction of immature forms of dengue vectors. One study of three also showed a reduction of adult indices. Three of four before and after studies demonstrated a reduction of immature stages. A long-term decline over two years has been reported by the two studies measuring such an extended period. The studies measuring human transmission showed a reduction in the number of human cases, however, this must be interpreted cautiously as these were before and after study designs without a control and thus subject to temporal trends in dengue transmission. Study design and quality were an issue, and geographical coverage of studies. "*The findings suggest that the use of larvivorous fish, used as a single agent or in combination with other measures, can reduce significantly infestations of the immature vector stages. However, there is no evidence to demonstrate any community effectiveness of larvivorous fish as a single agent*" (…especially when considering human transmission).

SRs for a service orientated purpose. Outbreak response may be the most commonly performed program undertaken by public health services, since routine control efforts are difficult to achieve and sustain. In a SR for outbreak response, both vector management and clinical response (Pilger *et al*, 2010), including both single and combined interventions were considered. The 24 included studies could be broadly classified into 1) studies focusing on transmission reduction, 2) studies focusing on mortality reduction and 3) studies describing both. It became clear that there are different organizational strategies for an outbreak response, but the most common is an inter-sectorial approach. Multidisciplinary response teams, with vector control personnel working with communities, including monitoring and evaluation, resulted in good perceived outbreak control. Combined responses with 1) vector control (larval habitats interventions with communities; insecticides, intra- and peridomestic) and 2) capacity training for clinical response are successful. Spatial spraying of insecticides as a single intervention was generally not effective. However, the evidence level is weak, especially given the poor quality of the included studies. The SR concluded that "*outbreak response has to be organised multidisciplinary and monitored/*

evaluated. During outbreaks the above-mentioned interventions have to be implemented as a combined set of interventions in order to achieve rapid control. Further research is needed especially linking effectiveness of outbreak response to human disease epidemiology".

SRs of the organizational context of vector control. There is a longstanding discussion of the optimal delivery of vector control services, primarily debating vertical *vs* horizontal programs. However, the question of how the services are delivered, including resources and quality of delivery, is not well defined in the literature. A SR on the organizational context of vector control, including qualitative methods and integrating stakeholders' views (Horstick *et al*, 2010), addressed this question. Most services combine numerous interventions and therefore further investigation of selected interventions was not pursued. Of 32 included studies, nine were assessed to have relatively high study quality, with a clearly defined methodology, while 16 had less strict criteria. Additionally, there were three guidelines and four country case studies included. Three of the first group of nine studies showed little change of control operations over time. There were, however, strategic changes (decentralization, inter-sectorial collaboration). Including the results of all studies, staffing levels, capacity building, management and organization, funding and community engagement were found insufficient. It becomes evident that vector control services are not regularly analyzed and/or audited. The study concluded that *the analysis underlined the need for: 1) operational standards, 2) evidence based selection/delivery of combinations of interventions, 3) development/ application of monitoring and evaluation tools, 4) needs driven capacity building*.

Cross-cutting issues of all SIs

Study quality varied in this series of SRs, for both study design, specified outcome measures and data analysis, particularly the application of appropriate statistical analysis. This was a recurrent observation, with a tendency towards more complex and higher quality of studies with RCTs and cRCTs over time.

There is a pattern that particularly carefully implemented studies are more successful, recurrently quality of delivery of the intervention is an important item. These studies have higher-level study design, are often larger in size and implemented over a longer time period. This is also underlined by the fact that those SRs that included studies with multiple study arms, often find inferior efficacy and community effectiveness compared to studies with only one study arm.

The results of this series of SRs on feasibility, acceptability and costs are limited, since these issues were not part of the original search. However, the topics are recurrently discussed in the articles included in the individual studies of the SRs. A pattern emerges that acceptability is considered as one of the most crucial elements for study authors. Feasibility is mostly addressed in the context of different methods of application of a particular vector control method. Costs are not addressed in any the included studies.

DISCUSSION

Overall, the results of the SRs demonstrate the variable impact of dengue vector control methods under real world conditions and highlight the heterogeneous organization and operation of vector control services. One of the most important findings of this analysis is that almost all of the dengue vector control methods studied may have a role in the control of dengue vectors. Only peridomestic space spraying failed to show positive results. This confirms the results of a previous meta-analysis, in which the authors concluded that vector control "*is effective in reducing vector control populations,*" but do not comment on the potential reduction in human disease. In a more recent meta-analysis and systematic review (Bowman *et al*, 2016), analyzing vector control studies with a focus on studies measuring indices of human transmission, the study authors conclude that there is a general lack of evidence to suggest that vector control can reduce disease incidence.

The SRs included in this review show that for each vector control method studied there are examples of very successful trials, highlighting the

potential efficacy and community effectiveness of each method. In contrast, Erlanger *et al* (2008) singled out biological control methods as more efficacious than others and IVM performed best, while Bowman *et al* (2016) favored house screening and combining community-based environmental management and water container covers to reduce dengue risk. The different approaches of the respective analyses clearly yielded different results. We hypothesize that the implementation of the intervention, including rigorous methods and widespread coverage, are crucial. When stratifying by large and well-conducted trials only, a clearer picture of the community effectiveness of vector control may emerge.

Our findings suggest that when developing a strategy of IVM, clearly the local context needs to be considered, but if well delivered, most vector control methods may play a role. Future research is urgently needed to determine which social, environmental and entomological factors define the "best possible combinations of vector control methods" for different geographical areas. Targeting larval and adult stages of mosquitoes should result in improved transmission control: targeting both also implies combinations of interventions, especially when considering potential synergies for IVM. No systematic answer can be derived from the analysis of the SR's, apart from the fact that different combinations of interventions may need separate trials to ensure clear definition of most efficacious and community effective combinations of interventions in their local context. In other words, the recommendations for IVM, as described by WHO (2004), need more evidence.

Despite the lack of evidence to guide implementation programs, some basic criteria needs to be met in order for vector control interventions to be efficacious or effective in the community. The primary determinant of effect seems to be the quality of delivery, be it through community involvement or centralized vector control services. This analysis seems to underline the importance of the latter, particularly because combinations of vector control methods, even under relatively strict study conditions, can be difficult to deliver in a rigorous manner as evidenced by the fact that such approaches often have inferior results compared to well delivered, "single" method studies. Perhaps it is simply easier and more effective to deliver one method well, than to deliver several methods sub-optimally.

This analysis has several limitations with the potential for publication bias being the most significant. The substantial operational experience of national vector control programs is often not documented. However, we attempted to mitigate this potential limitation in each SR by including a search of the grey literature and a thorough examination of the reference section of each of the included studies. Prominent dengue entomologists and program managers were queried and also provided the authors with additional evidence that may not be readily available.

A further limitation is "updating" of SRs, since the results of the SRs are only valid in the context of their dates of literature searches. A systematic approach to SR updating would be the ultimate solution to this bias of the overall analysis. However, the simple fact that the group of authors are well embedded in the research community and are aware of upcoming and published studies, especially considering "game changing" studies, should limit this bias.

In summary, when considering the analysis of the SRs and the existing meta-analyses, nearly all vector control methods showed excellent results in at least one study, although outcome measures varied significantly,. Furthermore, we conclude that:

• Although vector control can be effective, implementation remains an issue. No clear evidence exists for optimal delivery structures of vector control services (Horstick *et al*, 2010).

• Single interventions are probably not useful, efficacy varies between different interventions, but sustained community-effectiveness can almost never be demonstrated (Esu *et al*, 2010; Boyce *et al*, 2013, George *et al*, 2015; Han *et al*, 2015; Lazaro *et al*, 2015; Maoz *et al*,2017; Samuel *et al*, 2017).

Table 1. Evidence table of systematic reviews on vector control published by authors.

Author/Year	Focus of SR	Efficacy or community effectiveness	Single intervention or combination	Study quality	Databases	Inclusion/Exclusion criteria	Studies Identified Assessed Included	Outcome measures	Study quality included	Main outcomes	Further outcomes	Conclusions
								Single vector control methods				
Esu et al, 2010	Peri-domestic space spraying	Community effectiveness	Single	cRCTs, RCTs, Quasi RCTs, Before and after studies (with or without control), Post-intervention studies with control	Medline EMBASE LILACS Web of Science WHOLIS MedCarib CENTRAL	Peer-reviewed studies Study quality All languages	2102 / 78 / 15	Indoor adult mosquito landing/ resting catches, oviposition trap counts, BI, CI, HI Human cases	1 RCT, 8 Controlled before and after studies, 1 Post intervention with control, 5 before and after studies without a control	13 studies reported a reduction in entomological indices, for adult mosquitoes around 90 % post-spraying, not-sustained (including the RCT), mosquito populations returning to normal levels with a few days/weeks. 2 studies showed no reduction of entomological indices Only one study assessed human disease parameters, with a reduction of number of cases. However this study was a before and after study	Outcome parameters are heterogeneous, and so is study design. Human disease parameters are mostly not measured	No conclusive evidence for or against the use of peridomestic space spraying
Samuel et al, 2017	IRS	Community effectiveness	Single and combinations	cRCTs, RCTs, Quasi RCTs, Before and after studies (with control), Cross-sectional studies	PubMed, EMBASE, LILACS, Web of Science, WHOLIS, Cochrane, Google Scholar	Peer-reviewed studies, including control groups, pre- and post-intervention assessments, cross-sectional studies, no language restrictions	825 / 39 / 7	Larval indices: BI, CI, HI Adult indices: KD rates Adult mosquito densities % adult mosquito mortality Mean % mortality of adult mosquitoes Human transmission: Age adjusted dengue incidence Odds of secondary dengue infections Number of confirmed and reported cases	7 studies: 5 controlled studies, 2 for IRS and 3 for ISS (indoor space spraying) 2 non-controlled studies (1 each IRS/ISS) Controlled studies were subsequently classified into 4 intervention control studies and 1 cross-sectional time series	Both adult and immature mosquito stages were suppressed by often more than 90 %, and sustained. Two studies showed a decrease of new dengue cases	The analysis of the studies shows different study types and settings used, inappropriate use of statistical methods, relatively short study periods, lack of randomisation in most studies	Evidence obtained from this systematic review showed that the use of IRS either solely or in combination with other control measures can produce significant reductions of Aedes populations (mature and immature forms). IRS can also produce significant reductions in human dengue cases. However, evidence to suggest the effectiveness of IRS either on immature stages of Aedes or on human dengue cases as a single intervention is limited

Table 1. Evidence table of systematic reviews on vector control published by authors. (*Continue*)

