Adult Dengue

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Adult Dengue

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Editors

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PREFACE

Dengue is the most common mosquito-borne infection that burdens tropical and subtropical regions of the world, and the incidence of dengue infections in adulthood is currently increasing worldwide. In response to the WHO-announced goal of reducing dengue mortality by 50% and morbidity by 25% by 2020, we need improved monitoring of outbreaks to facilitate the allocation of resources to the endemic areas. In addition, improved triaging and monitoring of dengue patients would greatly reduce the human and economic costs associated with the complications of this disease.

The Infectious Disease Association of Thailand (IDAT) and the Pediatric Infectious Disease Society of Thailand (PIDST) have the main purposes of sharing their knowledge, conducting research on infectious diseases, and knowledge management. These professional bodies also provide training and advice on various infectious diseases for doctors, medical personnel, and the public. Accordingly, the Short Training Course on Adult Dengue was successfully organized during 2014 August 6-8 in Thailand by the IDAT and the PIDST.

These proceedings consist of most of the topics presented at the aforementioned course. In addition, some informative and useful articles have been added, and altogether 6 chapters with 19 articles are included. The updated and relevant topics are comprised of the epidemiology, pathogenesis, diagnosis, management, and prevention and control of dengue infection. The Practical Guidelines for Management of Dengue in Adults, 2014 from the Royal College Physician of Thailand has also been added in the Annex. We intend these proceedings to be a reference for physicians and scientists who are interested in adult dengue to provide the greatest benefit for its management.

These proceedings are one of the memorable events in 2015 of the 33th anniversary celebration of the IDAT and the 20th anniversary celebration of the PIDST.

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The editors would like to express their sincere appreciation to the Infectious Disease Association of Thailand (IDAT) and the Pediatric Infectious Disease Society of Thailand (PIDST) for organizing the informative Short Training Course on Adult Dengue from August 6 to 8, 2014 in Thailand, and their kind support in the publication of these proceedings. We are also grateful to the invited course speakers and the authors of these proceedings for contributing their time, experience, and knowledge.

The editors would also like to thank the World Health Organization (South-East Asia Regional Office) for supporting the delegates to participate in the aforementioned course.

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Dangue: overview and epidemiology

DENGUE: GLOBAL THREAT

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Abstract. Dengue is a mosquito-borne viral disease, which is currently an expanding global problem. Four closely related dengue serotypes cause the disease, which ranges from asymptomatic infection to undifferentiated fever, dengue fever (DF), and dengue hemorrhagic fever (DHF). DHF is characterized by fever, bleeding diathesis, and a tendency to develop a potentially fatal shock syndrome. Dengue infection with organ impairment mainly involves the central nervous system and the liver. Consistent hematological findings include vasculopathy, coagulopathy, and thrombocytopenia. Laboratory diagnosis includes virus isolation, serology, and detection of dengue ribonucleic acid. 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 and validated in many countries. There is no specific dengue treatment, and prevention is currently limited to vector control measures. The world's first, large-scale dengue vaccine efficacy study demonstrated its efficacy and a reduction of dengue disease severity with a good safety profile in a study of more than 30,000 volunteers from Asia and Latin America.

Keywords: dengue, global threat

INTRODUCTION

Dengue is one of the most devastating mosquito-borne viral diseases in humans. The disease, caused by the four dengue virus serotypes, ranges from asymptomatic infection, undifferentiated fever, dengue fever (DF), to severe and fatal dengue hemorrhagic fever (DHF). The clinical spectrum of the infection undermines surveillance activities because the majority of cases are asymptomatic and go undetected. These cases can be an important source of infection for dengue virus transmission via the mosquito vector. DHF is characterized by fever, bleeding diathesis, and a tendency to develop a potentially fatal shock syndrome. The disease is a major public health concern in several countries, and the disease could potentially spread to non-endemic areas. It is one of the leading causes of hospitalization, placing tremendous pressure on strained medical resources with an associated major economic and social impact in countries where dengue disease is prevalent (Hemungkorn *et al*, 2007; Capeding *et al*, 2013).

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EPIDEMIOLOGY

Dengue is the most common arboviral infection of humans transmitted by *Aedes* mosquitoes, principally *Aedes aegypti*. These mosquitoes largely breed indoors in clean water, mainly in artificially water containers, and feed on humans during the daytime. There are four antigenically distinct serotypes of dengue virus (DEN 1, 2, 3 and 4), which belong to the genus *Flavivirus* of the family Flaviviridae. Primary infection with a particular dengue serotype confers long-lasting immunity for that serotype (homotypic immunity) while the immunity it confers to other dengue serotypes (heterotypic immunity) lasts for only a few months, after which patients are susceptible to heterotypic infection. Four viral serotypes cause disease in proportions that change over time and from place to place, even within the same country. A review of dengue virus incidence from 1973 to 1999 in Bangkok found that all four dengue serotypes could be found circulating in any one year, with one predominant serotype emerging and re-emerging as the cause of the epidemic. The authors concluded that the pathogenesis of DHF is complex, and that it is a product of host determinants, dengue serotype, and environmental factors (Nisalak *et al*, 2003).

Global phenomenon such as urbanization and international travel are key factors in facilitating the spread of dengue. Documenting the type-specific record of dengue virus spread has important implications for understanding patterns in dengue hyperendemicity and disease severity as well as vaccine design and deployment strategies. A series of global maps on the distribution of confirmed instances of each dengue virus serotype from 1943 to 2013 shows the worldwide expansion of the dengue virus, the hyperendemicity of the disease, and its establishment as an increasingly important infectious disease of global public health significance (Messina *et al*, 2014). Dengue, along with the mosquito vectors that transmit it, is now endemic in over 120 countries throughout the tropical and subtropical regions of the world (Bhatt *et al*, 2013; Thisyakorn and Thisyakorn, 2015). It is nearly ubiquitous in the tropics and has continued to emerge, or become hyperendemic, in new areas as the range of the *Aedes* mosquito vectors continues to expand.

Global dengue transmission has increased at least 30-fold in the past 50 years (WHO, 2009). The burden borne by the health and medical resources of affected countries is enormous, but nowhere is the burden greater than in the Southeast Asian and Western Pacific regions, where the incidence of dengue is already the highest in the world and continues to increase and cause epidemics. The estimated annual economic burden for Southeast Asia, excluding prevention and vector control, was nearly USD1 billion or USD1.65 per capita with two countries, Indonesia and Thailand, accounting for over 60% of this burden (Shepard *et al*, 2013). Currently, over 70% of the global population-at-risk for dengue lives in these regions (WHO, 2012).

The global increase in dengue cases and also the potential spread of the disease to non-endemic areas are due to factors such as atmospheric composition, climate change and human movement. Even with estimates of disease burden increasing, dengue is widely under-reported due to misdiagnosis and inconsistencies in diag-

nostics and surveillance systems. Dengue has spread into new geographical areas affecting both children and adults despite being significantly under-reported. Over half of the world's population lives in areas at risk of infection. About 70% of the overall disease burden, thought to have increased 30-fold in the last 50 years, is reported in the Asia-Pacific region. In recent years, there has been an increase in dengue cases in rural settings and also a shift towards increased incidence in older age groups in many countries where dengue is endemic. The trend has important implications for control and prevention (Thisyakorn and Thisyakorn, 2015). Vertical transmission of dengue virus from mother to child has also been reported for the first time in English literature (Thaithumyanon *et al*, 1994).

The severity of DF manifestations increases with age. DF causes fever, rash, muscle or joint pain, headache, eye pain but is rarely fatal. DHF is considered a distinct disease characterized by increased vascular permeability leading to leakage of plasma and dengue shock syndrome (DSS). Unusual manifestations of dengue patients with severe organ involvement such as liver, kidney, brain, or heart associated with dengue infection have been increasingly reported in patients with dengue infection. These manifestations may be associated with co-infections, co-morbidities, or complications of prolonged shock. Exhaustive investigations should be done in these cases (Innis *et al*, 1990; Thisyakorn and Thisyakorn, 1994a,b; Thisyakorn *et al*, 1999; Hemungkorn *et al*, 2007).

Research into the pathogenesis of dengue infection has exploded over the last half century. Issues that were considered simple have become more complex as additional data have been found. This has led to the development of a number of controversies that are being studied globally and debated in the literature as follows: the 1997 World Health Organization (WHO) case definition of DHF is not useful; DHF is not significantly associated with secondary dengue infection; DHF results from infection with a virulent dengue virus; DHF is caused by abnormal T-cell responses; DHF results from auto-immune responses; and DHF results from direct infection of endothelial cells. A clinically and physiologically applicable case classification that will allow robust pathological research into the different levels of disease severity is a major priority (Thisyakorn and Nimmannitya, 1993; Sosothikul *et al*, 2007; Halstead, 2012).

DIAGNOSIS

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

Clinical manifestations

- Fever: acute onset, high and continuous, lasting two to seven days in most cases.
- Any of the following hemorrhagic manifestations including a positive tourniquet test, petechiae, purpura, ecchymosis, epistaxis, gum bleeding, and 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,

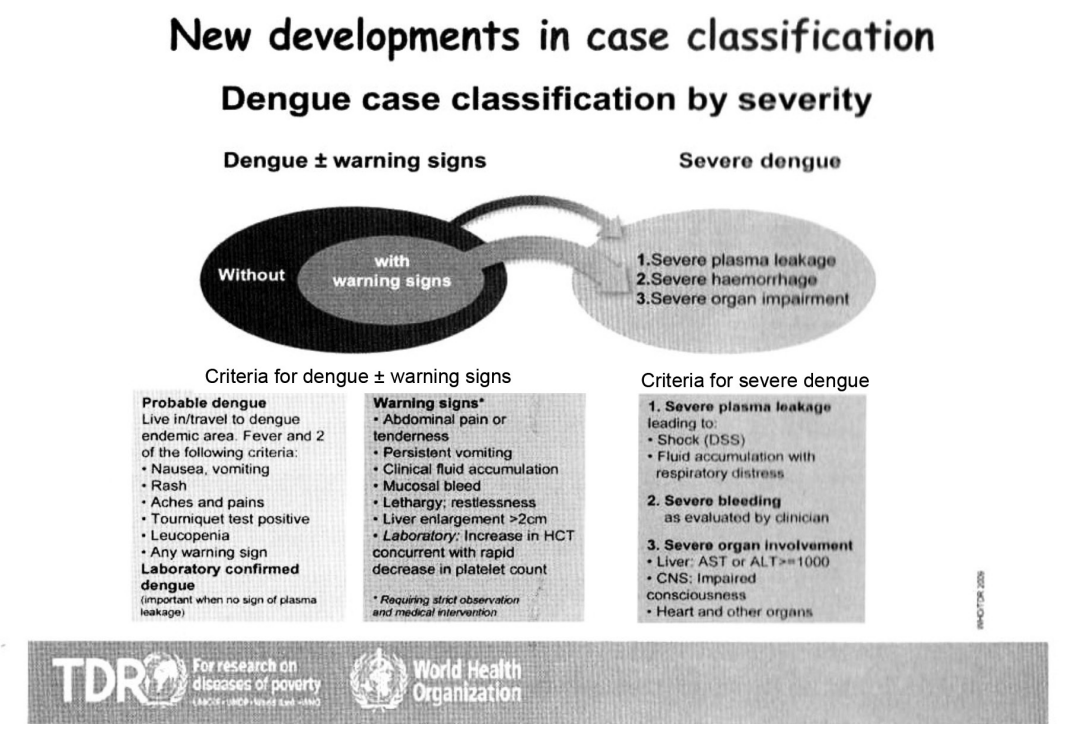

Fig 1–The 2009 WHO dengue case classification.

poor tissue perfusion with weak pulse and narrowed pulse pressure or hypotension with the presence of cold, clammy skin and/or restlessness.

Laboratory findings

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

The 1997 WHO case classification system for dengue was revised because of differences across the broad geographical areas and the age groups affected by dengue.

However, the current 2009 WHO classification (Fig 1) has yet to be definitively proved to be effective. The question remains, therefore, whether this latest classification requires further modification (Hadinegoro, 2012).

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

Hematological findings include vasculopathy, reduction of several coagulation factors, reduced platelet count, and platelet dysfunction. The tendency towards bleeding should be monitored in any dengue patient because it may cause severe and uncontrollable hemorrhage. The pathogenesis of bleeding in a dengue patient is not fully understood. The extent of endothelial

cells involvement, coagulation, and fibrinolysis activation in children with dengue infection seems to be correlated with dengue disease severity (Mitrakul and Thisyakorn, 1988; Setrkraising *et al*, 2007).

The laboratory diagnosis of dengue infection can be confirmed by serological tests, isolation of the virus, and detection of viral RNA by reverse transcriptase polymerase chain reaction. Commercial kits for dengue diagnosis are also available for routine use. A pilot evaluation of diagnostic values of ELISA and reverse transcription polymerase chain reaction from oral specimens yielded promising results. Collection of oral specimens is less invasive and may be more acceptable (Hemungkorn *et al*, 2007).

Clinical manifestations of dengue infection vary with age as 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 are associated with greater risk of mortality (Panpitpat *et al*, 2007; Tantawichien, 2012).

TREATMENT

Treatment of dengue infection is symptomatic and supportive. In most cases, early and effective replacement of lost plasma with fluid and electrolyte solutions, plasma, and/or plasma expanders results in a favorable outcome. The outcome depends on early recognition of infection and careful monitoring. 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 decline in the 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 carried out as a routine precaution for every patient in shock.

The rate of fluid infusion needs to be carefully tailored according to the patient's vital signs, hematocrit, and urine output. In general, there is no need for fluid therapy beyond 48 hours after the cessation of shock. Reabsorption of extravasated plasma takes place, manifesting 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 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, 1994c).

PREVENTION

Prevention of dengue depends on the control of the mosquito vector by limiting its breeding places and treatment of stored water with larvicide. These measures against dengue are effective only with a high level of government commitment, education, and community participation. Development of a dengue vaccine is seen as the best hope to fight this potentially fatal disease (Thisya-

korn and Thisyakorn, 2015).

A phase III efficacy trial of a recombinant, live, attenuated tetravalent dengue vaccine (CYD-TDV) in highly dengue-endemic areas in Asia and Latin America in more than 30,000 children demonstrated that this dengue vaccine is efficacious when given as a 0-6-12 month schedule to children. Severe dengue episodes were avoided with a reduction in hospitalization. Higher efficacy was observed in the immunogenicity subset seropositive at baseline. The safety profile was consistent with the good safety profile observed in previous studies over the 25-month follow-up period, showing no evidence of antibody dependent enhancement in partial or completely vaccinated individuals.