Author/ Year	Focus of SR	Efficacy or community effectiveness	Single intervention or combination	Study quality	Databases	Inclusion/ Exclusion criteria	Studies Identified Assessed Included	Outcome measures	Study quality included	Main outcomes	Further outcomes	Conclusions
George et al, 2015	Temephos	Community effectiveness	Single and combinations	cRCTs, RCTs, Intervention Control trials, Before and after studies	PubMed, WHOLIS, GIFT, CDSR, EMBASE, Wiley	Peer-reviewed studies, study design including cRCTs, RCTs, intervention control trials, before and after studies	18439 54 27	Adult mosquito density, BI, CI, HI, PPI, reduction of breeding sites, positive ovitraps, mean number of larvae, % prevalence of larvae. No parameters to measure human cases	11 studies using a single intervention: seven intervention control studies, four before and after studies 16 studies with combinations: 3 cRCTs, 4 intervention control trials, 9 before and after studies	11 studies using a single intervention: All 11 studies reported a post-intervention reduction in the immature stages, with a prolonged effect of 4 - 8 weeks in the dry season and 6 - 12 weeks in the wet season, if re-application has been respected. 18 studies with combinations, mostly with health education and information, environmental management and the use of malathion, Bti, larvivorous fish: 10 studies reported a reduction of immature mosquito stages, 3 failed and 3 had some effect only	Operational issues may be important, including surveillance and coverage, regular application, mode of application, acceptability and limited residuality of Temephos	While there is little doubt concerning the effectiveness of Temephos in controlling Aedes breeding sites, the same level of effectiveness was not clear from the studies using Temephos combined with other interventions. This could be due to operational issues, delivering several interventions
Maoz et al, 2017	Pyri- proxyfen	Community effectiveness	Single and combinations	cRCTs, RCTs, Non RCTs with control: Quasi RCTs, Intervention Control trials Before and after studies	PubMed, Web of Science, EMBASE, LILACS, WHOLIS, Cochrane, Google Scholar	Peer-reviewed studies, study design as mentioned under study quality	698 49 17	Adult emergence BI, CI, HI, PPP, Pupal mortality, larval mortality 2 studies human disease parameters: serological surveys (IgM) and dengue incidence	1 cRCT 3 RCTs 1 Quasi RCT 12 Non RCTs	Container treatment studies: 6 studies showed a reduction above 80 %, 2 RCTs showed a limited effect. 2 Fumigation studies in combination with Permethrin showed a good inhibitory effect Studies measuring autodissemination should good results of reduction of adult emergence between 20 and 85 %, Combination with adulticides seemed to increase effectiveness Human transmission data were weak and could not show a good effect.	The evidence presented suggests that pyriproxyfen can effectively control the adult emergence of immature stages of dengue vector mosquitoes in a variety of breeding sites in a community setting. There is a clear consensus that pyriproxyfen effectively inhibits Aedes adult emergence at concentrations of <1 ppb	More and larger studies with appropriate study designs and relevant, standardised outcome measures are needed; also, tolerance/resistance of vectors to pyriproxyfen has been reported

Table 1. Evidence table of systematic reviews on vector control published by authors. (*Continue*)

Author/Year	Focus of SR	Efficacy or community effectiveness	Single intervention or combination	Study quality	Databases	Inclusion/ Exclusion criteria	Studies Identified Assessed Included	Outcome measures	Study quality included	Main outcomes	Further outcomes	Conclusions
Boyce et al, 2013	Bti	Community effectiveness	Single intervention	cRCTs, RCTs, NRCTs	Medline, EMBASE, Global Health, Web of Science, CDSR, WHOLIS	Experimental design, Bti as a single agent, minimum follow up of 20 days	355 / 45 / 14	BI, CI, HI, average larval free period, oviposition index. 1 study measured routinely collected surveillance data	3 cRCTs, 1 RCT, 10 NRCTs	Bti eliminated all larvae from treated containers within 24 hours, for most containers there was a prolonged effect of 14 days. The study that measured an effect on human transmission showed only 1 case in the intervention area, compared to 15 in the control	Different formulations did not show superiority in the 4 studies testing this. Higher doses of Bti showed a prolonged effect in 1 study. Study design and quality need to be improved in future studies	There is evidence that Bti is effective in reducing the density of immature dengue vectors when it is applied to targeted containers as demonstrated by the efficacy studies. However, the evidence to suggest that Bti is effective as a single agent, when used in a community setting, is limited
Han et al, 2015	Larvivorous fish	Efficacy and community effectiveness	Single intervention or combination	cRCTs, RCTs, NRCTs	PubMed, EMBASE, Web of Science, WHOLIS, Wiley, LILACS, GIFT (WHO database), CDSR	Experimental design	4914 / 28 / 13	Number of breeding sites, positive for Aedes, Adult mosquito density, BI, CI, HI, infestation rates. 2 studies measured surveillance data	9 intervention control studies, 4 before and after studies	All intervention control studies - but 1 – showed a reduction of immature forms of dengue vectors. 1 study showed a reduction of adult indices, of 3 measuring. 3 of 4 before and after study showed a reduction of immature stages. A long-term decline over 2 years has been reported by 2 the studies, measuring such an extended period. The studies measuring human transmission showed a reduction of human cases, however these were before and after studies only	Study design and quality is an issue, and geographical coverage of studies	The findings suggest that the use of larvivorous fish, used as a single agent or in combination with other measures, can reduce significantly infestations of the immature vector stages. However, there is no evidence to demonstrate any community effectiveness of larvivorous fish as a single agent

Table 1. Evidence table of systematic reviews on vector control published by authors. (*Continue*)

Author/ Year	Focus of SR	Efficacy or community effectiveness	Single intervention or combination	Study quality	Databases	Inclusion/ Exclusion criteria	Studies Identified Assessed Included	Outcome measures	Study quality included	Main outcomes	Further outcomes	Conclusions
Lazaro et al, 2015	Copepods	Community effectiveness	Single intervention	cRCTs, RCTs, NRCTs	PubMed, EMBASE, CDSR, WHOLIS, Web of Science, LILACS	Experimental design, Copepods as a single intervention	1222 29 11	Adult density, BI, CI, HI, PPI, 4 studies measured serological surveillance	11 Intervention Control trials	Copepods used were mostly Mesocyclops spp Copepods controlled larval Aedes populations up to 100%. At household level, reductions of households positive for Aedes larvae between 30 – 97 % were observed. Adult mosquito landing rates, and oviposition: reductions to zero. Adult Aedes per household: reductions between 30 -100 %. Adult mosquito indices reductions from 0.12-1.16 to 0-0.01 per community after a period of three years In 3 studies dengue transmission data were measured: results ranged from 0 reported cases in intervention and control communities to a 76.7 % reduction of dengue incidence, confirmed by a reduction of serological parameters	Study design and quality are issues	Copepods as a single intervention may be a community effective and sustainable dengue vector control method to control dengue vectors and dengue transmission. However, this is perhaps only possible provided several specific criteria are met: as clearly shown in the fives studies conducted in Vietnam, these would include rigid delivery of intervention; development of community management committees and collaborators; efficient mobilisation and sustained interest of the community residents

Table 1. Evidence table of systematic reviews on vector control published by authors. (*Continue*)

Author/Year	Focus of SR	Efficacy or community effectiveness	Single intervention or combination	Study quality	Databases	Inclusion/Exclusion criteria	Studies Identified Assessed Included	Outcome measures	Study quality included	Main outcomes	Further outcomes	Conclusions
Systematic reviews for a service orientated purpose												
Pilger et al, 2010	Outbreak control	Community effectiveness by definition, since only concerning real time outbreaks	Single interventions and combinations, could be clinical or vector management	Descriptive epidemiological studies (prospective or retrospective), before and after studies, evaluations using mixed methods	Medline, EMBASE, CDSR, LILACS, WHOLIS	Any study design, any intervention during outbreak, Outcome described and supports by data	1134 / 63 / 24	A: studies focusing on transmission reduction; B: studies focusing on mortality reduction C: studies describing both	All studies descriptive studies without control, 1 study with control	With different organisational strategies for an outbreak response, most common is an inter-sectorial approach. Multidisciplinary response teams, with vector control working with communities, including monitoring and evaluation, resulted in good outbreak control. Combined response with 1) vector control (larval habitats interventions with communities; insecticides intra- and peri-domestic) and 2) capacity training for clinical response are successful. Spatial spraying of insecticides as a single intervention is not effective	The evidence level is weak, also due to study design	Outbreak response has to be organised multidisciplinarily and monitored/evaluated. During outbreaks the above-mentioned interventions have to be implemented as a combined set of interventions in order to achieve rapid control Further research is needed especially linking effectiveness of outbreak response to human disease epidemiology
Organisational context of vector control												
Horstick et al, 2010	Vector control service organi- sation	Community effectiveness by definition, since only concerning real time services analysed	Combination of interventions	Any study that is not only an expert opinion	PubMed, WHOLIS, WHO regional databases	1) Studies with clear methodology and/result section analysing vector control services 2) Reports/ articles describing existing vector control services, without clear methodology/ results section, but can be derived (in the text)	2148 / 46 / 32	Service organisation: Staffing levels, capacity building, finances, material available	9 defined methodology, 16 not defined, 3 guidelines and 4 case studies	3 of 9 studies showed little change of control operations over time. There were however strategic changes (decentralisation, inter-sectorial collaboration). Staffing levels, capacity building, management and organisation, funding and community engagement were insufficient	Analysis of vector control services is not common and/ or not reported	The analysis underlined the need for: 1) operational standards, 2) evidence based selection/delivery of combinations of interventions, 3) development/ application of monitoring and evaluation tools, 4) needs driven capacity building

• Combinations of interventions have mixed results, largely related to the logistical challenges of implementing multiple interventions (George *et al*, 2015).

• In real world outbreaks, multiple interventions are often applied although the effectiveness is questionable (Pilger *et al*, 2010).

• One of the key elements for more effective vector control measures may be timely alerts of outbreaks, as indicated by surveillance systems, followed by immediate vector control interventions, including health promotional campaigns.

• Careful implementation of vector control measures may be more important than the actual choice of vector control method.

ACKNOWLEDGEMENTS

CONFLICS OF INTEREST

None declared.

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PROSPECTS FOR THE DEVELOPMENT OF A DENGUE VACCINE

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Abstract. Dengue is a mosquito-borne viral disease, which is currently an important and rapidly growing health problem across the globe. Four closely related dengue serotypes cause the disease, which ranges from asymptomatic infection to undifferentiated fever, dengue fever (DF), and dengue hemorrhagic fever (DHF). Specific antiviral medications are not available for dengue, and successful treatment that is mainly supportive depends on early recognition of the disease and careful monitoring for shock. Prevention of dengue depends on the control of the mosquito vector, which has had only limited success. Development of a dengue vaccine is seen as a new tool to prevent this potentially fatal disease. The scope and intensity of dengue vaccine development has increased dramatically in the last decade. A live-attenuated tetravalent dengue vaccine based on chimeric yellow fever dengue virus has progressed to licensure in several dengue endemic countries in 2015 after its Phase III efficacy study involving more than 30000 volunteers from Asia and Latin America. Several other dengue vaccine candidates are currently being evaluated in clinical and preclinical studies including other live-attenuated vaccines, subunit, DNA purified inactivated vaccine candidates, as well as virus-vectored and virus-like particle-based vaccines. Since dengue poses a heavy economic cost to the health system and society, the potential economic benefits are associated with promising dengue prevention interventions such as dengue vaccine and vector control innovations.

Keywords: dengue, vaccine development

INTRODUCTION

Dengue is a mosquito-borne viral disease that is currently an expanding global problem. Successful treatment, which is mainly supportive, depends on early recognition of the disease and careful monitoring for shock.

A severity-based revised dengue classification for medical interventions has been developed by the World Health Organization (WHO) and has been adopted in most countries. Dengue is a disease entity with different clinical manifestations often with unpredictable clinical evolutions and outcomes. Four closely related dengue serotypes cause the disease, which ranges from asymptomatic infection to undifferentiated fever, dengue fever (DF), and dengue hemorrhagic fever (DHF). The severity of DF manifestations increases with age. DF causes fever, rash, muscle or joint pain, headache, and eye pain, but DF is rarely fatal. DHF is considered a distinct disease characterized by fever, bleeding diathesis, and increased vascular permeability leading to leakage of plasma with a tendency to develop potentially fatal dengue shock syndrome (DSS). Although shock and plasma leakage seem to be more prevalent as age decreases, the frequency of internal hemorrhage rises as age increases. Increases in liver enzymes found in both children and adults indicate liver involvement during dengue infections. Pre-existing liver diseases, which are more common in adults, such as chronic hepatitis, alcoholic cirrhosis, and hemoglobinopathies can aggravate the liver impairment in dengue patients.

Dengue with organ impairment mainly involves the liver and the central nervous system. Consistent hematological findings include vasculopathy,

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coagulopathy, and thrombocytopenia. Laboratory diagnosis includes virus isolation, serology, and detection of dengue ribonucleic acid. The age of dengue cases in several countries has increased from children to adolescents and adults (Thisyakorn and Thisyakorn, 2015a).

Antiviral medications are not available for dengue and successful treatment, which is mainly supportive, depends on early recognition of the disease, bleeding tendency, and careful monitoring for signs of circulatory failure. Adults have a higher prevalence of underlying diseases, *eg*, coronary artery disease, peptic ulcer, hypertension, diabetes mellitus, cirrhosis, and chronic kidney diseases, which should be considered in dengue management. A severity-based revised dengue classification for medical interventions has been developed and adopted in many countries (Tantawichien, 2015). Prevention using vector control has had limited success, and dengue vaccine is thus seen as one of the major tools in effectively controlling dengue diseases (Horstick and Ranzinger, 2015; Thisyakorn and Thisyakorn, 2015b).

DENGUE VACCINE DEVELOPMENT

Dengue virus is a positive-sense, single-stranded, 11kb RNA *Flavivirus* consisting of three structural proteins [premembrane/membrane (prM/M), envelope (E), as well as capsid (C) and seven non-structural proteins]. There are four antigenically distinct serotypes (DENV-1, 2, 3, and 4). The pathogenesis of DHF is not clearly understood. The uniqueness of the dengue viruses and the spectrum of diseases resulting from infection have made dengue vaccine development difficult.

Several different approaches have been tried to develop a dengue vaccine. Because dengue is a unique and complex disease, developing a dengue vaccine has proven equally complex. However, there is an advanced pipeline of vaccine research currently in clinical and preclinical studies including live-attenuated vaccines, subunit, DNA, purified and inactivated vaccine candidates, as well as virus-vectored and virus-like particle-based vaccines. (Thisyakorn and Thisyakorn 2014a; Prommalikit and Thisyakorn 2015; Vannice *et al*, 2016).

LICENSED DENGUE VACCINE

The first dengue vaccine, CYD-TDV (Dengvaxia®, Sanofi Pasteur: Lyon), is a live-attenuated tetravalent dengue vaccine with a yellow fever backbone, and all four dengue serotype components are chimeric (prM and E proteins). This vaccine has now been licensed by several dengue endemic countries in Asia and Latin America for use in persons aged 9-45 or 9-60 years and is under regulatory review in several others (WHO, 2016b).

The first Phase III efficacy trial for CYD-TDV in highly dengue-endemic areas of five Asian countries in 10,275 children demonstrated that this dengue vaccine is efficacious when given as a 0-6-12 month schedule to 2-14 year-old children. The vaccine showed a 56.5% (95% CI: 43.8-66.4) overall efficacy with the contributions of each of the four serotypes, and more than 80% of severe dengue episodes were avoided with a two-third reduction in hospitalization. Higher efficacy was observed in the immunogenicity subset seropositive at baseline. A good safety profile was observed with an interesting finding that vaccine efficacy was higher for participants who were seropositive for dengue than for those who were seronegative. Furthermore, vaccine efficacy increased with age, which could be a marker of previous exposure to dengue (Capeding *et al*, 2014).

A second Phase III clinical trial in Latin American countries involving 20,875 children and adolescents aged 9-16 years demonstrated a 60.8% (95% CI: 52.0-68.0) overall efficacy with the contributions from each of the four serotypes. Additional results showed a significant reduction of the risk of hospitalization by 80.3%. Higher efficacy was observed in the immunogenicity subset seropositive at baseline with a good safety profile (Villar *et al*, 2015). The burdens of dengue were substantial in both regions and in all age groups. Burdens varied widely according to country, but the rates were generally higher and the disease more frequently severe in Asian

countries than in Latin American countries (L'Azou *et al*, 2016).

Assessment of the incidence of hospitalization for virologically-confirmed dengue as a surrogate safety end point during follow-up in years 3 to 6 of two Phase III trials and a Phase IIb trial showed the risk among children 2 to 16 years of age was lower in the vaccine group than in the control group. However, the unexplained higher incidence of hospitalization for dengue in year 3 among those children younger than 5 years needs to be carefully monitored during long-term follow-up (Hadinegoro *et al*, 2015).

The WHO Strategic Advisory Group of Experts on Immunization (SAGE) recommends countries consider introduction of CYD-TDV in geographic settings where dengue is highly prevalent. SAGE recommends that vaccination should be considered as an integrated strategy together with communication strategy, well-executed and sustained vector control, the best evidence-based clinical care for all patients with dengue, and robust dengue surveillance (WHO 2016a). The observed vaccine efficacy against asymptomatic dengue infection is expected to translate into reduced dengue virus transmission if individuals are vaccinated in endemic areas (Olivera-Botello *et al*, 2016).

The WHO published its first position paper on a dengue vaccine based on the available evidence of CYD-TDV or Dengvaxia®, the only dengue vaccine to have received regulatory approval, in the Weekly Epidemiological Record. Since December 2015, Dengvaxia® has been approved by the regulatory authorities of several countries in Latin America and Asia. The WHO recommends that countries should consider the introduction of the dengue vaccine Dengvaxia® only in geographic settings (national or subnational) where epidemiological data indicate a high burden of disease, and dengue vaccine introduction should be a part of a comprehensive dengue control strategy, including well-executed and sustained vector control, evidence-based best practices for clinical care for all patients with dengue illness and strong dengue surveillance (WHO, 2016b).

DENGUE VACCINES IN THE PIPELINE

Two dengue vaccine candidates at advanced stages of clinical development are both live-attenuated vaccines and both have one or more chimeric serotype components. TDV (Takeda: Osaka) has one component that is attenuated but not chimeric (DEN-2) and three chimeric components (prM and E proteins) while TV003/TV005 (National Institutes of Health: Bethesda, MD) has three attenuated components and one chimeric component (a DEN-4 backbone with DEN-2 prM and E proteins). Both are at Phase II and III efficacy trial stages (Asia Dengue Summit, 2016).

Increasing knowledge of dengue vaccine development is providing more insights into improved vaccine design. Recent advances in vaccine science have greatly increased the technological options for dengue vaccine development as several promising dengue vaccine candidates are in preclinical and clinical development. In parallel, molecular biology and system biology permit more specific analysis of vaccine-induced immunogenicity and safety. However, possibly the most intriguing finding in dengue vaccines over the past year comes from the first licensed CYD-TDV. There are also three inactivated whole virus dengue vaccines with incorporated adjuvants for enhancing immunogenicity at the Phase I trial stage.

The preclinical dengue vaccine pipeline covers a broad range of approaches both in antigen as well as in delivery and presentation. Second generation vaccines may improve upon first generation vaccines in overall and strain-specific vaccine efficacy, in particular in immunologically naïve subjects, and in more favorable immunization schedules. Carefully designed studies in non-human primates should allow prioritization of preclinical candidates for human subject trials (Thisyakorn and Thisyakorn, 2014a; Thisyakorn and Thisyakorn, 2015b; Vannice *et al*, 2015). Future clinical development of dengue vaccine candidates needs to consider that CYD-TDV (Dengvaxia®, Sanofi Pasteur: Lyon) has been introduced into many endemic countries.

ASIA DENGUE SUMMIT

During 13-14 January 2016, the Asia Dengue Summit (ADS) co-organized by the Asian Dengue Vaccination Advocacy (ADVA), the Dengue Vaccine Initiative, the Southeast Asian Ministers of Education Organization Tropical Medicine and Public Health Network, and the Fondation Mérieux was held in Bangkok, Thailand. This meeting focused on improving strategies for dengue prevention and control.

The ADS and the ADVA workshop presentations provided a foundation from which to form an outcome statement and call to action. Because dengue vaccine is potentially game changing, the events of 2016 will impact the future for populations in dengue endemic areas around the world.

The second part of the meeting comprised of discussions of a set of statements drafted by ADVA based on the key messages from the ADS as described below.

Call to action

Within the broader context of the outcomes of the Asia Dengue Summit, we…
- Recognize that dengue continues to be a major global public health threat and the problem is growing.
- Recognize that vaccines would be a useful addition to current prevention and control efforts that, in most cases, are inadequate for full impact.
- Are aware of the licensure of the first dengue vaccine and imminent availability of other promising candidates for preventive vaccination.
- Are cognizant of the strong leadership role provided by the WHO, guided by the Global Strategy for Dengue Prevention and Control, to reduce dengue mortality by 50% and morbidity by 25% by the year 2020.
- Are informed by the urgent need for adequate resources, for integrated surveillance, for sustainable vector control methods, for adequate preparedness for vaccination

programs that include school-based vaccination, appropriate evaluation and monitoring of interventions, as well as for new methodologies and high-quality point-of-care diagnostic tests.
- Recognize the central role of good science, good communications, the media, strong political leadership, and public support.
- Are sensitive to the need to ensure equity, sustainability, ethics, and social justice.

The co-hosts of the Asia Dengue Summit make the following call to action: we…
- Call on countries, where appropriate, to develop and implement a carefully controlled stepwise programmatic introduction of dengue vaccine(s), including school-based vaccination and catch-up campaigns, which are closely integrated with other control strategies and the needs and constraints of health systems. These activities should be based on close consideration of the country's own disease epidemiology, capacities, health infrastructure, financial resources, and decision on vaccine registration.
- Call on countries to ensure that vaccine implementation programs are monitored and tracked, and evaluated for safety, effectiveness, and acceptance through sound risk communication and management plans with good communication, active surveillance, laboratory support, and clinical management.
- Call on various related initiatives to work closely together through global efforts such as the Global Dengue and *Aedes*-transmitted Diseases Consortium to monitor developments in vaccine and vector control implementation as well as to perform high-level advocacy with governments and international organizations for vaccine introduction in endemic countries. This should be guided by the need for better integration and synergy of strategies to avoid fragmentation and duplication.
- Call for the political will and commitment to accelerate effective dengue prevention

and control interventions, including strengthening health systems and ensuring sustainable financing.

- Call on relevant organizations and institutions to continue performing and supporting research to further enhance the impact of vaccination, including biomedical and clinical research, mathematical modeling, implementation and operational research, as well as post-licensure studies.