An interesting finding of this trial was 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. Results confirm the potential public health impact of the vaccine and support the vaccines potential at reducing the public health burden of dengue. It should be recognized as the dawn of a new era of dengue control because the potential use of this vaccine could be a major turning point for global dengue control. The interesting finding of this trial was that vaccine efficacy was higher for participants who were seropositive for dengue than for those who were seronegative (Capeding *et al*, 2014; Dengue Vaccine Initiative, 2014; Wilder-Smith, 2014). The results from Latin America complement those in Asia and provide a more global picture of the vaccine's potential to contribute to reaching the 2020 WHO target of reducing the global burden of dengue by decreasing morbidity by 25% and mortality by 50% (WHO, 2012).

A dengue vaccine could greatly alter the disease landscape, but this goal can only be realized if the many challenges to its implementation are addressed effectively. We have waited a long time for an effective intervention against dengue. Informed decisions must be made for each setting to determine how dengue vaccination should be implemented into existing national vaccinations programs: catch-up campaigns must be delivered; optimal vaccination strategies must be defined; and post-approval safety and efficacy must be monitored. Countries must plan for the vaccine's introduction. In particular, how the vaccine will complement existing vector management programs, and how dengue surveillance can be strengthened, which will be essential to assess the appropriate dengue vaccination strategy for each epidemiological setting. It is good news that a safe and effective dengue vaccine is on the horizon. While this stands to be a critically important achievement in the fight against dengue, we need to understand how to implement this new tool effectively, and this will require firm commitments from all affected countries if the WHO objectives are to be met (Thisyakorn *et al*, 2014b).

An independent scientific and educational Association of Southeast Asian Nations (ASEAN) Members States Dengue Vaccination Advocacy Steering Committee (ADVASC) was established in 2011 to address the practical challenges faced by ASEAN countries as they prepare for the eventual introduction of a dengue vaccine. The ADVASC convened workshops that drew together public health representatives

and dengue experts from ASEAN countries in order to make practical recommendations to improve current surveillance and diagnostics for dengue to enable countries to consistently assess and accurately communicate the impact of a dengue vaccine (Thisyakorn, 2012; Thisyakorn *et al*, 2014a).

A 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 groups to be vaccinated, which will lead to universal dengue vaccine implementation in the future.

In summary, 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 a dengue vaccine and vector control innovations (Suaya *et al*, 2009).

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SUGGESTED READING:

Villar L, Dayan GH, Arredondo-García JL, *et al*. Efficacy of a tetravalent dengue vaccine in children in Latin America. *N Engl J Med* 2015; 372: 113-23.

DENGUE PATIENTS AT PHOTHARAM HOSPITAL: A CLINICAL TRIAL SITE OF DENGUE VACCINE

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Abstract. Between 2005 and 2013, 1,868 dengue patients, 916 male and 952 female, were admitted to Photharam Hospital, Ratchaburi, Thailand. Among these patients, there were 1,209 with dengue fever (DF), 598 with dengue hemorrhagic fever (DHF), and 61 with dengue shock syndrome (DSS) with 1 death. The disease was seen all year round with a higher incidence in the rainy season. A trend of shift in age group towards older children and adults was seen during the study period. These data show that dengue patient admissions to Photharam Hospital are common, causing a heavy burden on the health system. Only one death was seen during the period of study, indicating that early recognition and effective management of dengue patients occurred. The trend towards higher age in dengue patients during the study period is a problem of concern and needs further clarification.

Keywords: dengue, epidemiology, dengue vaccine, clinical trial

INTRODUCTION

Dengue, along with the mosquito vectors that transmit it, is now endemic in over 120 countries throughout the tropical and subtropical regions of the world (Bhatt *et al*, 2013; Thisyakorn, 2014). It is nearly ubiquitous in the Tropics and has continued to emerge or become hyperendemic in new areas as the range of the *Aedes* mosquito vectors continues to expand. Global dengue transmission has increased at least 30-fold in the past 50 years (WHO, 2009a). The burden borne by the health and medical resources of affected countries is enormous, but nowhere is the burden greater than in the Southeast Asia and the Western Pacific regions, where the incidence of dengue is already the highest in the world and continues to increase and to cause epidemics. The estimated annual economic burden for Southeast Asia excluding prevention and vector control was nearly USD1 billion or USD1.65 per capita with two countries, Indonesia and Thailand, accounting for over 60% of this burden (Shepard *et al*, 2013). Currently, over 70% of the global population-at-risk for dengue lives in these regions (WHO, 2012). The global increase in dengue cases and also the potential spread of the disease to non-endemic areas are due to factors such as atmospheric composition, climate change, and human movement. Even with increasing estimates of disease burden, dengue is widely under-reported due to misdiagnosis as well as inconsistencies in diagnostics

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and surveillance systems. Dengue has spread into new geographical areas affecting both children and adults despite being significantly under-reported. Over half of the world's population lives in areas at risk of infection.

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, and dengue hemorrhagic fever (DHF) causes abnormal hemostasis and increased vascular permeability with severe cases leading to dengue shock syndrome (DSS) and death. Patients with severe organ involvement such as liver, kidneys, brain, or heart associated with dengue infection have been increasingly reported in DHF and also in dengue patients without evidence of plasma leakage. These manifestations may be associated with co-infections, comorbidities or complications of prolonged shock. Exhaustive investigations should be done in these cases. The clinical spectrum of the infection undermines surveillance activities because the majority of cases are asymptomatic and go undetected.

Complex disease presentation and sudden development of hemorrhagic symptoms in seemingly stable patients can cause fatal outcomes even in well-prepared hospitals. There is currently neither an approved preventive vaccine nor a specific anti-viral treatment against dengue. Main public health preventive interventions consist of mosquito control, which is currently used in endemic countries, and use of vector repellents; both generally have had only limited success. Development of a dengue vaccine is seen as the best hope to fight this disease. In Thailand, a dengue patient was first seen in Bangkok, Thailand in 1958 and then others appeared in other parts of the country (Thisyakorn, 2014). The aim of this study is to describe the epidemiological pattern of dengue patients admitted to Photharam Hospital, Ratchaburi Province, Thailand, a clinical trial site of dengue vaccine.

MATERIALS AND METHODS

Analysis of the data of dengue patients admitted to Photharam Hospital, a provincial hospital in Ratchaburi Province, Thailand from January 2005 to December 2013 was done after the approval of an ethics review committee. Photharam Hospital is among ten clinical trial sites for a dengue vaccine (Capeding *et al*, 2014). The hospital is in Ratchaburi Province, which is approximately 100 kilometer west of Bangkok, and it is among the ten provinces in Thailand with the highest dengue incidences (Capeding *et al*, 2013). Hence, it provides a suitable site for clinical trials of candidate dengue vaccines. A field-site for large scale clinical trials for dengue vaccines has been developed in Ratchaburi from 2005 up to the present time (Sabcharoen *et al*, 2012). 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).

RESULTS

Between 2005 and 2013, there were 1,868 dengue patients, 916 male and 952 female, admitted to Photharam Hospital in Ratchaburi, Thailand. According to the

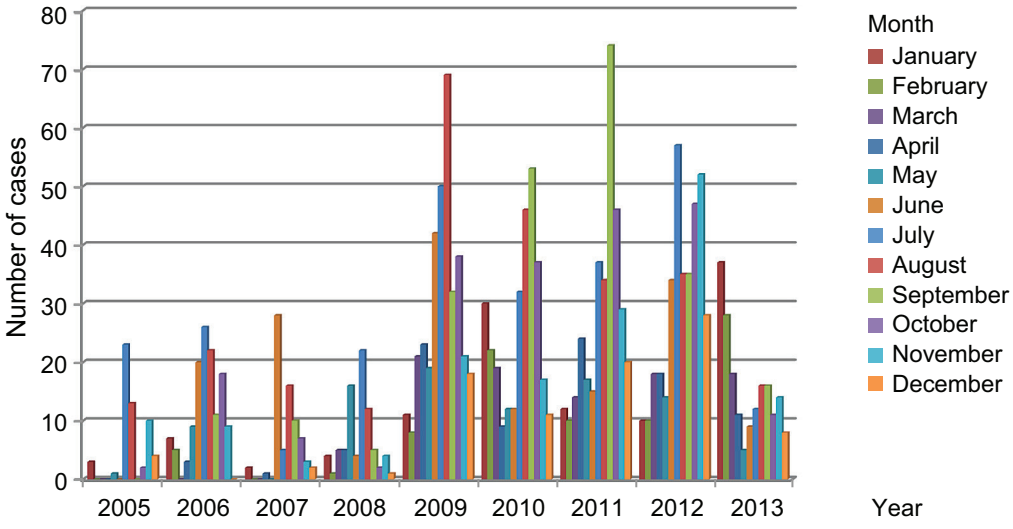

Fig 1–Seasonal distribution of dengue patients in Photharam Hospital, Thailand between 2005 and 2013.

1997 WHO case classification of dengue, there were 1,209 cases of DF, 598 of DHF, and 61 of DSS. The only patient who died in this study was a 17-year-old Down syndrome boy with refractory DSS, hematemesis, and organopathy involving the liver and central nervous system.

The disease was seen all year round and started to increase during the dry hot months of April to June. A higher incidence was observed in the rainy season, and this usually peaked 2-4 weeks after the arrival of the rains, which began anytime between June and September. The rainy season usually finished in October, but could last into November (Fig 1).

Fig 2 shows incidence by age group. Rates were constantly high among children below 15 years old with a trend of increasing mean age with time. Rates in older children and adults increased dramatically throughout the period of study.

Fig 3 shows the severity of dengue disease by age group. It demonstrates that all degrees of dengue severity can be seen in all age group with the trend of higher DSS cases in children in comparison to adults.

DISCUSSION

During the past decades, dengue epidemics are known to have occurred regularly in Ratchaburi, Thailand causing a heavy burden on the healthcare system. Population growth together with a remarkable degree of urbanization has allowed dramatic expansion of the mosquito population through an increase in urban breeding sites (Tanayapong *et al,* 2013). This explains the explosive increase in reported cases. A greater awareness and high reporting behavior could have contributed to some of the increase over time. The reasons for the apparent upsurge in dengue are probably multifactorial. Vector

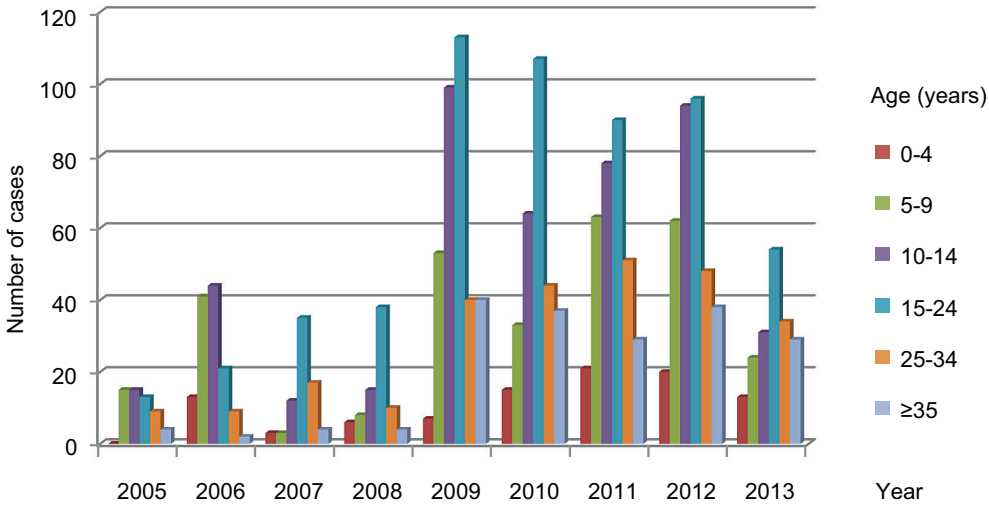

Fig 2–Age distribution of dengue patients in Photharam Hospital, Thailand between 2005 and 2013.

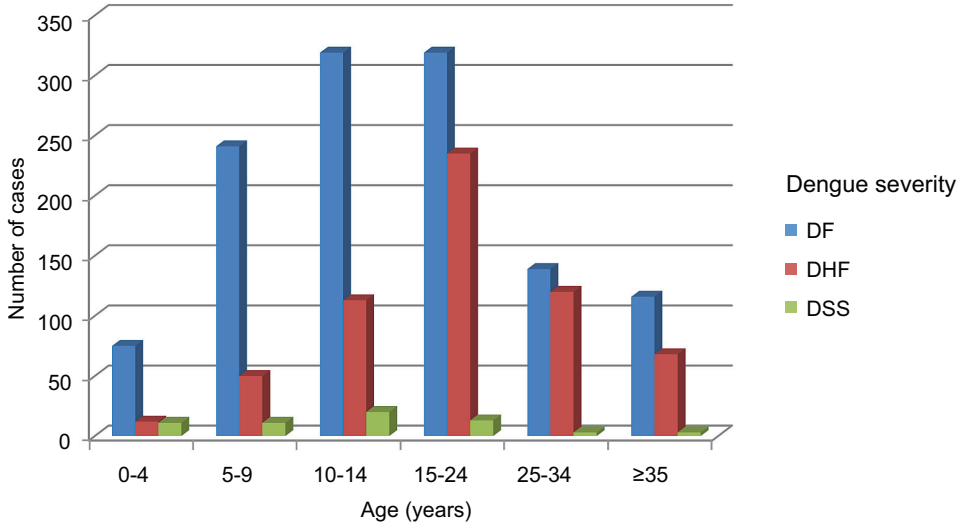

Fig 3–Severity of dengue patients by age group in Photharam Hospital, Thailand between 2005 and 2013.

efficiency of *Aedes aegypti* increases with increasing temperature for dengue virus (Tanayapong *et al,* 2013). This may explain the increasing number of dengue patients during the dry hot season. Global warming may contribute to the wider spread of dengue infection. The availability of more water and higher humidity, including higher biting rates may augment the epidemic during rainy period. Changes in weather patterns with increases in average temperatures and rainfall are classically seen as possible causes (Tanayapong *et al,* 2013). Many factors influence the epidemiologic patterns of dengue besides climate such as movements of mosquitoes, the types of

circulating dengue viruses, and environmental factors such as temperature and humidity as well as human behavior and development (Tanayapong *et al*, 2013).