- Call on the WHO and other global and regional organizations and initiatives to give higher priority to dengue prevention and control, to provide continued leadership, guidance, and technical support to countries on the possible introduction of dengue vaccine, as well as to assist with implementation strategies and obtaining sustainable financial support for countries (Thisyakorn *et al*, 2014b; Asia Dengue Summit, 2016).

The 2nd ADS will be held in Manila, Philippines during 1-2 March 2017 (2nd Asia Dengue Summit, 2017).

ASEAN DENGUE DAY

ASEAN Dengue Day is an advocacy event held every 15 June to increase public awareness of dengue, to mobilize resources for its prevention and control, and to demonstrate the region's commitment to tackling the disease. The advocacy event was agreed upon during the 10th ASEAN Health Ministers Meeting in 2010. The first regional event was held in 2011 in Jakarta, Indonesia. During 13-15 June 2016, the 6th ASEAN Dengue Day was held in Bangkok, Thailand where health experts called for collective regional action to fight the fastest-growing burden of dengue in the ASEAN region (ASEAN Dengue Day, 2016).

CONCLUSION

The global burden of dengue is increasing rapidly, driven by population growth, urbanization, globalization, and ecological changes. A dengue vaccine is needed as part of an integrated approach to dengue prevention and control. One dengue vaccine, CYD-TDV, has been licensed in several dengue endemic countries in Latin America and Asia since 2015 for use in 9-45 or 9-60 year-old individuals. Two other vaccines are at advanced stages of clinical development at Phases II and III. Several other vaccines are at varying stages of preclinical and clinical development.

CONFLICT OF INTEREST

The authors declare no conflicts of intrest in preparing this article.

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THE ESTIMATED IMPACT AND COST-EFFECTIVENESS OF DENGUE VACCINATION

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Abstract. Dengue infection is considered a significant global health threat, especially in Thailand. Dengue vaccination is one of promising methods to prevent dengue infection. Recently, the Dengvaxia™ (CYD-TDV) has become available in the market. Furthermore, some new dengue vaccines may soon become available. In this paper, we reviewed published studies focusing on cost-effectiveness of the dengue vaccine. Results from this review would help key stakeholders for making their decisions in adding the vaccine into the National List of Essential Medicines (NLEM) Thailand.

Keywords: cost-effectiveness analysis, dengue vaccine, health economics

PATHOPHYSIOLOGY OF DENGUE INFECTION

Dengue virus (DENV) is a single-stranded, positive-sense RNA viruses of the genus *Flavivirus* (family Flaviviridae) (Simmons *et al*, 2012). DENV can be classified into four antigenically diverse serotypes (DENV1-4) (Simmons *et al*, 2012). Based on data of the years 1994 to 2006, the distribution of dengue serotype in Thailand was DEN-1 (36%), followed by DEN-3 (27%), DEN-2 (23%) and DEN-4 (14%) (Fried *et al*, 2010).

The primary vector of DENV is the *Aedes aegypti* mosquito, which is widely distributed in tropical and subtropical countries (Lambrechts *et al*, 2010). The clinical presentation of dengue infection can range from asymptomatic (inapparent) dengue infection, undifferentiated fever, dengue fever, dengue hemorrhagic fever, or dengue shock syndrome (Simmons *et al*, 2012).

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BURDEN OF DENGUE INFECTION

Dengue infection is considered a significant global health threat, especially in Asian countries. Not only does dengue infection results in significant morbidity and mortality, but it also results in high resource utilization. A recent study using new statistical methods and geo-located techniques was conducted to accurately predict the global burden of dengue infection (Bhatt *et al*, 2013). From that study, the global estimates of overall dengue infection and apparent dengue infection were 390 (95% CI: 284-528) and 96 (95% CI: 67-136) million per year, respectively. Of these, nearly 400 million episodes of dengue infection, approximately 70% of them occurred in Asian countries. Moreover, a recent observational study conducted in three Southeast Asian countries (Thailand, Indonesia, and Vietnam) revealed that dengue infection is the most common cause of community-acquired sepsis and severe sepsis among hospitalized patients (Southeast Asia Infectious Disease Clinical Research Network, 2017).

Dengue infection is also one of the most common causes of acute febrile illness in Thai children. A past seroprevalence study reported that 50% of 4- to 16-year-old students at a Bangkok school had antibodies to at least one dengue serotype (Burke *et al*, 1988). However, more recent

evidence reported that the crude attack rate of virologically-confirmed dengue infection among Thai children aged 2-14 years old was only 5.9 per 100 person years (Nealon *et al*, 2016).

EFFICACY OF DENGUE VACCINE

Dengvaxia™ (Sanofi Pasteur: Lyon) the first licensed dengue vaccine is a recombinant, live-attenuated tetravalent dengue vaccine (CYD-TDV). It was approved by the Thai Food and Drug Administration in October 2016.

The vaccine efficacy has been well documented in two landmark phase III trials, namely CYD14 and CYD15. The CYD14 was conducted among 10,275 healthy children aged 2-14 years from five countries in the Asia-Pacific region including Thailand (Capeding *et al*, 2014) while the CYD15 was conducted among 20,869 healthy children between aged 9-16 years from five Latin America countries (Villar *et al*, 2015). Based on results from the long-term follow-up of these two clinical trials, the pooled vaccine efficacy against symptomatic dengue virus infection during the first 25 months were 65.6% (95% CI: 60.7-69.9) for children under 9 years of age and 44.6% (95% CI: 31.6-55.0) for the older population. Furthermore, the pooled relative risks of hospitalization for dengue were 0.84 (95% CI: 0.56-1.24) among all participants, 1.58 (95% CI: 0.83-3.02) among those under the age of 9 years, and 0.50 (95% CI: 0.29-0.86) among those 9 years of age or older (Hadinegoro *et al*, 2015).

The CYD-TDV seems to be more effective among younger or previously immune populations. Despite the relatively high vaccine efficacy, the absolute risk reduction of CYD-TDV for symptomatic dengue infection was only 0.1 - 0.2% per year (Hadinegoro *et al*, 2015). Although the CYD-TDV efficacy was not proven in two phase II trials of patients aged 2-45 years, the meta-analysis including seven studies of patients aged between 2-45 years confirmed the clinical efficacy of the CYD-TDV of 59% (95% CI 15-80), or relative risk of 0.41 [95%CI 0.2-0.85] (da Costa *et al*, 2014). Given these findings, the CYD-TDV vaccine was approved for use in patients aged 2-45 years.

THAILAND'S NATIONAL LIST OF ESSENTIAL MEDICINES

In 2011, Thailand became an upper-middle income economy by the World Bank classification [USD4,036 - USD12,475 gross national income (GNI) per capita]. Nevertheless, affordability is still one of the important factors for policy makers to make decisions in adopting new vaccines or new treatment options. Currently, there are several mechanisms for resource-limited countries to procure vaccines at affordable prices (Burchett *et al*, 2012). For example, countries classified as low-income countries by the World Bank (USD1,025 or less GNI per capita) can secure remarkably lower price vaccines via the Global Alliance for Vaccines (GAVI) negotiation process. Furthermore, United Nations International Children's Emergency Fund established a vaccine procurement program to make some vaccines more affordable for GAVI ineligible-countries (Kaddar *et al*, 2013).

Although Thailand is not eligible for those aforementioned mechanisms, the Thai government has systematically instituted price negotiation mechanisms before adding necessary medicines and vaccines into the National List of Essential Medicines (NLEM) Thailand (Teerawattananon and Tritasavit, 2015). One of the most important steps in price negotiation is to conduct an economic analysis on such medicines or vaccines. If the given medicine does not represent good value for money, the projected price to make such medicine become good value is requested. According to the suggestion of World Health Organization (WHO), the willingness to pay (cost-effectiveness threshold) should be three times the per capita gross domestic product (GDP) per disability-adjusted life-year (DALY) averted (Bertram *et al*, 2016). Unfortunately, the threshold that is currently used for NLEM of Thailand is approximately USD 5,000 per one DALY averted or only one time of Thailand per capita GDP.

COST-EFFECTIVENESS OF DENGUE VACCINATION IN THAILAND

There has been a number of health economic analyses evaluating the impact and economic

burden of the dengue vaccine (Shepard *et al*, 2004; Lee *et al*, 2011; Durham *et al*, 2013; Yeo *et al*, 2015; Shim, 2016; Flasche *et al*, 2016). However, the results from a study conducted in one country may not be applicable to another country due to differences in many important aspects (*ie*, the difference in vaccine efficacy across patients with differences in ethnicity, incidence of dengue infection, mortality rate or cost of treatment.

An economic analysis of the dengue vaccine using the context of Thailand was conducted in 2011 before the CYD-TDV vaccine was available in the market (Lee *et al*, 2011). The authors constructed a decision tree using the Markov model. The model started with two options to choose; vaccination versus no vaccination. Vaccinated subjects would have a lower chance of acquiring a dengue infection including asymptomatic dengue infection, dengue fever, dengue hemorrhagic fever, dengue shock syndrome and death based on vaccine efficacy. Costs for vaccination, treatment for infection, treatment for vaccine side effect and school-day or work-day missed were all calculated from the Societal perspective. Sensitivity analyses were subsequently performed using a broad range of variables such as vaccine efficacy, cumulative incidence of dengue (dengue risk) and disease characteristic (*ie*, % of hospitalization and % of outpatient visit). By using the model with a dengue risk of 5%, a vaccine efficacy of 50%, and a cost-effectiveness threshold of one per-capita GDP, the dengue vaccine would be cost-effective if the vaccination cost is less than USD 60 per course. In a situation using three times of per-capita GDP as the cost-effectiveness threshold, the dengue vaccine would be cost-effective if the vaccination cost is less than USD 200 per course. Unfortunately, the current market price of Dengvaxia™ in Thailand is approximately USD 300 per course.

CONCLUSION

In an ideal situation, one would prefer to employ all treatment or preventive options that would increase the life expectancy of a patient. However, more than half of population in the world are in resource-limited countries. Because the CYD-

TDV is currently available in the market, and some new dengue vaccines may soon become available, results from cost-effectiveness analyses would help key stakeholders for making their decisions and assisting in the price negotiation process.

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Severe Dengue

CHAPTER 6
Adult dengue cases

- Hemophagocytic lymphohistiocytosis and acute kidney injury with severe nephrotic syndrome in dengue patients: a case report

HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS AND ACUTE KIDNEY INJURY WITH SEVERE NEPHROTIC SYNDROME IN DENGUE PATIENTS: A CASE REPORT

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Abstract. Dengue virus infection (DVI) has been rarely reported to have association with secondary hemophagocytic lymphohistiocytosis (HLH) in adults. Moreover, biopsy-proven nephrotic syndrome in the same patients has never been reported. Therefore, we describe a case of severe DVI-associated HLH and biopsy-proven renal involvement.