Well-targeted operations such as population-based epidemiological studies with clear operational objectives are urgently needed to progress control and prevention. Dengue remains predominantly a pediatric disease, but the trend towards higher rates in older children and adults during the last decade is incompletely understood (Tanayapong *et al,* 2013). This trend may be due to the lesser frequency of epidemics in the last decades, which may have caused second exposures to dengue virus to be postponed (Tanayapong *et al,* 2013). The only fatal case in this study had severe dengue according to the 2009 WHO dengue case classification with the patient deteriorating to multiple organ failure despite vigorous intensive therapy. The low mortality seen throughout the period of study indicates early recognition and effective management of dengue patients in Photharam Hospital.

Prevention of dengue depends on the control of the mosquito vector by limiting its breeding places and treatment of stored water with larvicide. These measures against dengue are effective only with a high level of government commitment, education, and community participation (Thisyakorn, 2014). Ultimately, the utilization of an effective and long-lasting vaccine is needed. Due to the unique challenges of dengue, including the need to provide protection against the four antigenically-distinct serotypes of the viruses, no vaccine is yet licensed to protect against this disease despite more than six decades of research.

The 1997 WHO dengue case classification, which classifies by clinical manifestations and laboratory findings may not clearly be correlated with disease severity. Therefore, the establishment of a new validated classification system in which cases are categorized by levels of severity has been recommended (WHO, 2009b). Several studies were done to compare the two classification systems regarding applicability in clinical practice and for surveillance. The new classification has shown a high potential for facilitating dengue case management and surveillance, but further evaluation is necessary (Hadinegoro, 2012).

A global strategy aimed at increasing the capacity for surveillance and outbreak response, changing behaviors, and reducing the disease burden using integrated vector management in conjunction with early and accurate diagnosis has been advocated. Antiviral drugs and vaccines that are currently under development could also make an important contribution to dengue control in the future (WHO, 2012).

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

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FOCAL DENGUE VIRUS TRANSMISSION IN KAMPHAENG PHET, THAILAND AND IMPLICATIONS FOR MANAGEMENT

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Abstract. Dengue is the most globally prevalent vector-borne viral disease. However, our understanding of dengue virus (DENV) transmission is incomplete. Prospective longitudinal cohort and cluster studies in Kamphaeng Phet, Thailand have contributed much to our understanding of DENV transmission. These studies have demonstrated the spatiotemporal heterogeneity of DENV transmission with highly focal transmission at small scales in a rural setting. Geographic cluster studies have suggested the presence of small "hotspots" of transmission at the house level that may have a disproportionately high impact on local spread. These hotspots should be considered when planning overall vector control interventions. The combined cohort and cluster design have shown that clinically inapparent DENV infections from prospective longitudinal cohorts likely consist of a clinical spectrum of infections from asymptomatic to mildly symptomatic with and without fever. The proportion of all DENV infections that are completely asymptomatic may be substantially lower than those considered to be inapparent in cohort studies. In addition, some of these inapparent infections from cohort studies have viable DENV and may potentially contribute to virus transmission. These findings require further validation in other settings and in adults and children. A more comprehensive understanding of DENV transmission will be critical to inform prevention, prognostication and management strategies.

Keywords: asymptomatic, cluster, cohort, dengue, focal, inapparent, spatiotemporal, transmission

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INTRODUCTION

Dengue is the most common vector-borne viral disease globally with approximately 2.5 billion people living in areas at-risk for infection. In the past several decades, dengue has expanded rapidly, with dengue virus (DENV) recently estimated to cause 390 million infections per year worldwide and with 96 million symptomatic cases (Bhatt *et al*, 2013). Four closely related, but antigenically distinct DENV serotypes

(DENV 1-4) from the genus *Flavivirus* in the family Flaviviridae are known to cause human disease (Gubler, 2002; Lindenbach and Rice, 2003).

These serotypes often co-circulate in endemic regions leading potentially to both disease mitigation and disease enhancement among different serotypes (Reich *et al*, 2013). However, despite the widespread circulation and disease burden caused by DENV, our understanding of transmission dynamics is incomplete but is critical to inform prevention, prognostication, and management strategies. In order to improve our knowledge of DENV transmission, the Armed Forces Research Institute of Medical Sciences (AFRIMS) in close partnership with other collaborating institutions has conducted prospective dengue studies in Kamphaeng Phet, Thailand since the 1990's (Gibbons *et al*, 2013).

Prospective longitudinal cohort studies conducted over multiple years can provide useful information about the true incidence of infections within a defined cohort, elucidate the full clinical spectrum of infections including clinically inapparent infections, and clarify the spatiotemporal distribution of infections (Endy *et al*, 2010). Geographic cluster studies can additionally indicate finer scale spatiotemporal dimensions of transmission and can be more sensitive in detecting mildly symptomatic and asymptomatic infections (Yoon *et al*, 2013). Cohort and cluster studies, therefore, provide useful platforms from which to study the different elements of DENV transmission. Here, we present some of the contributions to our understanding of DENV transmission from two sequential cohort/cluster studies conducted in Kamphaeng Phet, Thailand.

DESCRIPTION OF TWO COHORT/ CLUSTER STUDIES IN KAMPHAENG PHET, THAILAND

Two sequential prospective longitudinal cohort studies were conducted from 1998-2002 (called KPSI) (Endy *et al*, 2002a,b; Endy *et al*, 2011) and 2004-2007 (called KPSII) (Mammen *et al*, 2008; Yoon *et al*, 2012a) in Mueang District of Kamphaeng Phet Province in rural north-central Thailand (Fig 1). Dynamic cohorts of approximately 2,000 primary school children in grades 2-6 (KPSI) or kindergarten to grade 6 (KPSII) were followed by active school absence-based surveillance for acute febrile illnesses from June to November each year.

Acute blood samples were collected from cohort subjects who reported fever in the previous seven days; convalescent blood samples were collected two weeks later. Acute samples were tested by semi-nested reverse transcriptase polymerase chain reaction (RT-PCR) to detect DENV RNA (Lanciotti *et al*, 1992; Klungthong *et al*, 2007). Acute/convalescent sample pairs were tested by an in-house dengue/ Japanese encephalitis (JE) IgM/IgG capture enzyme-linked immunosorbent assay (ELISA) (Innis *et al*, 1989) and dengue/JE hemagglutination inhibition (HAI) assay (Clarke and Casals, 1958).

Virus isolation was attempted in *Aedes albopictus*-derived C6/36 cells from selected DENV PCR-positive samples (Jarman *et al*, 2011). Cohort subjects also underwent scheduled phlebotomy in January, May, August and November of each year (KPSI), or May and December/January of each year (KPSII). These scheduled blood samples

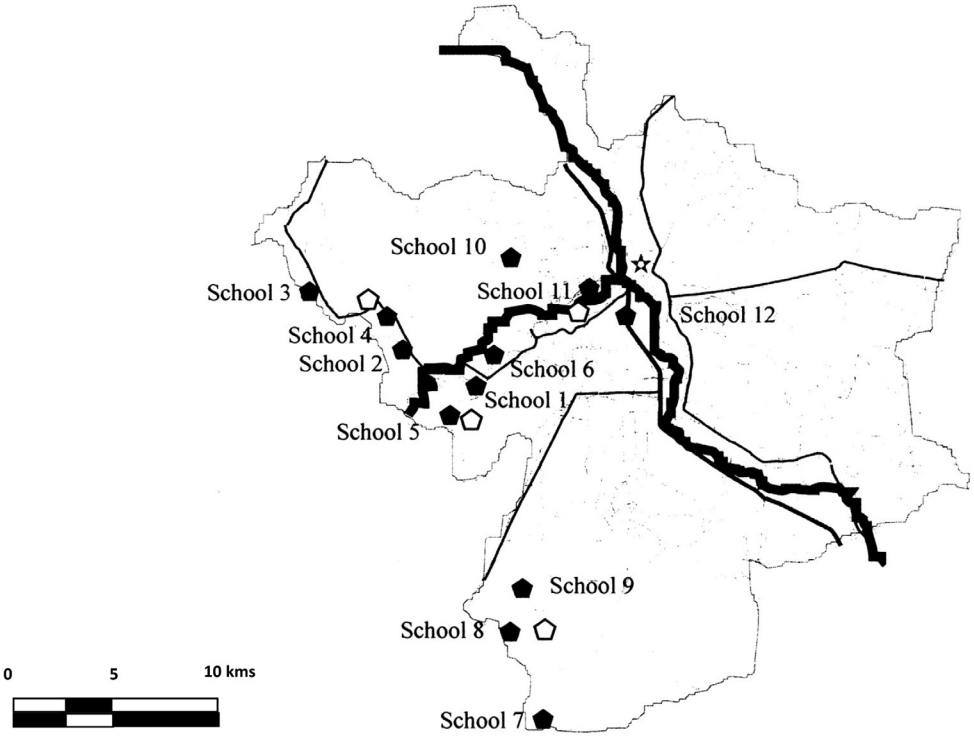

Fig 1–Map of Mueang District, Kamphaeng Phet Province, Thailand. Black pentagons indicate the 12 schools that participated in the first prospective cohort study (KPSI). Adapted from: Endy *et al,* 2002a.

were tested by dengue/JE HAI for four-fold rise in dengue HAI titers, and, if positive, were confirmed by dengue/JE plaque reduction neutralization test (PRNT) (Russell and Nisalak, 1967; Salje *et al*, 2014).

'Symptomatic' DENV infection in a cohort subject was defined as an acute febrile illness with positive DENV PCR in the acute sample and/or positive dengue ELISA/HAI in the acute/convalescent sample pair. "Inapparent" DENV infection was defined as four-fold rise in dengue HAI and PRNT titers between scheduled blood samples but without symptomatic infection during the intervening surveillance period.

In conjunction with the second cohort study (KPSII), geographic cluster investigations were additionally performed (Yoon *et al*, 2012a; 2013). KPSII cohort subjects with DENV PCR-positive acute samples served as index cases for 'positive' geographic cluster investigations around the index house. DENV PCR-negative cohort subjects served as index cases for 'negative' cluster investigations. Contact subjects aged six months to 15 years living within 100 meters of the index case were clinically evaluated at days 0, 5, 10, and 15. Blood samples were also collected on days 0 and 15 and tested for DENV infection by DENV PCR and ELISA/HAI.

Contact subjects with DENV infection could have had fever history, no fever history but other non-fever symptoms, or

no detectable symptoms at all (asymptomatic). In addition, adult female *Aedes aegypti* mosquitoes were collected on day 1 of each cluster investigation from all houses within a cluster, and immature *Aedes aegypti* (larvae and pupae) were collected from water-holding containers. The heads/thoraces of captured adult mosquitoes were tested for DENV infection by DENV PCR (Yoon *et al*, 2012b).

HETEROGENEITY OF DENGUE VIRUS INFECTION

Dengue incidence in the cohort subjects at different schools participating in KPSI and KPSII demonstrated marked heterogeneity in both space and time (Endy *et al*, 2002a,b; Yoon *et al*, 2012a). Dengue incidence was cyclical in each school. However, these cycles did not necessarily coincide among different schools even when the schools were in close proximity (within 5 km). This heterogeneity included not just dengue incidence but also the predominant circulating serotypes, which could be quite variable among different schools during the same year. The ratios of inapparent-to-symptomatic infections were also highly variable among schools.

An analysis of these ratios from KPSI showed that the most important determinants at a given school were the incidence of DENV infection in a given year and the incidence in the preceding year suggesting serotype cross-reactive immunity with disease mitigating effects at the school level (Endy *et al*, 2011). When geographic cluster investigations were added in KPSII, the heterogeneous pattern of DENV infections was demonstrated to occur within individual villages.

Contact subjects in positive clusters had a mean DENV infection rate of 16.0% compared to only 1.1% in negative clusters despite the fact that paired positive and negative clusters were typically performed within the same village and within 5 days of each other (Yoon *et al*, 2012b). Even among the positive clusters, the infection rates varied widely among individual clusters ranging from 0-to-65% (Fig 2).

SPATIOTEMPORAL DIMENSIONS OF FOCAL TRANSMISSION

Given the heterogeneity of DENV infections among different schools and within individual villages, the spatiotemporal dimensions of transmission risk were further evaluated within the boundaries of the positive clusters. By analyzing infections in contact subjects living at various distances from DENV PCR-positive index cases, infection rates were noted to differ substantially even within the 100 meter radius of the clusters (Mammen *et al*, 2008; Yoon *et al*, 2012a).

The DENV infection rate was approximately 35% among contact subjects living in the same house as an index case and about 30% in houses within 20 meters of the index case. However, the infection rate decreased to less than 10% in houses 80-100 meters away (Fig 3). Within the same positive clusters, the DENV infection rate in adult female *Aedes aegypti* mosquitoes was greater than 8% in index houses, 2%-3% in houses within 40 meters of the index case, and under 1% in houses >40 meters away.

Taken together, these findings indicated that DENV transmission between humans and mosquitoes largely took place within a 40 meters radius during the two-

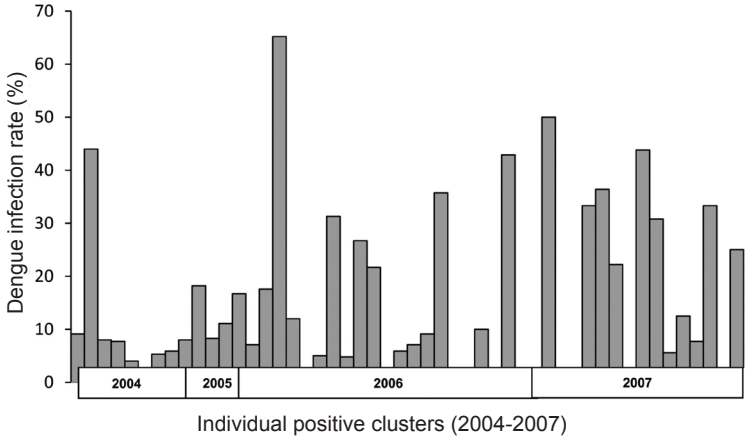

Fig 2–Dengue virus infection rates in individual positive clusters. Fifty positive cluster investigations were conducted from 2004-2007 in the second prospective cohort/cluster study (KPSII). Adapted from: Yoon *et al,* 2012b.

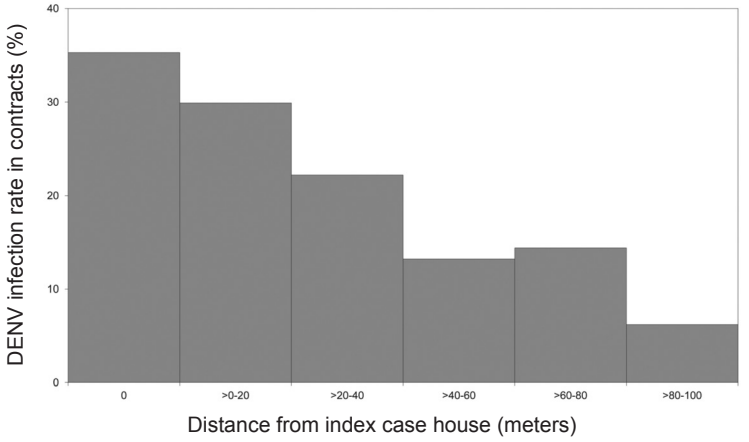

Fig 3–Dengue virus (DENV) infection rates in contact subjects living in houses at various distances from a DENV PCR-positive index case. Adapted from: Yoon *et al*, 2012a.

week interval of each cluster investigation. Therefore, within this dengue hyperendemic rural setting in Thailand, DENV transmission was found to be very highly focal in space and time.

PRESENCE OF DENGUE VIRUS TRANSMISSION "HOTSPOTS"

The data available from the KPSII geographic cluster investigations allowed for further detailed evaluation of DENV infection risk in different houses. Entomological indices and human infection rates were analyzed in houses from which DENV-infected mosquitoes were captured, and in houses from which no infected mosquitoes were captured but were located in clusters either with or without infected mosquitoes (Table 1). The infection rate was about 47% in contact subjects in houses with infected mosquitoes, 28% in houses with-

Table 1
Characteristics of houses with and without DENV-infected mosquitoes in positive clusters.

Parameter	Houses with infected mosquitoes	Houses without infected mosquitoes within clusters with infected mosquitoes	Houses in positive clusters without infected mosquitoes	*p*-value
Houses, *n*	16	158	324	-
Adult female *Aedes aegypti* per person (SD)	1.57 (2.05)	0.49 (1.00)	0.45 (0.89)	<0.001
Aedes aegypti pupae per person (SD)	5.84 (11.59)	1.86 (3.76)	1.22 (2.56)	<0.001
Human adults per house (SD)	2.94 (1.18)	2.92 (1.31)	2.70 (1.44)	0.239
Children per house (SD)	1.75 (0.86)	1.68 (0.88)	1.77 (1.04)	0.598
DENV-infected child contacts/all enrolled contact subjects (%)	9/19 (47.4)	56/195 (28.7)	64/591 (10.8)	<0.001

DENV, dengue virus; *n*, number; SD, standard deviation. Adapted from: Yoon *et al*, 2012a.

out infected mosquitoes located in clusters with infected mosquitoes, and 10% in positive cluster houses without infected mosquitoes.

Interestingly, the numbers of adult female mosquitoes and pupae per person were elevated only in houses with infected mosquitoes despite the fact that the human infection rate was quite high (28%) in houses without infected mosquitoes located in clusters with infected mosquitoes. This suggested that houses with elevated entomological indices served as DENV transmission hotspots that increased the risk of human infection not only in the same house but also in neighboring houses without elevated entomological indices.

Given the relatively small spatial scale of these hotspots (house level), it may be difficult to identify these hotspots in the course of wide scale vector control efforts. Because hotspots may have a disproportionately high impact on local transmission, vector control interventions that miss these hotspots may have limited effectiveness in reducing overall DENV transmission.

ROLE OF INAPPARENT INFECTIONS IN DENGUE VIRUS TRANSMISSION

The clinical spectrum of DENV infection can vary from asymptomatic, or inapparent, infection to mild dengue fever, dengue hemorrhagic fever, dengue shock syndrome, and other forms of severe dengue. Inapparent infections can account for varying proportions of all infections depending on the specific cohort study design, location and year (Endy *et al*, 2010). Much of this variability is affected by the method of surveillance and how inapparent infections are defined (Yoon *et al*, 2013;

Grange *et al*, 2014). In KPSII, approximately 65% of all DENV infections in the cohort were inapparent using the school absence-based active surveillance cohort design. However, in the geographic cluster investigations, only 40% of all DENV infections in contact subjects were 'inapparent' if this was defined as having no fever history (with or without other symptoms).

Alternatively, if inapparent was defined as being completely asymptomatic, then a mere 20% of all infections in contact subjects could be considered "inapparent". Because cluster investigations are generally more sensitive in detecting symptoms (and assuming that the KPSII cohort and clusters had similar dengue epidemiology as they were done concurrently), then about 40% of inapparent infections in the cohort may, in fact, have had fever history. Alternatively, about 70% of cohort inapparent infections may have had some symptoms (with or without fever history). These percentages are merely hypothetical since DENV infections in contact subjects did not correspond to the actual same inapparent infections from the cohort.

However, because the cohort and clusters investigations were conducted in the same area during the same time period, some inapparent infections in the cohort were, in fact, detected as contact subject infections in the cluster investigations. Sixteen such overlapping cases were identified in KPSII, with 12 of these 16 reporting fever histories as a contact subject. Despite the fever history, these 12 infections did not lead to school absence resulting; therefore, in characterization of the infection as inapparent in the cohort. Of the 16 overlapping cases, nine also had detectable DENV RNA by PCR with mean viremia of 4.84 log RNA copies/ml (range, 2.80-7.14). All nine had viable virus by culture and fever history. These nine cases demonstrated that at least some inapparent infections in the cohort had the potential to contribute to DENV transmission. To what degree they might play such a role in actuality would depend on other factors to include the level and duration of viremia (Nguyet *et al*, 2013).

INCREASING AGE OF DENGUE IN KAMPHAENG PHET

Because KPSI and KSPII were studied in children, the direct applicability to adults is not clear. In particular, the proportion of inapparent infections in adults and the role of adult inapparent and symptomatic infections in DENV transmission require more study. The mean age of dengue in Thailand has increased over the past 10-20 years with decreasing force of infection possibly due to decreasing birth and death rates (Cummings *et al*, 2009). Despite this decreasing force of infection, the potential for infection (as indicated by the basic reproduction number) may, in fact, have changed very little as supported by a comparison of age-stratified dengue seroprevalence assessments conducted in Rayong, Thailand in 1980 and 2010 (Rodriquez-Barraquer *et al*, 2014). As in other provinces of Thailand, the mean age of dengue in Kamphaeng Phet has also increased over the past 20 years (Yoon, submitted). Further cohort and cluster studies in Kamphaeng Phet are being planned in order to clarify the clinical spectrum of dengue in adults and to investigate the role of adults in DENV transmission.

CONCLUSION

Prospective longitudinal cohort and cluster studies in Kamphaeng Phet have contributed significantly to our understanding of DENV transmission. These studies have demonstrated the spatiotemporal heterogeneity of DENV transmission with highly focal transmission at small scales in this rural setting. Small easily missed hotspots of transmission may exist which can have a disproportionately high impact on local spread. Therefore, these hotspots should be taken into account when planning overall vector control strategies. Inapparent DENV infections as defined by prospective longitudinal cohort studies consist of a clinical spectrum of infections from asymptomatic to mildly symptomatic with and without fever. The proportion of all DENV infections that are completely asymptomatic may be substantially lower than those considered to be inapparent. Some of the inapparent infections from cohort studies have viable DENV and may, therefore, potentially contribute to virus transmission. These findings require further validation in other settings (for example, urban areas) and in adults and children to develop a more comprehensive understanding of DENV transmission.

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Chapter 2.

Dangue pathogenesis

ASSOCIATION BETWEEN MANNOSE-BINDING LECTIN GENE POLYMORPHISMS AND SUSCEPTIBILITY TO DENGUE VIRUS INFECTION: A PRELIMINARY REPORT

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Abstract. Mannose-binding lectin (MBL) can bind with a wide range of pathogens and can activate through lectin pathway or enhances opsonophagocytosis. MBL is encoded by the *MBL2* gene and single-nucleotide polymorphisms (SNPs) in the promoter and exon have functional effects on serum levels of MBL. MBL deficiency has been shown to predispose to infectious diseases. We assess whether or not, the variant MBL alleles are associated with susceptibility to dengue infection. Patients with confirmed dengue infection who were admitted to King Chulalongkorn Memorial Hospital during a calendar year were studied. Controls were patients without dengue infection. Deoxyribonucleic acid (DNA) was extracted from 50 µl of peripheral blood mononuclear cell (PBMC) using the DNA Blood Mini Kit. The SNPs in the promoter (-221 *X/Y*) and exon 1 (codon 54 *A/B*) of *MBL2* gene were genotyped by using 2 separate cycling reactions of the TaqMan allele discrimination system. Serum levels of MBL were determined by double-antibody sandwich ELISA. Chi-square was used for statistical analysis. Serum MBL levels and genotypes were determined in 110 dengue patients (mean age 18.1 years; 62 males and 48 females) and 42 controls (mean age 25.8 years; males: females = 1:1). Our study showed that *YB* haplotype is associated with low serum levels of MBL. There was no association between *MBL2* gene polymorphisms and susceptibility to dengue infection. The higher frequency of *YB* in dengue patients than in controls suggesting the likelihood of an association. Further studies are warranted.

Keywords: dengue, mannose-binding lectin, single-nucleotide polymorphism

INTRODUCTION

Dengue virus infections cause a spectrum of illness ranging from asymptomatic, mild undifferentiated fever, dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS) (Hemungkorn *et al,* 2007). Individual susceptibility to dengue infection seems to be variable. For decades, two distinct hypotheses to explain the mechanism of DHF have been debated between secondary infection or immune enhancement and viral virulence (Prommalikit *et al,* 2004). Host genetic factors may also be relevant

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to this infection (Lan and Hirayama, 2011). Mannose-binding lectin (MBL) is an acute phase protein primarily produced by the liver which plays a critical role in the innate immune response before the production of antibodies, and it can bind with multiple carbohydrate recognition domains on microbial surfaces. MBL can activate the complement system via lectin pathway and promote opsonophagocytosis.

MBL is encoded by the *MBL2* gene on chromosome 10. *MBL2* has four exons. Single-nucleotide polymorphisms (SNPs) in the promoter and coding regions have functional effects on serum levels of MBL (Worthley *et al,* 2005). Three missense mutations of coding region in Exon 1 at codons 52 (allele *D*), 54 (allele *B*), and 57 (allele *C*) disrupt the collagen-like helical structure of the MBL peptides and interfere with oligomerization of MBL peptides, or assembly of MBL peptide triplets into multimers, resulting in low serum MBL levels (Madsen *et al,* 1994). An *MBL2*-coding region carrying any of the *B*, *C* or *D* mutant alleles is referred to as *O*, and the wide-type is referred to as *A*.

The MBL concentrations are also dependent on SNPs in the promoter region of *MBL2*. In particular, a SNP at position -221 (allele *Y* or *X*) has a significant effect on serum MBL levels, with the allele *Y* and allele *X* being responsible for high and low MBL-expressing activity, respectively (Madsen *et al,* 1995). With the three structural SNPs in exon 1, the -221 alleles form the haplotypes *YA*, *XA*, *YB*, *YC,* and *YD*. The haplotypes *YB* are more frequent in individuals with low MBL levels than in those randomly selected or in those with high MBL levels (Crosdale *et al,* 2000). MBL deficiency has been shown to predispose to infectious diseases (Worthley *et al,* 2005).

The envelope of dengue virus has complete high-mannose glycans and non-structural proteins that may be opsonized by MBL (Chan, 1997). An activation of complement system is a constant finding in patients with DHF (Avirutnan *et al,* 2006). Thus, MBL may be a candidate gene for dengue infection. However, the data on MBL association with dengue infection is scarce in the literature and absent in Thai population. We assess whether or not, the variant MBL alleles are associated with susceptibility to dengue infection in an ethnically homogeneous population born in Thailand.

MATERIALS AND METHODS

Patients with serologically confirmed dengue infection by an enzyme-linked immunosorbent assay (ELISA) admitted to King Chulalongkorn Memorial Hospital during a calendar year were studied. Controls were patients with negative dengue serology. Informed consent was obtained from the subjects and controls recruited into the study or from their parents. Deoxyribonucleic acid (DNA) was extracted from 50 µl of peripheral blood mononuclear cell (PBMC) using the QIAamd DNA Blood Mini Kit® (Qiagen, Hilden, Germany) and stored at -20ºC until used for analysis.

The SNPs in the promoter (-221 *X/Y*) and exon 1 (codon 54 *A/B*) of *MBL2* gene were genotyped by using two separate cycling reactions of the TaqMan® allelic discrimination system (Applied Biosystems, Foster City, CA) as described in previous studies (Ip *et al,* 2004, 2005). The *MBL2* mutant structural allele *B*; however, is in

Table 1
Serum MBL levels and MBL genotypes with or without the mutant allele B in dengue patients and controls.

Variable	Mean levels of serum MBL in dengue patients (ng/ml)	Mean levels of serum MBL in controls (ng/ml)
MBL genotypes		
YA/YA, YA/XA, XA/XA	2,738.97 (673.89-7,446.40)	2,521.24 (717.71-6,160.89)
YA/YB, XA/YB, YB/YB	327.52 (undetectable-723.73)	960.79 (216.58-3,346.33)

Table 2
Frequencies of MBL genotypes and YB haplotype in dengue patients and controls.

Variable	Dengue patients (%)	Controls (%)	*p*-value
MBL genotypes			0.783
YA/YA	37 (33.6)	19 (45.2)	
YA/XA	36 (32.7)	12 (28.6)	
XA/XA	8 (7.3)	3 (7.1)	
YA/YB	24 (21.8)	7 (16.7)	
XA/YB	3 (2.7)	1 (2.4)	
YB/YB	2 (1.8)	-	
YB carrier	29 (26.4)	8 (19.1)	0.466

linkage disequilibrium with the promoter polymorphism *X/Y*, so that *B* only occurs with *Y* (Madsen *et al,* 1995). The data from the two separate TaqMan polymerase chain reactions in the present study were combined to give three haplotypes: *YA*, *YB* and *XA*. *YB* is commonly referred as a mutant haplotype *O* (Neth *et al,* 2001).