Keywords: dengue virus infection, hemophagocytic lymphohistiocytosis, nephrotic syndrome

INTRODUCTION

Hemophagocytic lymphohistiocytosis (HLH) is disease of inappropriate macrophage activation. It is characterized by the presence of hemophagocytosis. This is an engulfment of blood cells by the patient's self-macrophages in the bone marrow, followed by excessive production of inflammatory cytokines, leading to a sepsis-like syndrome and multi-organ failure (Henter *et al*, 2007). Dengue virus infection (DVI) is recognized as one of the causes of secondary HLH, although seemingly rare (Rueda *et al*, 2002; De Koninck *et al*, 2014; Phuakpet *et al*, 2015). Renal involvement has been reported in both conditions but in separate case reports.

CASE REPORT

A previously healthy 23-year-old Thai man was referred to King Chulalongkorn Memorial Hospital (KCMH) due to high-grade fever and oliguria for 4 days. Two weeks earlier, he had had all-day high-grade fever without other specific symptoms

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when he first went to a referring public hospital. The physical examination at KCMH revealed high-grade fever (BT 39.0 °C) without other remarkable abnormalities. His complete blood count (CBC) showed mild hemoconcentration and thrombocytopenia (Hb 15.7 g/dl, Hct 48%, WBC 5,800/mm^3, and platelet 40,000/mm^3). He was suspected to have DVI. Thus, dengue NS1 antigen was requested which later returned positive on the same day. After 2 days of admission, his fever had partially resolved, and he was discharged.

He came back to KCMH again with recurrent high-grade fever, malaise, and oliguria for 4 days. On admission, he was drowsy and mildly confused. His vital signs showed fever, tachycardia, and hypertension (BT 38.5 °C, PR 110/min, RR 18/min, and BP 150/90 mmHg). He also had pale conjunctivae and a palpable spleen. At this time, he had anemia and thrombocytopenia (Hb 10.2 g/dL, Hct 31%, WBC 10,200/mm^3, neutrophil 84%, lymphocyte 8.6%, and platelet 84,000/mm^3). He also developed azotemia, metabolic acidosis, elevated liver enzyme and hyperCKemia (BUN 72 mg/dl, Cr 9.4 mg/dl, Na 136 mmol/l, K 3.8 mmol/l, Cl 100 mmol/l, HCO$_3$ 15 mmol/l, total bilirubin 1.74 mg/dl, direct bilirubin 1.36 mg/dl, AST 2913 IU/l, ALT 558 IU/l, ALP 124 IU/l, and CPK 21,779 IU/l).

The urinalysis revealed dark-colored urine with proteinuria and microscopic hematuria without

identifiable dysmorphic RBC under phase-contrast microscopy (SpGr 1.010, pH 6.5, protein 1+, glucose negative, blood 3+, RBC 10-20/hpf, and WBC 2-3/hpf). Serology studies for DVI of dengue IgG and IgM were done, both of which showed positive results.

He was diagnosed as DVI with rhabdomyolysis, hepatitis, and severe acute kidney injury (AKI). Because the patient had oliguria with uremic encephalopathy, metabolic acidosis, and refractory volume overload, sustained low efficiency dialysis was initiated at intensive care unit (ICU).

After 2 days of hospital admission, he had persistent high-grade fever with splenomegaly, cytopenia (anemia and thrombocytopenia), and liver injury following acute viral infection, which led to the suspicion of hemophagocytic syndrome. All further blood tests showing LDH 7,280 IU/l, serum ferritin 33,437 ng/ml, and fasting triglyceride 392 mg/dl supported our suspicion. We then performed bone marrow aspiration that revealed mildly increased cellularity with moderately increased hemophagocytic activities. These findings determined the diagnosis of HLH associated with the antecedent DVI.

There was no significant hemodynamic collapse that would cause either pre-renal AKI or ischemic acute tubular necrosis (ATN) in this patient, and the urine examination also showed urine sediments together with proteinuria. His urine-protein-creatinine-ratio and urine-albumin-creatinine-ratio were 7.16 g/g creatinine and 3.42 g/g creatinine, respectively. These ratios together with hypoalbuminemia (serum albumin 2.5 g/dl) and generalized edema suggested the presence of glomerular involvement.

After correction of thrombocytopenia by platelet transfusion to raise platelet count higher than 200,000/mm^3, we performed percutaneous renal biopsy under real time ultrasound guidance in ICU for the indications of delayed renal recovery and dialysis dependence. The procedure went well without any complication related to procedure. We obtained two cores of renal tissue. Light microscopic findings revealed 10 glomeruli with diffuse mild mesangial expansion, but the findings were otherwise unremarkable. The renal cortical tubules showed epithelial cell degeneration and necrosis (Fig 1).

The findings were consistent with acute tubular necrosis, which could explain the azotemia. However, these findings were not explanatory of the concomitant nephrotic proteinuria, which was proven to be mainly albuminuria. The most likely
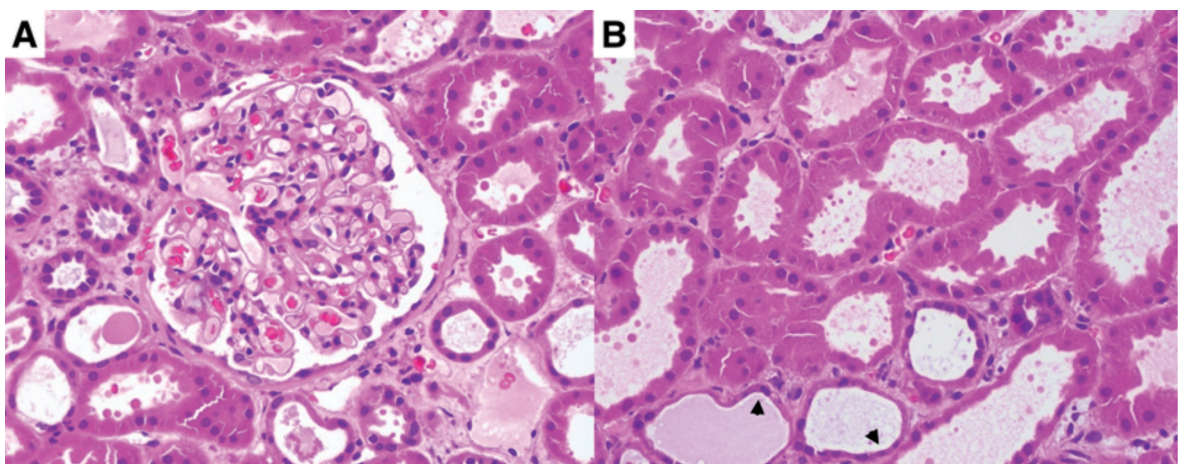

Fig 1–A: Renal specimens of the patient showing normal-looking glomerulus under light microscope (H&E staining, x 40). B: Proximal tubules were dilated and lined by flattened tubular epithelial cells (arrow heads), consistent with ATN (H&E staining, x 40).

possibility was acute podocytopathy in minimal change disease. Unfortunately, the expected diffuse foot process effacement could not be demonstrated owing to the lack of glomeruli in renal specimens submitted for transmission electron microscopy.

The patient was treated by a course of dexamethasone for HLH and best supportive care, including renal replacement therapy. He remained dialysis-dependent for 2 weeks before the recovery of renal function, anemia, thrombocytopenia, hepatitis, and rhabdomyolysis. After 4 weeks of admission, he was discharged safely with a serum creatinine of 3 mg/dl. At a follow-up visit 1 month later, his serum creatinine had returned to a normal level (0.9 mg/dl).

DISCUSSION

Our patient presented with classic clinical manifestation of DVI and HLH. Firstly, he presented with acute high-grade fever without any specific findings except for thrombocytopenia and positive dengue NS-1 antigen, both of which together made the diagnosis of DVI very likely. However, his fever persisted for more than 1 week, which was unusual for DVI. At this time, he also developed splenomegaly together with hypertriglyceridemia, elevated serum ferritin, and evidence of hemophagocytosis in bone marrow consistent with HLH by the HLH-2004 criteria (Henter et al, 2007).

HLH is an uncommon condition and comprises two major entities: a primary or familial HLH (FHL) mainly affecting children and secondary HLH that associated with wide arrays of diseases including infection, autoimmune disease, and malignancy (Henter et al, 2007). Apart from age at the onset of disease, other clinical features of both forms of HLH are similar and difficult to distinguish.

Clinical manifestations typically are fever, hepatosplenomegaly, generalized lymphadenopathy, and cytopenia while elevated liver enzyme, coagulopathy, hypertriglyceridemia, hyperferritinemia, and hypofibrinogenemia are also commonly found. According to HLH-2004 guidelines (Henter

et al, 2007), the diagnosis of HLH is made if 5 of the following 8 features are evident including 1) fever, 2) splenomegaly, 3) cytopenia affecting 2 of 3 lineages, 4) hypertriglyceridemia and/or hypofibrinogenemia, 5) hemophagocytosis in the bone marrow, spleen, or lymph nodes, 6) low or absent NK-cell activity, 7) ferritin ≥500 µg/l, and 8) soluble CD25 ≥2,400 U/ml.

We reviewed renal involvement in DVI separately. AKI was found in up to 33.3% of severe DVI cases [dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS)] (Mendez and Gonzalez, 2003; Wiwanitkit, 2005; Khan et al, 2008). Hemodynamic collapse seems to be responsible in most cases of DVI because AKI is typically present in association with hypotension, and a multivariate analysis from a retrospective series found that DSS was an independent risk factor for AKI in DHF patients (Lee et al, 2009). Other causes of AKI include rhabdomyolysis and hemolysis, leading to acute tubular injury (Lima and Nogueira, 2008).

Proteinuria is also prevalent in DHF, and it has been documented in 30% to 74% of patients, of which 1.1% even had nephrotic-range proteinuria (Garcia et al, 1995; Horvath et al, 1999). Unfortunately, renal pathology was not available in most of these cases. However, DVI can cause glomerulonephritis. In an animal model, dengue-infected mice developed mesangial and endocapillary hypercellularity with IgM deposition 2 days after the infection (Barreto et al, 2004). Renal biopsies from human patients with DVI with microscopic hematuria also yielded similar findings of mesangial cell hypertrophy together with glomerular deposition of immune complexes (Boonpucknavig et al, 1976).

Dengue antigen has also been detected in renal tissues obtained from DHF patients, but only in renal tubular epithelial cells (Jessie et al, 2004). This has led to the hypothesis of immune-mediated injury rather than direct viral cytopathic effects as the cause of glomerulopathy.

Our patient had generalized edema at second presentation together with nephrotic-range proteinuria and hypoalbuminemia, both of which

are compatible with nephrotic syndrome. He also had microscopic hematuria although this was not demonstrated to comprise of dysmorphic RBC, suggesting the possibility of proliferative glomerulonephritis. However, the renal pathology revealed 10 normal glomeruli with only minimal mesangial matrix expansion, which implied the presence of minimal change disease (MCD) or unsampled focal segmental glomerulosclerosis (FSGS), neither of which would not explain the presence of urinary sediments and AKI.

Thus, the concomitant findings of tubular epithelial necrosis consistent with ATN was likely responsible for the severe AKI in this patient. In our case, the pathophysiology of ATN may have been caused by tubular toxicity due to rhabdomyolysis with myoglobinuria and tubulointerstitial inflammation, resulting from the storm of cytokines in HLH because there was no clinically-evident hemodynamic collapse in this patient.

Conversely, HLH itself has been found to be associated with AKI and nephrotic syndrome. Recently, Aulagnon and colleagues (2015) reported the largest series of 95 HLH patients to date. Of 95 patients, 59 (62%) had AKI according to the KDIGO criteria (Khwaja, 2012). The major causes of AKI were ATN either by renal ischemia following hypoperfusion or nephrotoxic ATN (effect of cytokines). Glomerulonephritis as a cause of AKI was reported in 10 patients (17%), 9 of which had nephrotic-range proteinuria. Regrettably, all 10 cases were diagnosed solely on a clinical basis without renal biopsy.

There was a series of 11 patients by Thaunut and colleagues (2006), which first described the glomerular complications of HLH. Renal pathology was available in all cases yielding collapsing glomerulopathy in 5 patients, minimal change disease in 4 patients, and thrombotic microangiopathy (TMA) with podocytosis in 2 patients. In this report, malignant non-Hodgkin lymphoma was the major etiology of secondary HLH.

There were 2 cases of infection-associated HLH. The first patient was a 63-year-old Caucasian female whose HLH was secondary to cytomegalovirus infection. She experienced nephrotic syndrome with severe renal failure. The renal biopsy revealed glomerular thrombotic microangiopathy together with swollen and vaculolated podocytes, both of which were characteristic of damage to podocytes.

Another patient, a 16-year-old African female, suffered from leishmaniasis with secondary HLH and nephrotic syndrome associated with dialysis-dependent renal failure. The hyperplasia of podocytes compressing glomerular tufts and dilated tubules were noted, consistent with collapsing glomerulopathy and acute tubular necrosis, respectively.

To the best of our knowledge, there has never been a report of biopsy-proven MCD in DVI, However, this condition has been well recognized in HLH of various underlying etiologies including infection. We believe that HLH plays a major role in the development of MCD. MCD and FSGS including the collapsing variant are known to result from podocyte injury by unknown circulating permeability factors (Cho *et al*, 2007). In the setting of HLH, the hallmark is excessive release of cytokines by inflammatory cells including interleukin-6, tumor necrosis factor-alpha, and other inflammatory cytokines, one or more of which might be responsible for the injury of podocytes and contribute to the development of glomerulopathy.

In conclusion, we have presented a case of DVI whose clinical manifestation was atypical and interesting. If the patients with DVI have persistent fever of more than a week with multi-organ dysfunction not caused by leakage syndrome of typical DHF/DSS, the attending physician should raise the possibility of complication including DVI-associated HLH. This rare complication of DVI results in the storm of cytokines and can eventually contribute to AKI and nephrotic syndrome.

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1ST ASIAN DENGUE SUMMIT (ADS): ARE WE READY FOR THE NEW VACCINE ERA?

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Abstract. This is a summary of the Proceedings of the 1st Asia Dengue Summit on evaluating the preparedness of countries for dengue vaccine introduction in the Asia - Pacific region.

INTRODUCTION

Dengue is the most common vector-borne viral infection. The global burden is increasing rapidly, driven by population growth, urbanization, globalization, and ecological changes. Dengue vaccination is needed as part of an integrated approach to dengue prevention and control that also includes vector management and improved surveillance.

A milestone in dengue control was reached with the introduction of the first dengue vaccine in Asia (Philippines) and South and Central America (Mexico, Brazil and El Salvador) in 2015-2016. The vaccine has been shown to be safe and moderately effective, particularly at reducing severe disease and hospitalization (Capeding *et al,* 2014; Villar *et al*, 2015).

The first Asia Dengue Summit (ADS) was held on 13-14 January 2015 at the Shangri-La Hotel, Bangkok, Thailand, in conjunction with the Asia Dengue Vaccination Advocacy, the Dengue Vaccine Initiative, the Southeast Asian Ministers of Education Organization Tropical Medicine and Public Health Network, and Fondation Mérieux. The goal was to explore the preparedness for dengue vaccine introduction in the Asia-Pacific region. This article summarizes the *Proceedings of the 1st ADS* on the preparedness for dengue vaccine introduction of countries in the Asia-Pacific region.

WHO PERSPECTIVE AND GUIDANCE ON DENGUE

The World Health Organization's (WHO's) Global Strategy for Dengue Prevention and Control (2012-2020) aims to reduce the burden of dengue by reducing dengue mortality by ≥50% and morbidity by ≥25% by 2020 (WHO, 2012). The Global Strategy is based on five technical elements of:
- Diagnosis and case management,
- Integrated surveillance and outbreak preparedness,
- Sustainable vector control (*Aedes aegypti* and *Aedes albopictus*),
- Future vaccine implementation, and
- Basic operational and implementation research.

Five enabling factors support the technical elements:
- Advocacy and resource mobilization,

- Partnership, coordination and collaboration,
- Communication to achieve behavioral outcomes,
- Capacity building, and
- Monitoring and evaluation.

Burden estimation, case management and surveillance

The burden estimation program involves greater access to dengue data in selected countries (Brazil, Mexico, Sri Lanka, Maldives, and Cambodia) and integration of the data into the national health information system. Burden estimation includes real-time case tracking and estimation of the economic burden of dengue during outbreaks or epidemics. To estimate the true burden of dengue disease, factors of severity (including infection, fever, disease warranting medical attention, and death), cost, age, and laboratory diagnosis need to be incorporated. The gold standard for measuring dengue incidence is active detection through serology, but hospital-based case detection is more usually done.

Diagnostic tests include immunoglobulin (Ig) M-based, dengue virus non-structural 1 (NS1) antigen-based, and combination IgM/NS1-based tests, and molecular diagnostics. Laboratory networks have been established in some regions, and the intention is to form a global network. Challenges include the varied performance of rapid diagnostic tests across populations, the need for resources for diagnostic kits, and strengthening of dengue laboratory networks.

The 2009 WHO dengue classification has been refined and treatment algorithms have been developed aimed at reducing mortality and assisting with triage. Importantly, mortality has decreased in many countries, primarily due to better hospital case management.

Integrated surveillance is important for risk assessment and situation awareness, and can support outbreak preparedness and appropriate communication. As resources are often limited, national level surveillance techniques remain a priority while ensuring sustained surveillance and early identification of disease for local response.

Early outbreak detection and prediction enables prompt intervention to moderate the impact of the outbreak. Research into outbreak response and prediction variables (rainfall, relative humidity, and temperature), and identification of key parameters for each epidemiological setting is ongoing to predict outbreaks, improve data quality, and evaluate the effectiveness of outbreak responses.

One of the key elements of the Global Strategy is 'sustainability', as tools and strategies for dengue are needed in the long term. Multiple tools for sustainable vector management are available, and tools in development include genetically modified lethal insects, Wolbachia-based *Aedes aegypti*, toxic sugar baits, and a matrix for long-term larval control (Achee *et al,* 2015).

Introduction of vaccines and combined interventions

Results of the first successful phase 3 trials of a dengue vaccine have been published (Capeding *et al,* 2016; Villar *et al*, 2016), and several other vaccine candidates are in development. Challenges to vaccine implementation include selection of the target population, the administration schedule, acceptability, affordability, and long-term effectiveness.

Dengue is no longer solely an urban disease, partly due to the role of human movement in its transmission (Stoddard *et al,* 2009). Therefore, identification of hot spots is needed for a prompt response to suppress outbreaks, and integrated surveillance is key to intervention and prevention. The impact of environmental changes needs further study, but temperature increases favor vector and virus multiplication, and climate plays a role in transmission (Colón-González *et al,* 2013). Lack of piped water may aggravate dengue incidence if domestic water storage is increased. However, vector control is sustainable with good community participation (Andersson *et al,* 2015).

Globally, the burden of malaria is declining, with many countries on the verge of disease elimination, while that of dengue continues to increase. Dengue is endemic in 128 countries and 3.9 billion people

are at risk. *Aedes albopictus* has expanded its presence into several European countries. Thus, dengue is a disease of the future, with uncertain distribution and burden.

THE DENGUE VACCINE LANDSCAPE

There have been several different approaches to developing a dengue vaccine, all of which involve the envelope (E) structural protein — the key part of the virus responsible for the antigenic distinction between serotypes. Challenges to the development of a dengue vaccine include the four antigenic serotypes that interact with each other, often in unpredictable ways, resulting in protection, cross-protection, enhancement, and interference. Technical challenges involve imprecise biological assays to measure immune response, lack of a laboratory measurement for protection, and lack of valid animal models for preclinical research. However, there is a robust vaccine pipeline, with several vaccines in preclinical development (Table 1) (Vannice *et al,* 2015).

Licensed dengue vaccine

CYD-TDV (Dengvaxia®, Sanofi Pasteur, Lyon, France) has completed phase 2b and 3 trials (Sabchareon *et al,* 2012; Hadinegoro *et al,* 2015; Capeding *et al,* 2016; Villar *et al,* 2016), and is the first dengue vaccine to be licensed. CYD-TDV is serotype-specific, with good efficacy against DENV-3 and 4, moderate efficacy against DENV-1, and poor efficacy against DENV-2. CYD-TDV has greatest efficacy against severe dengue and in older children and dengue-primed individuals. However, there was increased risk in very young children during the third year after vaccination in the Asian trial (Capeding *et al,* 2016). Given the efficacy and safety profiles, Sanofi Pasteur applied for licensure in dengue endemic countries in Asia and Latin America. In 2015-2016, CYD-TDV was licensed in Philippines, Brazil, Mexico, and El Salvador for use in 9-45-year-old individuals in endemic areas.

Vaccines in development

Two vaccine candidates at advanced stages of clinical development are TAK-003 (Takeda, Osaka, Japan) and TV003/TV005 [National Institutes of Health (NIH), Bethesda, MD, USA]. CYD-TDV, TAK-003, and TV003/TV005 are all live-attenuated vaccines and all have one or more chimeric serotype component. CYD-TDV has a yellow fever backbone and all four serotype components are chimeric (prM and E structural proteins); TAK-003 has one component that is attenuated but not chimeric (DENV-2) and three chimeric components (prM and E structural proteins); and TV003/TV005 has three attenuated components and one chimeric component (DENV-4 backbone with DENV-2 prM and E structural proteins).

There are also several vaccines at earlier development stages. GlaxoSmithKline (Brentford, UK), Fiocruz (Rio de Janeiro, Brazil), and the US Army (Walter Reed Army Institute of Research, Silver Spring, MD, USA) have collaborated on a tetravalent purified formalin-inactivated whole virus vaccine (DPIV); the US Army has developed a tetravalent dengue virus purified inactivated vaccine (TDENV-PIV) and GlaxoSmithKline has manufactured an inactivated whole virus vaccine (PIV). The V180 vaccine (Merck & Co, Kenilworth, NJ, USA) is a tetravalent recombinant protein subunit vaccine based on a truncated E structural protein (DENV-80E) that is expressed in the Drosophila S2 expression system. The TVDV vaccine (Naval Medical Research Center, Silver Spring, MD, USA) is a tetravalent DNA plasmid vaccine with genes encoding prM and E structural proteins.

IS DENGUE CONTROL POSSIBLE?

Efforts to prevent the spread of dengue virus and control dengue disease have been unsuccessful despite the many methods of mosquito control, including space spraying, perifocal control, targeted source reduction, integrated vector management, community participation, bio-control, and genetic control. However, there are some promising new approaches.