Serum levels of MBL were determined by double-antibody sandwich ELISA in which a mouse monoclonal anti-human MBL antibody (HYB 131-01; Antibody Shop, Copenhagen, Denmark), either unlabelled or labelled with biotin, was used as the primary or secondary antibody, respectively. Horseradish-peroxidase (HRP)-conjugated streptavidin (R&D Systems, Minneapolis, MN) and substrate solution containing tetramethylbenzidine (Substrate Reagent Pack; R&D Systems, Minneapolis, MN) were used for detection of bound secondary antibody. Chi-square was used for statistical analysis. A *p*-value <0.05 was considered to be significant.

RESULTS

Serum MBL levels and genotypes were determined in 110 dengue patients (mean age 18.1 years; 62 males and 48 females) and 42 controls (mean age 25.8 years; males: females = 1:1). The SNPs in the promoter (-221 *X/Y*) and exon 1 (codon 54 *A/B*) of the MBL gene were genotyped and were identified as six genotypes in this study: *YA/YA*, *YA/XA*, *XA/XA*, *YA/YB*, *XA/YB* and *YB/YB*. *YB* haplotype is associated with low serum levels of MBL (Table 1 and Fig 1). There was no association

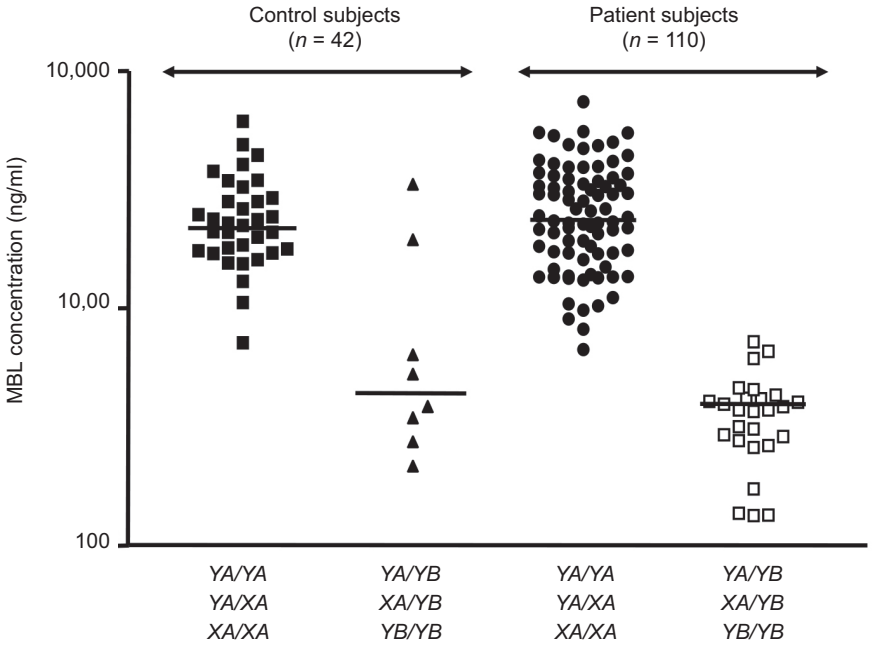

Fig 1–Serum MBL levels and MBL genotypes with or without the mutant allele B in dengue patients and controls.

between *MBL2* gene polymorphisms and susceptibility to dengue infection but the frequency of *YB* was higher in patients than in controls. Frequencies of MBL genotypes and *YB* haplotype in dengue patients and controls were shown in Table 2. When the analysis was performed comparing the incidence of *MBL2* polymorphisms between DF versus DHF and DSS, we found that there was no significant difference in the MBL genotype between the two groups and frequencies of MBL genotypes were shown in Table 3.

DISCUSSION

MBL interacts with wide range of pathogens, including many different bacteria, viruses, fungi and protozoa. MBL deficiency increases the susceptibility of an individual to infectious diseases including human immunodeficiency virus, influenza A, *Cryptosporidium parvum*, *Neisseria meningitidis*, severe acute respiratory syndrome coronavirus and is also associated with autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis (Worthley *et al,* 2005; Ip *et al,* 2005; Tsutsumi *et al,* 2001). MBL may be a candidate gene for dengue infection because the envelope of dengue virus has complete high-mannose glycans which MBL recognizes repetitive oligosaccharide moieties present on a wide array of pathogens.

In the present study, both heterozygous and homozygous for *YB* is associated with low serum levels of MBL, which is similarly found in Chinese and Caucasian populations. Although there was no associ-

Table 3
Frequencies of MBL genotypes in DF patients versus DHF and DSS patients.

Variable	DF (%) (*n* = 30)	DHF and DSS (%) (*n* = 80)	*p*-value
MBL genotypes			0.186
YA/YA	12 (40.0)	25 (31.2)	
YA/XA	8 (26.7)	28 (35.0)	
XA/XA	2 (6.7)	6 (7.5)	
YA/YB	6 (20.0)	18 (22.5)	
XA/YB	-	3 (3.7)	
YB/YB	2 (6.7)	-	

ation between *MBL2* gene polymorphisms and susceptibility to dengue infection, but the frequency of *YB* was higher in dengue patients than in controls, suggesting the likelihood of an association.

In recent years, several publications reporting correlations between *MBL2* polymorphisms and disease susceptibility or disease protection have been published. Alagarasu *et al* (2012) found that deficiency of MBL may be associated with primary DHF. Based on the cutoff value of 500 ng/ml, 50% of primary DHF cases had MBL deficiency as compared to 10% of primary DF cases (p=0.038, odds ratio (OR) 9; 95% confidence limits 0.84-120). This difference was not observed in secondary infections.

For disease protection, Acioli-Santos *et al* (2008) reported that dengue patients with lower MBL serum levels were less likely to develop thrombocytopenia than the MBL wide-type group, suggesting a protective role of *MBL2* allele *O* toward dengue-related thrombocytopenia, whereas the presence of the *MBL2* AA genotype was associated with augmented risk of thrombocytopenia. Loke *et al* (2002) have investigated MBL genotype in a Vietnamese cohort to identify possible correlation between the codon 54 *MBL2* polymorphism and DHF. There were no significant differences in MBL genotypes or allele frequencies between DHF patients and controls. However, the relatively low frequency of the variant allele in this population limits the statistical power of this analysis.

The findings of various studies concerning association between *MBL2* polymorphism and susceptibility to dengue infection were controversial. Further studies with increased sample size are warranted since the underpowered sample size may be unable to detect such association. In addition, demonstration of MBL binding to dengue virus and neutralization of infection is needed.

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DENGUE VIRUS VIRULENCE AND DISEASES SEVERITY

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

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

INTRODUCTION

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

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

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DENGUE VIRUS VIRULENCE AND DISEASES SEVERITY

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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HEMOSTATIC STUDIES IN DENGUE PATIENTS

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Abstract. The pathogenesis of hematologic changes in dengue patients is not clearly understood. Consistent hematological findings include vasculopathy, thrombocytopenia, and coagulopathy. There are evidences suggesting that dengue virus causes pathophysiological changes that involve all of the consistent hematologic findings resulting in vasculopathy, reduction in platelet number as well as platelet dysfunction, and reduction of several coagulation factors. Laboratory evidences of disseminated intravascular coagulation (DIC) are also demonstrated in all degrees of severity in dengue patients. Only in severe dengue cases is profound DIC aggravated, leading to uncontrolled bleeding and death. A study to determine the extent of the activation of endothelial cells and the hemostatic system in correlation with clinical severity and also to detect the best prognostic factor for severe dengue showed plasma von Willebrand factor antigen (VWF:Ag) to be the best indicator of progression to severe dengue.

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 pathogenesis of bleeding in dengue patients is poorly understood.

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HEMOSTATIC STUDIES IN DENGUE PATIENTS

The hemostatic changes, which have been shown to occur early in the course of illness in all severity of dengue, included the following:

Vasculopathy manifested by generalized petechiae and a positive tourniquet test.

Platelet abnormalities manifested by thrombocytopenia, which was one of the most consistent abnormal hemostatic tests, and this occurred in the febrile phase to reach its lowest values in the defervescence phase. The platelet count then increased during the convalescent stage to reach its normal values. Many cases had platelet counts higher than the normal

ranges during the second week of illness. The clinical severity also correlated with the degree of thrombocytopenia.

The possible causes of thrombocytopenia include megakaryocytic abnormalities, because the degenerative changes and decreased number of marrow megakaryocytes were noted in the early febrile phase. The megakaryocyte number was often normal or increased later on. Isologous platelet kinetic studies have shown a shortened half-life survival during the course of illness, which became normal later on. Surface counting of radiolabeled platelets revealed increased pooling of platelets to the liver more than to the spleen. In a normal spleen, liver platelet-pooling ratios returned to normal in the convalescent stage. This increased pooling of platelets in the liver and possibly their destruction; there might be another contributory factor to the thrombocytopenia noted in dengue patients. In addition to decreased quantity, platelet dysfunction (that is, impaired ADP-induced aggregation and ADP release) was reported.

Coagulopathy shown by mild to moderately prolonged partial thromboplastin time and prothrombin time. Assays of clotting factors showed variable patterns of reduction, mostly of mild to moderate degrees. Fibrinogen was the only factor that almost always decreased mildly to moderately. Minimal increase of fibrin degradation products was noted intermittently throughout the course of illness. In addition, increased consumption of fibrinogen was demonstrated, and 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 alteration of their cytokines production and barrier functions, 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. 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 illness. In DHF patients during the febrile phase, von Willebrand factor antigen (vWF:Ag), tissue factor (TF) and plasminogen activator inhibitor (PAI-1) were significantly elevated while platelet counts and ADAMTS 13 (a disintegrin and metalloprotease with thrombospondin repeats) were significantly low compared to those in DF patients.

In DHF patients during the toxic phase, soluble thrombomodulin (sTM), tissue plasminogen activator (t-PA), and PAI-1 were also significantly increased while ADAMTS 13 and thrombin activatable fibrinolysis inhibitor (TAFIa) were significantly low, compared to 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. Using logistic regression analysis, plasma VWF:Ag was found to be the best indicator of progression to DHF (Sosothikul *et al*, 2007).

Hematopoietic suppression is a very well known phenomenon that occurred during dengue infection. Bone marrow examinations showed markedly hypocellularity accompanied by decreases in all the hematopoietic cell precursors, namely megakaryocytes, erythroid, and myeloid precursors. Later on, recovery of hematopoiesis occurred, and the bone marrow showed hypercellular, accompanied by an increase in number of megakaryocytes, erythroid, and myeloid precursors. Despite the increase in normal number of megakaryocytes, these cells showed a sign of degeneration. The hemophagocytosis of young and mature erythroid and myeloid cells, including lymphocytes and platelets, were also observed (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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Chapter 3.

Diagnosis of dengue virus infection

REPORTING PROGRESS ON THE USE OF THE WHO 2009 DENGUE CASE CLASSIFICATION: A REVIEW

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Abstract. This review reports on the progress in the use of the WHO 2009 dengue case classification—dengue and severe dengue—following up on a previous review (Horstick *et al*, 2012). The previous review detailed Steps 1 - 5 in developing the 2009 WHO case classification. As a further step, a systematic review of published studies comparing the two classifications has been published with 12 studies and a further 10 expert opinion papers that recommend the use of the 2009 WHO dengue case classification for clinical management, epidemiology, and clinical research. Furthermore, a formal expert consensus was reached in La Habana, Cuba in 2013 with dengue experts from the Americas, sharing experiences that applied the 2009 WHO dengue case classification. The expert panel recommended to 1) update ICD10, 2) include the 2009 WHO case classification in country epidemiological reports globally, and 3) implement studies improving sensitivity/specificity of the dengue case definition.

Keywords: 2009 WHO dengue case classification, dengue and severe dengue, evidence

INTRODUCTION

The World Health Organization (WHO) with its Special Programme for Research and Training in Tropical Diseases (WHO/TDR) issued new dengue guidelines in 2009 (WHO/TDR, 2009), including the 2009 WHO dengue case classification: dengue and severe dengue (D/SD). Warning signs (WS) have been established for triage, to help clinicians with symptomatic cases in need of closer surveillance and/or hospitalization [dengue with warning signs (D+WS)].

Historically, the DF/DHF/DSS case classification of dengue (dengue hemorrhagic fever and dengue shock syndrome) was developed in 1975 by expert consensus, based on studies on Thai children in the 1950´s and 1960´s, with modifications in 1986 and 1997 (Bandyopadhyay *et al*, 2006). In the last modification of 1997 four grades of DHF were defined (DHF 1, 2, 3, 4) with 1, 2 being DHF and 3, 4 being DSS (WHO, 1997).

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This article refers in the following to the dengue case classification recommended by WHO in 2009 (D/SD) as the "2009 WHO case classification" and to DF/DHF/DSS as the "1997 WHO case classification".

The reasons for developing the 2009 WHO case classification were the shortcomings of the 1997 WHO case classification that were established in many studies. Based on the largest prospective multicenter dengue study, the Dengue and Control (DENCO) study (Alexander *et al*, 2011), the 2009 WHO case classification describes dengue as it currently occurs globally; focusing on severe dengue, defined as plasma leakage (shock or fluid accumulation with respiratory distress, which includes the former dengue shock syndrome), severe bleeding, or severe organ manifestation.

With the improved description of dengue cases, case reporting is facilitated. Warning signs have been empirically validated to some extent in the DENCO study. This review has the objective to report on the further evidence that has been reported on the use of the 2009 WHO case classification globally, because after its publication in 2009, a discussion evolved internationally on the usefulness and applicability of the 2009 WHO case classification compared to the 1997 WHO case classification.

METHODS

This article reports the process of the development and on further published evidence for or against the use of the 2009 WHO case classification, after a previous review in 2012 (Horstick *et al*, 2012). Additionally implementation aspects derived from the individual were analyzed, with a view towards practical public health recommendations.

RESULTS

In the previous review (Horstick *et al*, 2012), the steps were published for the development of the 2009 WHO dengue case classification . These steps included:

Step 1: A systematic literature review that highlighted the shortcomings of the 1997 WHO case classification, which were (1) difficulties in applying the criteria for DHF/DSS, (2) the tourniquet test has a low sensitivity for distinguishing between DHF and DF, and (3) most DHF criteria had a large variability in the frequency of occurrence (Bandhyopadhyay *et al*, 2006).