Table 1. Vaccines in active human clinical trials.

Category	Sponsor	Vaccine designation	Approach	Phase
Live attenuated	Sanofi Pasteur	CYD-TDV	YF 17D backbone and YF-DENV chimera	Phase 3 results published: safe and moderately effective Licensed in Mexico, Philippines, Brazil, and El Salvador
	Takeda	TAK-003	DENV-2 PDK-53 backbone and DENV-DENV chimera	Phase 3
	US NIH licensed to: Butantan VaBiotech Panacea Serum Institute of India Merck	TV003/ TV005	Direct mutagenesis and DENV-2/4 chimera	Phase 2 and phase 3
Protein subunit	Merck	V180	DENV 80% E protein recombinant with adjuvant	Phase 1
Inactivated whole virus	GlaxoSmithKline/ Fiocruz/US Army	DPIV	Formalin inactivated with adjuvant	Preclinical to Phase 1
DNA	US Navy	TVDV	Plasmid DNA with adjuvant	Phase 1
Heterologous prime-boost	US Army	TDENV-LAV + TDENV-PIV	Live attenuated/inactivated whole	Phase 1

DENV, dengue virus; E protein, envelope protein; NIH, National Institutes of Health; YF, yellow fever.

Vector control

New mosquito control tools include novel insecticides, genetic control methods, biological controls, spatial repellents, lethal ovitraps, and insecticide-treated materials. Residual insecticides of new non-resistant compounds could be effective replacements for dichlorodiphenyltrichloroethane that are suitable for indoor spraying and for treating oviposition sites and cryptic larval habitats. Lethal ovitraps have a place in an integrated prevention and control program, but may have a limited impact on the mosquito population. Vapor-active spatial repellents are designed to emit a chemical to prevent mosquitoes from entering an enclosed area. Insecticide-treated materials (curtains, screens) prevent human-mosquito contact, thus reducing dengue transmission (Manrique-Saide *et al,* 2015).

A new repressible dominant lethal gene has been developed for genetic control, by which all the male mosquitoes are born sterile, so cannot produce progeny. Although this method will rapidly reduce a mosquito population, it is self-limiting so needs repeated application. Trials have been promising (Harris *et al,* 2011; Carvalho *et al,* 2015).

Another positive development is a modified *Wolbachia pipientis* strain that infects *Aedes aegypti. W. pipientis* reduces transmission of the dengue virus by reducing the fecundity and survival of the mosquitoes. Several trials have been successful (Nguyen *et al,* 2015; Hoffmann *et al,* 2014).

It is unlikely that any of these methods used alone will control dengue. However, if successful at reducing the mosquito population, they will also control other mosquito-borne diseases.

Vaccination

The only licensed vaccine is CYD-TDV (licensed in Brazil, Mexico, and Philippines in 2015 and El Salvador in 2016). CYD-TDV has variable efficacy against the four DENV serotypes, with moderate overall efficacy of 56-61% (Capeding *et al,* 2016; Villar *et al,* 2015). There is increased efficacy in people who have had prior exposure to dengue infection. The vaccine has efficacy against severe disease, especially dengue hemorrhagic fever, and in reducing hospitalization. It has a good safety profile.

However, based on knowledge of dengue infection and immunity, a tetravalent vaccine may not be necessary. There is high seroprevalence in endemic countries as most people have had dengue disease at some point in their lives. Most cases of severe dengue disease occur during the first or second infections (Gibbons *et al,* 2007), and the third and fourth dengue infections tend to be mild or asymptomatic (Olkowski *et al,* 2013). Therefore, protection is most needed against the first two infections (bivalent protection).

The three lead live attenuated candidate vaccines may not provide balanced tetravalent protection, resulting in variable protection against the different serotypes. The public health rationale for use of moderately effective dengue vaccines in endemic countries is the priming effect of previous dengue infection on immunity. Most people in hyperendemic areas have already had at least one dengue infection, so vaccinees will be protected against two or more dengue serotypes and against severe disease. Other public health benefits include decreased dengue transmission, reduced magnitude and frequency of epidemics, and reduced risk of healthcare overload, resulting in better management of severe disease and decreased case fatality rate, severe disease and hospitalization, with the associated economic benefits. However, there is a lack of research on third and fourth infections and inadequate surveillance to distinguish infection sequence. Other reservations include the role of the virus strain and possible mutation, patient age as a surrogate for prior infection, temporal distribution of infections with different serotypes, and cellular immunity.

Long-term phase 4 studies might provide answers, but the vaccines could be introduced under controlled conditions and the safety and impact carefully monitored. Thus, step-wise introduction could be considered, with any safety issues being mitigated by an effective risk management program, active surveillance with high-quality laboratory support, and clinical management training. Notably, it is unlikely that vaccines alone will be effective in controlling dengue.

Integrating prevention and control

There are major challenges for dengue prevention and control in the form of expanding urbanization and increasing globalization, lack of resources to build capacity, and the need for political will for economic support and public health leadership. To support regional control of dengue, the Global Dengue and *Aedes*-transmitted diseases Consortium was formed to avoid duplication of efforts and resource use between groups. The goals are to:

- eliminate dengue as a public health problem
- promote development and implementation of innovative and synergistic approaches for prevention and control
- support the WHO global strategy for dengue control
- strengthen advocacy, capacity building, and networking
- work closely with vaccine early adopter countries
- promote integration and innovation.

Integration is a well-known concept, but synergy has been introduced to correspond with the new technologies in development. Vector control continues to be needed to reduce the mosquito population and vaccination will increase herd immunity; combining these technologies with clinical management, therapeutics, and community engagement forms a targeted control program (Fig 1). Targeted control programs use research to develop integrated vaccination and vector control, with the addition of tools suited to individual ecological environments. Importantly, none of the new tools are likely to be effective if used alone, and effective dengue prevention and control requires integration of vaccines with mosquito control and enhanced surveillance.

MODELING AS A PUBLIC HEALTH TOOL

Computer modeling is an underutilized research method with many useful applications. Models can test the empirically untestable with no ethical constraints, and questions can be answered that would not be possible in real-world research. Use of detailed modeling in the field of public health is a relatively new concept, although appropriate models can be constructed.

Models may be intuitive or quantitative and use input and output to answer a question. Quantitative models are more sophisticated, and are used to answer specific questions or those with more serious consequences. Statistical models are used to describe patterns, while mechanistic models predict and explain patterns. Mechanistic models are more complicated than statistical models, but are also more powerful.
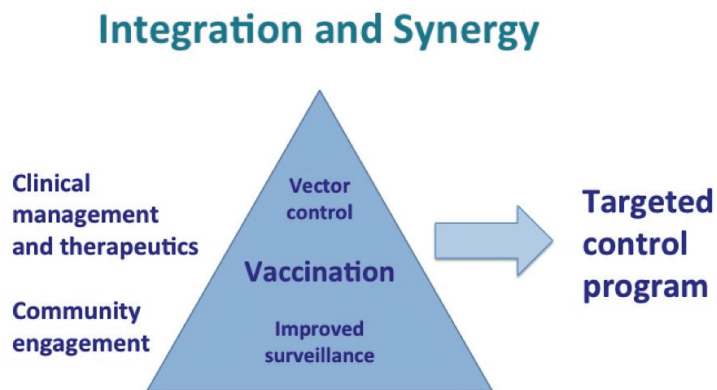

Fig 1–Global Dengue and *Aedes*-transmitted Diseases Consortium paradigm using new tools to control dengue.

All quantitative models have a similar structure of inputs (parameters), interactions between variables, and outputs. Parameters could include information about the speed of an event or duration of an infectious period. The interaction between variables could include transmission of disease by mosquitoes, perhaps on a seasonal basis. Outputs are the information produced by the model that can be compared to the real world, such as projected epidemic size.

There are several different approaches to model the spread of disease. Compartmental models (in which people are represented as counts in susceptible, infectious, and recovered groups) are the simplest type, while network models represent explicit population structure. Agent-based models are the most realistic, but also the most complicated to construct and interpret (Table 2).

Independent comparative modeling

A good model is one that makes sense, fits well to the data, is applied in ways that stay close to the fitted data, and is predictive. However, when constructing dengue models, events are being predicted that may be decades in the future. The data needed to test such ambitious forecasts are often unavailable. Thus, independent, comparative modeling can be the best option. Comparative modeling involves independent modelers, using different methods and assumptions, but collaborating and comparing results. If the results between groups are similar they are likely to be predictive (Penny *et al,* 2016). On-going dengue modeling work includes comparative modeling of dengue vaccine impact, supported by the WHO.

Epidemiology modelers working in isolation from clinicians, virologists, entomologists, and public health officials may produce models that are academically interesting, but are poorly informed, unrealistic, and cannot produce reliable predictions. Therefore, modelers need to be kept informed of the important questions and provided with accurate data to produce reliable results. Equally, modelers must specify their data needs to provide accurate answers for public health decision-making. Thus, modelers and clinical and public health communities must work together.

GLOBAL DENGUE VACCINE CONSIDERATIONS AND RECOMMENDATIONS

The WHO has supported the process of dengue vaccine development, and provided guidance and scientific consensus. During the pre-registration period, the WHO engaged in activities to support global vaccine guidance and introduction by developing regulatory standards. More recently, a dedicated technical advisory group consulted on the pivotal clinical trial results on behalf of the WHO to better understand the complex data from the trials and to ascertain the data needs for public health/policy recommendations. Post-registration, the most important activity is to provide recommendations for vaccine introduction and use, as well as guidance for monitoring vaccine effectiveness and safety.

Guidance for new vaccine introduction and use

The WHO Vaccine Position Papers include global recommendations for use of a specific vaccine (or vaccine class) (WHO, 2016a). Development of a position paper starts before registration of a vaccine by

Table 2. Model types by complexity.

Compartmental models	Network models	Agent-based models
Long history	Structured population	Most detailed and flexible
Most mathematically tractable	Sometimes mathematically tractable	Arbitrarily realistic
Everyone in a compartment is the same		Hard to understand
Deterministic/stochastic	Population structure is important and 'known'	Computationally intensive
	Deterministic/stochastic	Stochastic

national regulatory authorities and is issued after a vaccine is licensed. Position papers are endorsed by the Strategic Advisory Group of Experts (SAGE) on Immunization and published in The Weekly Epidemiological Record (WHO, 2016b). The information includes review of the evidence for key policy questions and review of the quality of evidence using the Grading of Recommendations Assessment, Development and Evaluation process. The position papers are updated regularly as new knowledge becomes available.

Much of the recommendation development is done by a dedicated SAGE working group, with input from other WHO advisory groups on specific issues. A background paper is produced and discussed by SAGE at an open meeting. The recommendations are reviewed by the WHO Director General, and tendered for broad stakeholder consultation before a position paper is developed. The process is rigorously evidence based, transparent, and inclusive. All the information that is critical for decision-making by SAGE is in the public domain or will be made public at the time of the SAGE meeting. The SAGE Working Group on Dengue Vaccines was established in March 2015.