Step 2: An analysis of regional and national dengue guidelines and their application in the clinical practice showed a need to re-evaluate and standardize guidelines because the actual ones showed a large variation of definitions, an inconsistent application by medical staff, and a lack of diagnostic facilities necessary for the DHF diagnosis in frontline services (Santamaria *et al*, 2009).

Step 3: A prospective cohort study in seven countries that confirmed the difficulties in applying the criteria of the 1997 WHO case classification, even in tertiary care hospitals; that this classification does not represent levels of disease severity; and that a clear distinction between severe dengue (defined by plasma leakage and/or severe hemorrhage, and/or organ failure), and (non-severe) dengue can be made using highly sensitive and specific criteria. In contrast, the sub-grouping of (non-severe) dengue into two further severity levels was

only possible with criteria that gave approximately 70% sensitivity and specificity (Alexander *et al*, 2011).

Step 4: Three regional expert consensus groups in the Americas and Asia concluded that "dengue is one disease entity with different clinical presentations and often with unpredictable clinical evolution and outcome" (Horstick *et al*, 2012), and that revising the results of Step 3, the 1997 WHO case classification is not related to disease severity (unpublished meeting proceedings in La Habana, Cuba and Kuala Lumpur, Malaysia 2007 and Heidelberg, Germany 2008).

Step 5: In a global expert consensus meeting at WHO in Geneva, the evidence collected in Steps 1-4 was reviewed, and a revised scheme was developed and accepted (unpublished meeting proceedings 2008); thereby distinguishing between: dengue with or without warning signs and severe dengue (D/D+WS/SD). Further field-testing and acquisition of further prospective evidence of the revised scheme was recommended.

Step 6: In 18 countries, the usefulness and applicability of the 2009 WHO case classification compared to the 1997 WHO case classification were tested showing clear results in favor of the former (Barniol *et al*, 2011).

In a further step (Step 7), a systematic review of the published studies comparing the two classifications has been published (Horstick *et al*, 2014a). These studies were performed after the publication of the 2009 WHO case classification, and most of the 12 studies included (prospective, post hoc analysis of existing datasets or reviewing existing medical charts, any qualitative design) were performed in Asia (Basuki *et al*, 2010; Chaterji *et al*, 2011; Kalayanarooj, 2011; Jayaratne *et al*, 2012; Van de Weg *et al*, 2012; Gan *et al*, 2013; Prasad *et al*, 2013; Thein *et al*, 2013; Tsai *et al*, 2013), with the exception of three studies: one which included 18 study sites worldwide (Barniol *et al*, 2011), one study from Nicaragua (Narvaez *et al*, 2011) and one study from Peru (Siles *et al*, 2013). Ten expert opinion articles were used for discussion (Srikiatkhachorn *et al*, 2011; Akbar *et al*, 2012; Hadinegoro, 2012; Halstead, 2012; Horstick *et al*, 2012; Farrar *et al*, 2013; Halstead, 2013; Horstick *et al*, 2013; Lin *et al*, 2013; Wiwantikit, 2013).

For the 2009 WHO case classification, studies show that : 1) determining severe dengue: sensitivity was measured between 59%-98% (88% and 98% for the two prospective studies), specificity 41%-99% (99% for the prospective study). When comparing the 1997 WHO classification, the sensitivity was lower with 24.8%-89.9% (24.8% and 74% for the prospective studies). Specificity for the 1997 WHO case classification was 25% and 100% (100% from the prospective study); 2) application of the 2009 WHO case classification is easy; 3) for (non-severe) dengue as defined in the 2009 WHO case classification, there may be a risk of monitoring increased case numbers of dengue cases; and 4) warning sign validation studies are needed to further validate the warning signs.

For epidemiological purposes and pathogenesis research, the following has been referenced (the information is derived only from the expert opinion papers): easy application, increased sensitivity (severe dengue), international comparability of the 2009 WHO case classification are advan-

tageous; the 3 SD criteria (severe plasma leakage, severe bleeding and severe organ manifestation) are useful research endpoints.

The 2009 WHO dengue case classification has been especially applied in the Americas and within the member states of the Pan American Health Organization (PAHO).

In a further study (Step 8), a formal expert consensus has been held as a side event of the biannual dengue course at the Instituto Pedro Kouri (IPK), La Habana, Cuba in 2013 (Horstick, *et al* 2014b). The two day expert consensus meeting aimed to 1) share experiences from PAHO member states applying the 2009 WHO case classification, 2) present national/local data using the 2009 WHO case classification, 3) agree - with a formal consensus group - on recommendations for/or against using the 2009 WHO case classification.

In this context, eight key questions were discussed, concluding that the 2009 WHO case classification: 1) is useful describing disease progression because it considers the dynamic nature of the disease; 2) helps defining dengue cases correctly for clinical studies because it defines more precisely disease severity and allows evaluating dynamically the progression of cases; and 3) describes correctly all clinical forms of severe dengue. Further standards need to be developed regionally, especially related to severe organ involvement. 4) the 2009 WHO case classification allows for pathophysiological research identifying—in a sequential manner—the clinical manifestations of dengue related to pathophysiological events; 5) the warning signs help identify early cases at risk of shock (children and adults; the pathophysiology of the warning signs deserves further study); 6) helps treating individual dengue cases and also the reorganization of health care services for outbreak management; 7) helps diagnosing dengue, in presumptive diagnosis and following-up of the disease, because of its high sensitivity and high negative predictive value; and, 8) there is currently no update of the International Disease Classification 10 (ICD10) to include the 2009 WHO dengue case classification; therefore, there are not enough experiences of epidemiological reporting.

Once it has been implemented in epidemiological surveillance, it allows 1) identifying the severity of dengue cases in real time, for any decision-making on action; 2) measuring and comparing morbidity and mortality in countries, but also globally; and 3) trigger contingency plans early, not only based on the number of reported cases, but also on the reported severity of cases.

CONSLUSIONS AND RECOMMENDATIONS

Based on the extensive work on the dengue case classifications, a list of recommendations can be drawn. These need to be seen firstly in the light of the limitations of this review. Limitations include publication bias; however, the inclusion of the systematic literature review (Step 7) on this issue should limit this bias. Experiences in practice may not be recorded, but the authors consulted extensively experts in the field, and especially Step 8, with the inclusion of more than 30 dengue experts, is a good example for this process.

Looking at the eight steps of a process encompassing more than ten years, the following practical recommendations can be

made, further to the recommendations in the previous review (Horstick *et al*, 2012).

• The systematic literature review indicated that the 2009 WHO case classification has clear advantages for clinical use; use in epidemiology is promising and research use may at least not be disadvantageous.

• When the experts in La Habana revised the evidence and complemented this evidence with their own experiences, the expert panel recommended to 1) update ICD10, 2), include the 2009 WHO case classification in country epidemiological reports globally, and 3) to implement studies improving sensitivity/specificity of the dengue case definition.

• Adaptations to this process may arise as further knowledge develops; especially the questions of the evidence base of the case definitions and warning signs have to be considered. Large prospective cohort studies are currently under way to strengthen the knowledge on these issues, and should be available in the near future (Jaenisch *et al,* 2013).

• The development for further elements of national capacity training for clinical management including the 2009 WHO case classification is recommended.

• Studies should attempt to include measuring dengue epidemiological data when considering the use of the 2009 WHO case classification and the related clinical algorithms.

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LABORATORY DIAGNOSIS OF DENGUE VIRUS INFECTIONS

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Abstract. Dengue diagnosis was one of the topics discussed at "the adult dengue" presentations. In this paper, a review is presented focusing on the main challenges of dengue laboratory diagnosis. 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. 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 the disease during febrile phase. In recent years, PCR (polymerase chain reaction) has become an important tool as a quick method for diagnosis of dengue, another is detection of NS1 antigen, using commercial ELISA kit. Serological studies, for primary infection, the dominant immunoglobulin isotype is IgM, anti-IgM may appear during febrile phase (50% of cases), the other half, it appears within 2-3 days of defervescence. Once detectable, IgM levels rise quickly and appears to peak about 2 weeks after the onset of symptoms, then they decline to undetectable level over 2-3 months. Anti-IgG appears shortly afterwards with 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, anti-dengue IgM appears in most instances, the level 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 co-circulation 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

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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.

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 had been passed 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 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; Greens *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 (≥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).

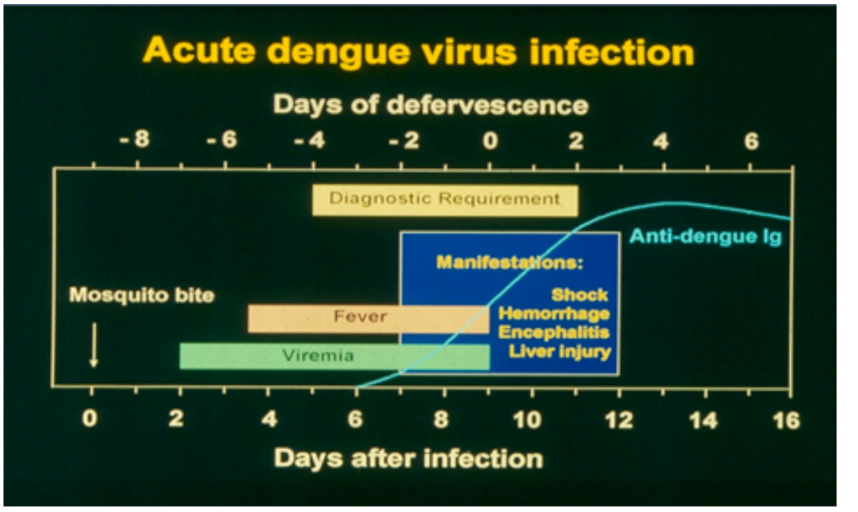

Fig 1–Diagnostic pathway in patients with suspected dengue.

Secondary dengue

Secondary dengue implies a 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 anti-dengue 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 ≥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 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 up to a week or more. 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 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 will 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 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 as-

sist in the differential diagnosis to reduce morbidity and mortality. For suspected dengue patients, serum specimens should be collected early in the febrile phase and stored at -70ºC for virus isolation/detection and at -20ºC 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 will be 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 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 DENV specifically are able to agglutinate certain types of erythrocytes. Normal erythrocytes in a suspension will settle to the bottom of the 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).

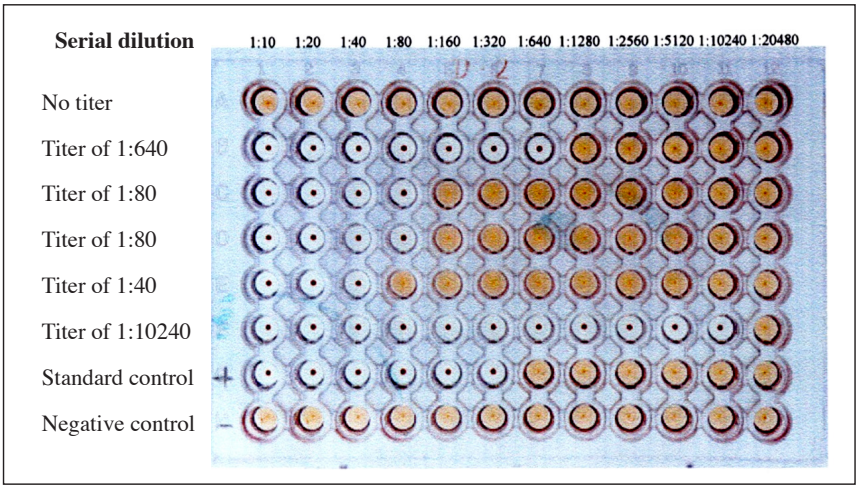

Fig 2–Hemagglutination Inhibition Assay.

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 goose red blood cells rather than human cells to remove non-specific inhibitors of hemaglutination. The techniques used for hemagglutination and HI as described by Clarke and Casals in 1958 have 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 a 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 a 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 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 buffer set at a specific pH for each dengue serotype prior to using them in the assay.

To prepare the test sera, non-specific inhibitors must be removed (that is, some human serum can inhibit agglutination without 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 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 4ºC 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 $PRNT_{50}$ 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

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.

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.

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 it's 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 concomitant IgM capture JE ELISA, eliminated 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 isotype-capture 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 a EIA plate reader. A binding index (BI) is calculated using the optical density (OD) 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 BI 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 to distinguish 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 -20ºC. Plates can be kept at -20ºC 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 IgG-HRP 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 serum-based 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 specimens 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 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 dip-

stick and upon exposure to DENV-specific antibody and a anti-human antibody detection system, 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 Pan-Bio, 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 *Ae. aegypti* (Gubler *et al*, 1979; Rosen and Shroyer, 1985; Rosen *et al*, 1985). *Toxorhynchites*

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* are non-blood feeders.

At the AFRIMS laboratory, *Toxorhynchites splendens* is used injecting 0.02 µl of human sera intrathoracically. 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 overlayed 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 an-

tibodies in an immunofluorescence assay or an antigen capture EIA format (Henchal *et al*, 1983). Common monoclonal antibodies used for serotype identification include 1F1 for DENV-1, 3H5 for DENV-2, 5D4 for DENV-3 and 1H10 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, 1F1 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 and others (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 cDNA 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 cDNA 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 cDNA-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 are essential 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 RT-PCR, which relies on the conversion of RNA into cDNA 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 Perkin-Elmer 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 cDNA sequence (follows 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 it's 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 were not detectable for 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). More over, their data suggested that the NS1 antigen was detectable during days 1-to-8 of illness (Shu *et al*, 2002).

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 remembered. 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 inexpensive so that developing countries with dengue epidemics might be able to utilize them. Likewise the assay must be simple to perform with minimal training and diagnostic equipment. Such an assay will 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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Chapter 4. Adult Dengue

Clinical features and management of dengue patients

DENGUE FEVER AND DENGUE HEMORRHAGIC FEVER IN ADULTS

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Abstract. Dengue fever and dengue hemorrhagic fever are re-emerging diseases that are endemic in the Tropics. The global prevalence of dengue cases has increased in South-East Asia, Africa, the Western Pacific, and the Americas. The increasingly widespread distribution and the rising incidence of dengue virus infections are related to increased distribution of *Aedes aegypti*, an increasingly urban population, and increasing air travel. Several Southeast Asian countries show that the age of the reported dengue cases has increased from 5-9 years, to older children and young adults. Dengue infection in adolescents and adults has also been recognized as a potential hazard to international travelers returning from endemic areas, especially Southeast Asia. Dengue is one disease entity with different clinical presentations; often with unpredictable clinical evolutions and outcomes. Bleeding manifestations in adult patients, including petechiae and menorrhagia were also frequently found; however, massive hematemesis may occur in adult patients because of peptic ulcer disease and may not be associated with profound shock as previously reported in children. Although shock and plasma leakage seem to be more prevalent as age decreases, the frequency of internal hemorrhage rises as age increases. Increase in liver enzymes found in both children and adults indicated liver involvement during dengue infections. Pre-existing liver diseases in adults such as chronic hepatitis, alcoholic cirrhosis, and hemoglobinopathies may aggravate the liver impairment in dengue infection. Fulminant hepatitis is a rare but well described problem in adult patients with dengue infection. Currently, no specific therapeutic agent exists for dengue. The early recognition of dengue infection, bleeding tendency, and signs of circulatory collapse would reduce mortality rates in adult patients with dengue infection.

Keywords: dengue fever, dengue hemorrhagic fever, dengue infection, adult

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INTRODUCTION

Dengue fever (DF) and dengue hemorrhagic fever (DHF) are re-emerging mosquito-borne viral infections caused by four closely related dengue viruses (serotypes 1-4) of the genus *Flavivirus*. These dengue viruses are transmitted

principally by the *Aedes aegypti* and *Ae. albopictus* mosquito species. The dengue virus has 4 antigenically similar but immunologically distinct serotypes. Infection confers lifelong immunity to the infecting serotype; therefore, a person can be infected with dengue virus up to four times during his or her lifetime. The World Health Organization (WHO) predicted that there are more than 2.5 billion people living in tropical and subtropical countries, mostly in large and small cities, at risk of dengue infection with one or more dengue viruses. The global prevalence of dengue cases has increased in South-East Asia, Africa, the Western Pacific, and the Americas. The increasingly widespread distribution and the rising incidence of dengue virus infections is related to increased distribution of *Ae. aegypti*, and to the increase in urban population in the cities of Southeast Asia and air travel. Ideal conditions for increased transmission of dengue virus in tropical urban centers have been created by substandard housing and crowding, as well as deterioration in water, sewer, and waste management systems, all of which are intimately associated with unplanned urbanization (Barbazan *et al*, 2002; Guzman and Kouri, 2003; Nakhapakorn and Tripathi, 2005; Anyamba *et al*, 2006). Without an effective vaccine or antiviral agent, an effective vector control program is the only means to reduce dengue infection in endemic areas.

Dengue virus infection is an important public health problem in Asia, and mainly occurs in children less than 15 years of age. The age distribution is different in the Americas, where these syndromes occur in all age groups. However, the majority of fatalities during epidemics in the Americas occur in children. Several Southeast Asian countries have shown that age of the reported dengue cases has increased from 5-9 years to older children and young adults (Charoensook *et al*, 1999; Pancharoen *et al*, 2002; Pongsumpun *et al*, 2002; Kulanatne *et al*, 2005). In Thailand, affected adults aged over 15 years old are reported to comprise 20%-40% of dengue virus infected cases according to the Epidemiological Surveillance System (Patumanond *et al*, 2003; Department of Epidemiology, 2012). Morbidity and mortality rates of dengue have been highest in children, especially in the 5-9 year age group. At present, the morbidity rate of DHF in Thailand has declined to 0.15% while the average age of dengue patients is increasing.

The evidence of rising age of DF/DHF cases has been explained by association with demographic transition, modern housing, and commercial development (Kyle and Harris, 2008; Cummings *et al*, 2009). Dengue infection in adolescents and adults has been recognized as a potential hazard to tourists as various reports have been published on dengue virus infection in international travelers returning from endemic areas, especially Southeast Asia (Jelinex, 2000; Brien *et al*, 2001; Stefen *et al*, 2002; Pongsumpun *et al*, 2004). The increase in international air travel and the increasing transmission of dengue in the tropics mean that healthcare providers in Western countries are more likely to be confronted with travel-acquired dengue infections. Dengue now appears to occur more frequently than malaria among travelers returning from any region except Africa and Central America (Schwartz *et al*, 2008; Burdino *et al*, 2011; Wilder-Smith, 2012; Leder *et al*, 2013).

In returning travelers, the discrepancy between the incidences of infection and clinical expression is comparable with observations in areas of endemicity, where infections may go unnoticed. The proportionate morbidity associated with dengue is especially high among travelers returning from Southeast Asia and the Caribbean. Thus, we emphasize the need for continued dengue surveillance in non-endemic countries with careful evaluation and follow-up of febrile patients who have returned home after visiting a country in which dengue is endemic (Freedman *et al*, 2006; Massed and Wilder-Smith, 2009).

Although rarely documented, dengue virus transmission without a mosquito vector has been reported. Documented dengue transmissions through needle-stick, receipt of infected blood component, tissues or organs transplantation, and transplacental infection are quite rare; however, they may be more widespread than previously recognized (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).

Dengue is one disease entity with different clinical presentations and often with unpredictable clinical outcomes. Dengue virus infection produces a spectrum of clinical illness ranging from undifferentiated fever, dengue fever (DF), which is a self-limiting febrile illness associated with fever, headache, myalgia, and thrombocytopenia, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), which may be fatal. DHF/DSS is characterized by rapid onset of capillary leakage accompanied by thrombocytopenia, altered hemostasis, which is characterized by hemoconcentration (hematocrit increased > 20%), thrombocytopenia (platelet count, <100 $x10^9$/l), vascular collapse, abdominal pain, and hemorrhagic manifestations (WHO, 1997). The 1997 WHO definition further subdivides DHF into four grades (grade I-IV) on the basis of the presence of spontaneous bleeding and the presence and severity of shock (grade IV; DSS). Despite the clinical classification of DF and DHF as distinct entities, they are likely to be a continuum of the same disease process with divergent outcomes with regards to the perturbation of vascular integrity.

The low accuracy of the WHO case definition and the difficulty to identify early clinical predictors of dengue infection in adults has been described (Srikiatkhachorn *et al*, 2010; Hadinegoro, 2012). Due to many reports about difficulty of using the 1997 WHO classification in clinical management, the WHO released new guidelines with a new classification in 2009, which is dengue without warning signs, dengue with a warning signs and severe dengue (WHO, 2009; 2012). The warning signs mark the beginning of the critical phase in dengue infection. These patients become worse around the time of defervescence, when the temperature drops to 37.5-38ºC or less and remains below this level, usually on days 3 to 8 of illness. Being an adult is also a risk factor for mortality in DF/DHF because of delayed diagnosis and treatment, comorbidity, and the increasing frequency of internal hemorrhage with age.

Physicians should be aware of warning signs (persistent or severe vomiting, abdominal pain or tenderness, liver enlargement, drowsy or alteration of consciousness, fluid accumulation with re-

spiratory distress, epitaxis, gum bleeding, gastrointestinal bleeding, retinal hemorrhage, oliguria, and hemoconcentration with severe thrombocytopenia) in patients with dengue infections before the patients develop severe dengue infections (shock from plasma leakage, severe bleeding, hepatic failure, acute renal failure, and encephalopathy) (Leo *et al*, 2011; Horstick *et al*, 2012; Prasad *et al*, 2013). The outcome of dengue depends largely on early diagnosis, the immediate replacement of fluid, and intensive supportive care.

There are several factors that may influence disease severity in dengue virus infection, including host factors, virus serotype or genotype, sequence of virus infection and differences in dengue cross-reactive antibody and T-cell responses (Green and Rothman, 2006). Age-related differences in dengue severity are poorly understood, and the data on clinical features in dengue adults are limited (Guzman *et al*, 2002; Hammond *et al*, 2005; Kittigul *et al*, 2007; Tantawichien, 2012; Namvongsa *et al*, 2013; Souza *et al*, 2013). Plasma leakage, as well as DSS and leakage appear to be less frequent in adults than children, possibly reflecting age-dependent differences in intrinsic vascular permeability, but anecdotal evidence suggests that bleeding manifestations, especially internal hemorrhage and hepatic dysfunction, are both more common in older age groups (Gamble *et al*, 2000; Wichmann *et al*, 2005; Guilarde *et al*, 2008; Tantawichien, 2012).

Previous studies have described the severity of clinical bleeding found in adult dengue patients. The emergence of DF with unusual bleeding and DHF in the adolescent, adult and elderly populations has been a cause of an apparent increase in the complications of dengue infection (Tsai *et al*, 1991; Anuradha *et al*, 1998; Agarwal *et al*, 1999; Tantawichien *et al*, 2000; Rongrungruang and Leelarasamee, 2001; Wichmann *et al*, 2005; Pungjitprapai and Tantaurcheen, 2008). Older age has previously been reported to be a risk factor for mortality in patients with DF or DHF because ageing, co-morbidity, and waning immunity pose a substantial risk for fatality in elderly patients with active infection (Rigau-Perez and Laufer, 2006; Kuo *et al*, 2007; Lee *et al*, 2008; Gautret *et al*, 2012; Pang *et al*, 2012).

CLINICAL MANIFESTATION

Dengue is a common cause of fever in the Tropics, but its contribution to the total burden of febrile illnesses that presents to primary health facilities in endemic regions such as Thailand is largely unknown. In the early stage of dengue infection, diagnosis from clinical manifestation alone is difficult, especially in adults. Dengue has numerous differential diagnoses, including malaria, leptospirosis, rickettsial diseases, typhoid, chikungunya, other viral hemorrhagic disease, and so forth (Leelarasamee *et al*, 2004; Phuong *et al*, 2006). Dengue infection should be suspected if the patients have a fever of 10 days or less with myalgia, arthralgia, bone pain, headache, peri-orbital pain, flushing, nausea or vomiting with no obvious respiratory tract symptoms or signs and no organ specific symptoms of other infectious diseases.

After an incubation period of 4 to 7 days, the febrile period is accompanied by severe headache, retro-orbital pain, myalgia, arthralgia, nausea, and vomiting. Tantawichien *et al* (2000) described the clinical manifestations of 140 adult patients

infected with dengue virus during the epidemic of dengue infection in Bangkok from 1997 to 1998, and he reported that there was fever (3 to 8 days), nausea/vomiting, headache, and myalgia in both DF and DHF; however abdominal pain, and severe or widespread bleeding manifestations were less frequent in DF.

Over one-quarter of infected people reported a rash during the febrile phase that was initially macular or maculopapular, and some became diffusely erythematous, sparing small areas of normal skin ("islands of white in a sea of red"). Minor hemorrhagic manifestations such as petechiae, epistaxis, and gingival bleeding do occur, but severe hemorrhage leading to shock through blood loss rarely occurs. Attempts to differentiate DF clinically from other acute febrile illnesses are unlikely to be successful although the diagnosis is aided if laboratory examination indicates leukopenia, neutropenia, thrombocytopenia, or mildly elevated AST levels, as well as a positive tourniquet test.

The tourniquet test has been used as a clue for dengue infection for a long time and has been considered by the WHO in 2009 as one of the criteria for probable dengue infection. Unfortunately, the sensitivity and specificity of tourniquet test from previous report, especially in children, were not excellent, ranging between 34%-56% and 68%-94%, respectively. However, this test was regarded to be a cheap and simple clinical method that is suggestive of dengue when positive, but a negative test does not exclude the disease (Phuong *et al*, 2002; Gregory *et al*, 2011; Mayxay *et al*, 2011; Halsey *et al*, 2013).

Laboratory tests, such as reverse transcription polymerase chain reaction (RT-PCR) or dengue nonstructural protein 1 antigen, capture assay (NS1 Ag assay) are usually used to diagnose the dengue infection antigen during the early phase of acute infection, and serological ELISA is used to detect specific IgM or IgG antibodies. However, those tests are not always available in all parts of dengue endemic countries. DF is usually a self-limiting condition, and death as a result is uncommon. Nevertheless, patients who have severe nausea/vomiting, severe hemorrhage (for example, hematemesis, hematochezia, or abnormal vaginal bleeding), hypotension, a platelet count of ≤20,000/mm^3 (≤20x10^9/l), AST or ALT >500 U/ml, renal failure, liver failure, heart failure, drowsiness, severe hypoxemia, pregnancy, and no opportunity to be followed up in an out-patient setting should be hospitalized in Thailand (Figs 1 and 2).

DHF is the most serious manifestation of dengue. Adults may be at lower risk of developing DHF compared to children and adolescent due to differences in capillary permeability (Gamble *et al*, 2000). During the transition from the febrile to afebrile phase, DHF patients with increased capillary permeability may manifest with the warning signs, mostly as a result of plasma leakage. The cardinal features that distinguish DHF from DF are increased vascular permeability (plasma leakage syndrome), and marked thrombocytopenia (< 100 x10^9/l) associated with bleeding and hepatomegaly and/or abnormal liver function (WHO, 1997).