Key considerations for policy

Key considerations for dengue vaccine policy include safety, efficacy, and programmatic aspects (Table 3). As the dengue vaccine is new, there may not be sufficient data to answer all the considerations, hence a need for mathematical modeling to inform and underpin policy recommendations. Comparative modeling of dengue vaccine public health impact will provide additional information for SAGE recommendations by assessing various vaccination scenarios and their impact on public health.

Comparative modeling of dengue vaccine impact has evaluated the following parameters: routine introduction at 9 years; catch-up vaccination at 10-17 years; Asian and Latin-American reference country scenarios and different transmission intensities; and vaccine impact on infection, clinical cases, severe cases, and death. The vaccine impact was modeled overall, by age group, and by 10- and 30-year time horizons. An exploratory economic evaluation was also done, although this will be more accurate if done by each country to suit their specific circumstances. The economic evaluation included traditional cost-effectiveness analysis (costs per clinical case and costs per disability-adjusted life year averted); delivery costs adapted from human papillomavirus vaccine delivery experience; and literature appraisal of the broader economic impact.

WHO global policy on dengue vaccine

In April 2016, recommendations on the use of the CYD-TDV vaccine were discussed by SAGE (WHO, 2016b). The first WHO Vaccine Position Paper on dengue vaccines was published in July 2016 (WHO, 2016a).

Table 3. Key considerations for dengue vaccine policy.

Parameter	Consideration
Vaccine safety	Reactogenicity and serious adverse events, adverse events of special interest
	Long-term safety and risk of hospitalization/severe dengue
Vaccine efficacy	Overall, by age, by serostatus, by serotype
	Efficacy against laboratory-confirmed dengue, severe disease
	Duration of protection
Programmatic aspects	Dose scheduling
	Co-administration
	Vaccine introduction strategies, including outbreak response
	Vaccine impact and cost-effectiveness
	Criteria for country decision-making

Development of vaccine policy is done at the global, regional, and national levels. The global recommendations from the WHO are intended to inform country decision makers and provide general orientation.

Considerations for vaccine introduction (Table 4) include disease factors (high morbidity with low mortality, outbreaks and burden on health system, school or work absenteeism, and alternative or additional preventive methods, *ie,* vector control) and vaccine factors (availability, price, programmatic costs, economic impact, national budget and vaccine affordability, and funding gaps and sustainability) (WHO, 2014a). The strength of the immunization program and the health system in the country are also considered. Important considerations include overall readiness for a new vaccine, school readiness, and implementation readiness (WHO, 2013), as well as tracking of vaccination status. Lessons can be learned from other vaccination programs in this age group such as human papillomavirus (HPV).

The use of both vector and vaccination strategies is essential, and communication, community mobilization, and advocacy remain important for both vector control and vaccination.

CURRENT SCHOOL-BASED VACCINATION PROGRAMS AND PLANS IN ASIA

School-based human papillomavirus vaccination program in Malaysia

Malaysia has low uptake of cervical cancer screening and delayed diagnosis and treatment, with most women seeking treatment at stage 2 or above. Thus, there is a need for cervical cancer prevention measures. When the WHO endorsed the HPV vaccine, Malaysia made it available to all girls aged 13 years (WHO, 2014b), with the aim of reducing the incidence of cervical cancer.

The vaccine was made available in the private sector in 2006, and implemented into the public healthcare system in 2010. The strategy was to deliver the vaccine as part of the Cervical Cancer Prevention and Control Program and integrate it into the Expanded Program of Immunization (EPI). The operational policy was for voluntary free school-based HPV vaccination delivery to Malaysian schoolgirls at age 12-13 years, with a target of three doses for 95% of the target population, which was exceeded at 98% completion. There was strong commitment and support from the Ministry of Education (MoE).

Factors contributing to the success of the HPV immunization program included:
- Political will and commitment,
- Public trust in the Malaysian EPI,
- Availability of school health services infrastructure,
- Existing strong relationship with the MoE,
- Effective risk communication strategy,
- Addressing religious issues, and
- Competitive procurement mechanism.

Integrating the HPV vaccine into the School Health Program made it part of the immunization package. The guiding principles of adding a new program into the school health service are:
- New service introduction must not affect existing services performance,
- Implementation must be approved by the MoE,
- Implementation must not interfere with the school schedule, and
- Participation must be voluntary, with parental approval.

There are several factors to consider before integrating a new vaccination program into school health activities (Table 4). Preparation and planning is key to the success of the program.

School-based immunization program in Philippines

There are many advantages of school-based immunization programs. Booster doses can be given to ensure high levels of protection, some vaccines are more effective if delivered at a specific age, and compliance is high. The current vaccinations delivered to Philippines schoolchildren are measles-rubella, tetanus-diphtheria, HPV and a deworming program.

Table 4.   Factors to consider when integrating a new vaccination program into school health activities.

Factor	Requirements
School health infrastructure and resources	Initial budget to include implementation, *eg*, cold-chain, transportation Resource mobilization
New program objectives and expected impact	Long-term/short-term impact Coverage (>95% for HPV)
Capacity building	Training and introduction phase Updates (*eg,* policy changes)
Monitoring and evaluation	Track implementation and impact
Dealing with public expectation	Health promotion campaign budget Crisis management Demand for service
Parental acceptance	Confidence in new program Vaccine safety and efficacy Vaccine combination (*eg*, HPV and tetanus toxoid)
Will the new program effect students' performance	Which cohort to choose from (consideration of examinations, prophylaxis status of HPV vaccine)
Compliance to schedule/follow-up	Completion within one schooling period (timing of doses)

HPV, human papillomavirus.

Guidelines for the implementation of school-based immunization were introduced in 2015. The guidelines comprise both general and specific recommendations on the vaccine use, storage and transport, immunization safety, recording and reporting, and AEs following immunization. The Department of Health (DoH) provides the vaccines and immunization logistics for routine distribution, training, and pharmacovigilance reporting. The Department of Education facilitates the implementation in schools, informs participants, screens students, and submits reports to the local health units. Other governmental and local level departments organize the vaccination team and provide healthcare personnel. The Parents–Teachers Association plays a role in raising awareness.

There are several components to the dengue prevention and control program, including surveillance, integrated vector management, case management, social mobilization and communication, outbreak response, and research. The existing dengue case definition and case fatality rate is based on the recommendations of the WHO. Laboratory surveillance will enable monitoring of serotypes circulating in different areas. Mechanisms for sharing data are in place (UNITEDengue; https://www.unitedengue.org/index.html). Dengue surveillance is incorporated into an integrated disease surveillance system.

An evidence-based integrated vector management strategy has been implemented with community involvement. Vector resistance is monitored regularly. There is laboratory support for case management and a referral network system in both the public and private sectors. Communication for behavioral impact (COMBI) training has been implemented and the COMBI approach disseminated and promoted. There is a dengue outbreak standard operating system and national early warning/dengue surveillance system. Tools and strategies for dengue control and case management will be evaluated regularly.

Philippines is the first country in the Asia-Pacific region to register the dengue vaccine, on 22 December 2015. The vaccine will be delivered via the school-based immunization program to children aged 9 years, in accordance with the results of the phase 3 trials (Capeding *et al,* 2014; Villar *et al,* 2015).

Table 5.   Expanded program of immunization in Thailand and Indonesia.

Age	Thailand	Indonesia
Birth	BCG, HB1	HB
1 month		BCG, OPV1
2 months	OPV1, DTP-HB1	DPT-HB-Hib 1, OPV1
3 months		DPT-HB-Hib 1, OPV2
4 months	OPV1, OPV2, DTP-HB2	DPT-HB-Hib 1, OPV3, IPV
6 months	OPV3, DTP-HB3	
9 months	MMR1	Measles
1 year	JE1-2	
18 months	OPV4, DTP4	Measles, DPT-HB-Hib
30 months	MMR2, JE3	
4 years	OPV5, DTP5	
7 years	BCG, dT, OPV, MR	
12 years	dT	
Pregnant women	dT	
Healthcare personnel and risk groups	Influenza	

BCG, Bacillus Calmette–Guérin; dT, diphtheria and tetanus; DTP, diphtheria, tetanus, and pertussis; HB, hepatitis B; Hib, *Haemophilus influenzae* type b; IPV, inactivated polio vaccine; JE, Japanese encephalitis; MMR, measles, mumps, and rubella; MR, measles and rubella; OPV, oral polio vaccine.

The vaccine will be implemented in three highly endemic regions with high-risk populations. Training of healthcare providers, active surveillance for AEs following immunization, and a recording and reporting system will be implemented. Good communication will be needed to explain why only certain regions have the vaccine. The DoH will provide all logistical items. Prevention strategies will continue in conjunction with the vaccine implementation initiative.

Operational research will include a post-authorization phase 4 study and collection of data on access to care, cost-effectiveness, and policy to support expansion of the vaccine to other parts of country.

School-based immunization program in Bangkok, Thailand

The Bangkok Metropolitan Administration healthcare providers run 68 public health centers, which are responsible for school-based vaccination, and eight hospitals. The Ministry of Public Health has 36 hospitals and 135 health units, and there are 95 hospitals and 466 clinics run by private healthcare providers. Thailand has a very full EPI (Table 5).

There are several optional vaccines recommended by the Infectious Disease Society of Thailand, including whooping cough (pertussis), *Haemophilus influenzae* type b, and HPV. School-based vaccination is well accepted with high coverage. Strengthening of capacity building is an important step for a successful school-based vaccination program.

School-based immunization program in Indonesia

The Indonesian constitution states that health is the right of all Indonesian people. Routine immunization services are available for infants, children younger than 5 years, schoolchildren, and women of childbearing age. Additional immunization is done for catch-up programs and campaigns, national immunization days, and outbreak response. Optional immunization includes those vaccines not provided by the government.

The policy and operational strategy is to achieve:
- high immunization coverage, that is equally distributed via a static and accessible EPI service and services in hard-to-reach areas,
- continuous quality improvement through skilled personnel, quality vaccine and cold chain system, and correct vaccination procedure,
- community mobilization and participation.

The target for the EPI is shown in Table 5.

The Usaha Kesehatan Sekolah (SHP) runs health education, health service delivery through schools, and the Bulan Imunisasi Anak Sekolah (School Immunization Month Program; BIAS). The objective of the school immunization program is to provide long-term protection against EPI target diseases of measles, diphtheria, and tetanus. The BIAS is a well-designed program, with operational guidelines for health workers and teachers, roles and responsibilities of each Ministry, health center budgets, and vaccine and supplies provided by central government. There is high coverage in all schools where the program is conducted. There are cost and financing issues, with limited resources for operational costs, monitoring and evaluation, and advocacy to local government. However, coverage is >90%.

The role of the Ministry of Health is development of policy and guidelines for technical matters, preparation and implementation of immunization services at schools, and monitoring and evaluation. The role of the MoE is mobilization of teachers in public and private schools to support the program, and coordination with schools and parents. The role of the Ministry of Religion is socialization and mobilization of teachers in faith-based public and private schools. The role of the Ministry of Home Affairs is advocacy to local governments for logistics and supplies and operational costs for program implementation.

The challenges include how to institutionalize the BIAS, improve parents' awareness, and integrate new vaccines such as dengue into the program. However, global disease elimination and eradication is a public health strategy.

ACKNOWLEDGEMENTS

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