During the transition from the febrile to afebrile phase, DHF patients with increased capillary permeability may manifest with the warning signs, mostly as a result of plasma leakage. A 20% increase in hematocrit

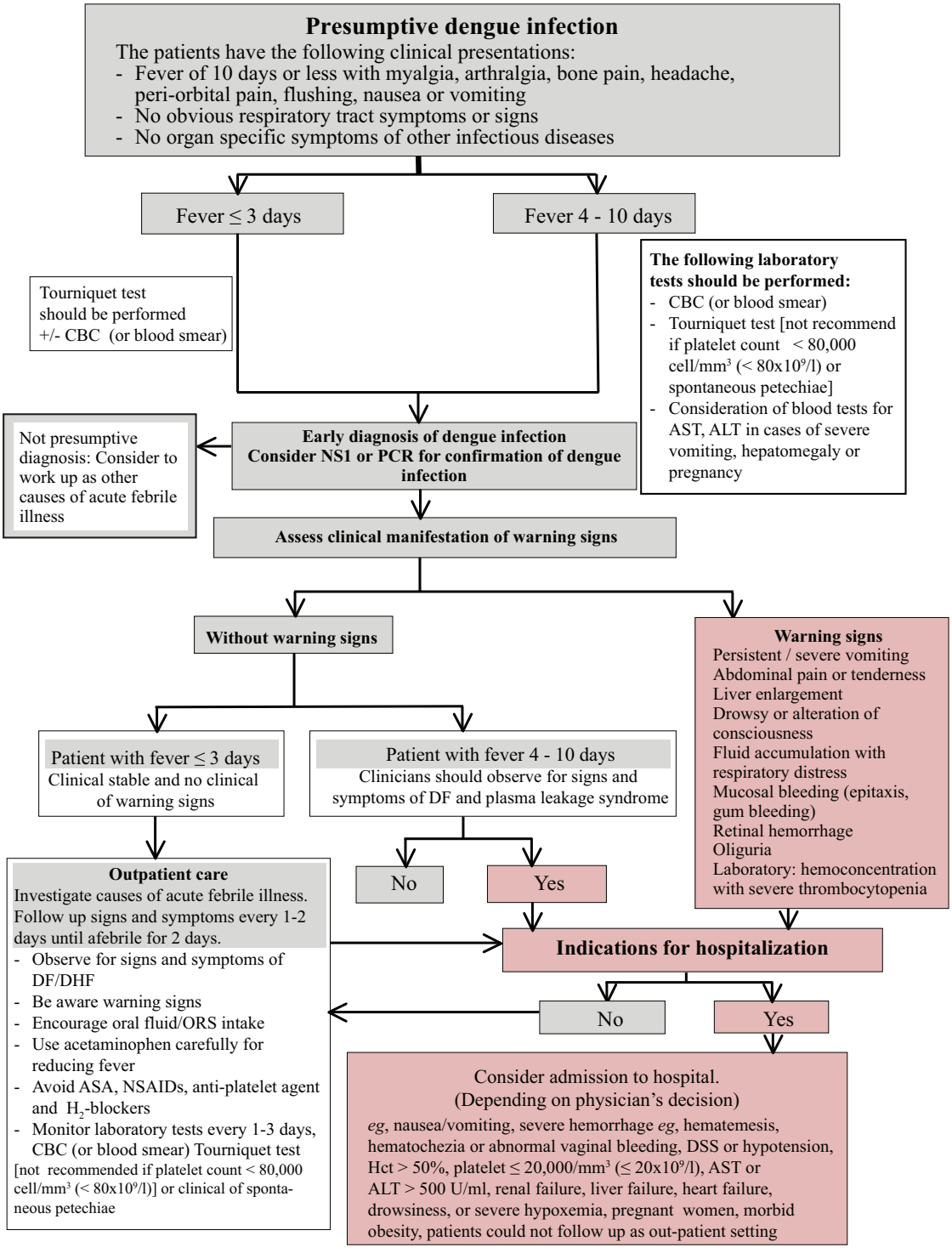

Fig 1–Guideline for outpatient management of dengue infections in adult (adapted from guidelines of Royal College Physician of Thailand, 2012).

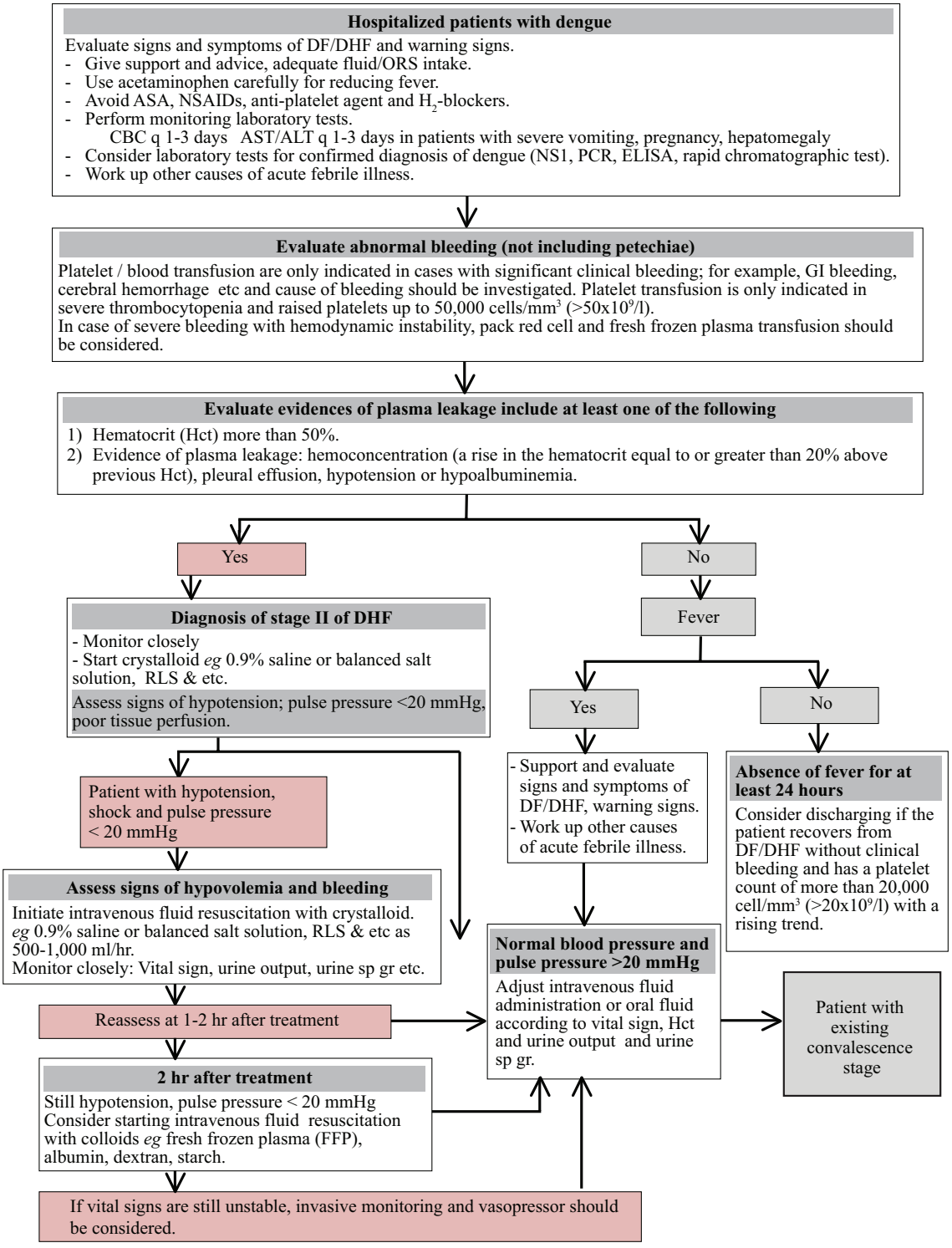

Fig 2–Guideline for inpatient management of dengue infections in adult (adapted from guideline of Royal College Physician of Thailand 2012).

over baseline is generally accepted as indicative of plasma leakage. The degree of hemoconcentration above the baseline hematocrit reflects the severity of plasma leakage; however, this may be reduced by early intravenous fluid therapy. Hematocrit readings can be affected by factors other than plasma leakage such as fever, dehydration, and bleeding. Furthermore, failure to obtain repeated measurements needed to calculate the degree of hemoconcentration often leads to difficulties in classifying dengue adult cases. Pleural fluid detected by right lateral decubitus chest roentgenogram, ultrasound detection of free fluid in the chest or abdomen, or gall bladder wall edema has been interpreted as evidence of plasma leakage, and ascites are usually only clinically detectable after intravenous fluid therapy unless plasma leakage is significant (Setcawan *et al*, 1995; Srikiatkhachorn *et al*, 2007; Wang *et al*, 2007a).

Plasma leakage syndrome and extreme decreases in platelet counts associated with bleeding frequently occur 3 to 7 days after the onset of illness. The period of clinically significant plasma leakage usually lasts 24-48 hours. In cases with a benign course of illness, blood pressure and pulse may be maintained, but a rapid and weak pulse, narrowing of the pulse pressure to less than 20 mmHg, or an unobtainable blood pressure in the most extreme cases establish DSS. If plasma loss continues and becomes excessive, the patient's situation can progress into profound shock. Clinical indicators of impending DSS include severe abdominal pain, change from fever to hypothermia, restlessness, sweating, prostration and tender hepatomegaly. DSS was an independent risk factor (odds ratio 220) for development of acute renal failure in adult patients with DHF (Lee *et al*, 2009). Cardiac involvement was observed in few patients requiring hospitalization with clinical manifestation ranging from mild elevation of cardiac biomarkers to myocarditis and/or pericarditis and death (Miranda *et al*, 2013).

Acute respiratory failure is a rare complication in adult dengue patients but has a high mortality rate (Wang *et al*, 2007b). In agreement with previous reports, Tantawichien *et al* (2000) reported that adults with DHF had a very low frequency of DSS compared with children with DHF. Although children are more likely to develop hypovolemic shock than adults in DHF characterized by increased microvascular permeability, a high mortality rate is seen in the adults and elderly with dengue virus infection.

Hemorrhage contributes to dengue morbidity and mortality, especially during the severe thrombocytopenia and the toxic hemorrhagic stage (3 to 5 days after the onset of illness) (Chuansumrit and Chaiyaratana, 2014). The pathogenesis of hemorrhage may be multifactorial and encompasses vasculopathy, platelet deficiency and dysfunction, as well as blood coagulation defects. Platelet counts begin to fall during the febrile stage and reach their lowest levels during the toxic stage. Bleeding complications usually occur in 5 to 8 days after onset in dengue infection. The abnormal bleeding is associated with low numbers of platelets and abnormalities of walls of vessels. Risk factors of bleeding are platelets ≤20,000/mm^3 (≤20x10^9/l), increased AST or ALT, prolonged PT, severe dengue hemorrhagic fever, patients with DIC, or liver failure (Chamnanchanunt *et al*, 2012).

Age had a non-linear relation to the

risk of bleeding. In almost all DHF patients, defects of coagulation activation such as a prolonged activated partial thromboplastin time (APTT) or thrombin time can be found. In addition, a decreased fibrinogen level and increased levels of fibrinogen degradation products indicating hyperfibrinolysis may also occur in DHF/DSS patients. There are typical coagulopathies of increased APTT and low fibrinogen levels in most patients, but severe thrombocytopenia and platelet dysfunction are probably the major cause of clinical bleeding. Massive bleeding such as hematemesis may occur in adults with DF or DHF. Minor hemorrhagic manifestations such as petechiae, epistaxis and gingival bleeding do sometimes occur in DF although they are rarely associated with severe hemorrhage leading to shock. Of the dengue patients with DHF, severity of hemorrhagic manifestations varied markedly with spontaneous petechiae, hematemesis, metrorrhagia, melana, and epistaxis.

Bleeding manifestations including petechiae and menorrhagia were also frequently found in adults. Bleeding from the nose, gums and upper gastrointestinal tract are not uncommon. However, massive hematemesis may occur in DF or DHF patients because of peptic ulcer disease in adults, and it is not associated with profound shock in adults as previously reported in children. Upper gastrointestinal bleeding is the most common type of severe hemorrhage in DF. In the few reports of endoscopic findings for dengue patients with upper gastrointestinal bleeding, hemorrhagic gastritis was the most common finding (40.9%-58.5%), followed by gastric ulcer, and duodenal ulcer (Tsai *et al*, 1991). In patients with preexisting peptic ulcer disease, severe and even fatal gastric bleeding can be precipitated. The role of endoscopic therapy in upper gastrointestinal bleeding of dengue patients is still unknown (Wung *et al*, 1990). Therefore, blood transfusion therapy with concentrated platelets, packed red cell, and fresh frozen plasma to correct the bleeding tendency, anemia, coagulopathy, and hypovolemia is still the mainstay of treatment of upper gastrointestinal bleeding in dengue patients.

Females had a greater tendency to develop abnormal bleeding such as menorrhagia during hospitalization. Tantawichien *et al* (2000) reported that vaginal bleeding (menorrhagia) was the most common site of bleeding (24.6%) in female adults with dengue virus infection. Hormonal therapy, such as premarin, primolute N, or oral contraceptive pills is suggested for females exhibiting excessive menstrual bleeding. Uterine hemorrhage resulting in spontaneous abortion and severe postpartum bleeding has also been reported in pregnant women (Thaithumyanon *et al*, 1994).

Life-threatening subcapsular splenic bleeding and ruptures are rare but can happen spontaneously or as a result of trauma, which may be minor or unnoticed. Splenectomy is still the treatment of choice for splenic rupture, but numerous recent reports have documented favorable outcomes with conservative treatment (Imbert *et al*, 1993; Pungjitprapai and Tantawichian, 2008). Early diagnosis and treatment are needed to avoid a fatal outcome. Surgical procedures performed on patients with dengue infection may unmask dengue-induced hemostatic defects, resulting in unexpected hemorrhage in post-operative period that is difficult to control. Therefore,

in the case of onset of labor, the route of delivery should be considered under obstetric indications. During hemorrhaging, patients should receive blood transfusions with concentrated platelets, packed red blood cells, and fresh-frozen plasma to correct bleeding, anemia, and hypovolemic shock. Close monitoring of vital signs and hematocrit levels to assess the severity of hemorrhage are required to reduce morbidity and mortality. Most patients with dengue infection recover spontaneously, and all abnormal hemostasis normalizes during the convalescent stage or within 1-2 weeks after defervescence.

Liver involvement during dengue virus infection in adults has been described (Kuo *et al*, 1992; Kalayannarooj *et al*, 1997; Souza *et al*, 2004; Trung *et al*, 2010). Increase in liver enzymes (AST and ALT) found in both children and adults indicated liver involvement during dengue virus infections. The pathogenesis of liver involvement during dengue infections is still poorly understood. Potential mechanisms of hepatic injury involve a variety of potential insults including direct effects of the virus or host immune response on liver cells; compromised circulation and/or hypoxia caused by hypotension or localized vascular leakage inside the liver capsule; hepatotoxic effects of drugs such as acetaminophen or traditional herbal remedies; and tissue tropism of particular viral serotypes or genotypes (Parkash *et al*, 2010). Dengue antigens and viral RNA have been demonstrated in some of these fatal cases, and dengue viruses have been isolated occasionally from hepatic tissue. However, biopsy specimens are rarely obtained from less severe cases, and the relevance of these findings to the broad spectrum of dengue infections remains uncertain. In endemic or epidemic areas, dengue infection should be included in the differential diagnosis of acute viral hepatitis.

Unlike conventional viral hepatitis, dengue infections have a higher level of AST than that of ALT. It has been suggested that this may be due to excessive release of AST from damaged myocytes during dengue infections. Attention must therefore be given to the use of hepatotoxic agents such as acetaminophen, antibiotics, and antiemetic drugs, all of which have the potential to aggravate liver damage in some cases of dengue. Liver transaminase should be measured in adult patients with dengue infection, especially when hepatitis is suspected, or a history of acetaminophen use of more than 2 grams per day has been noted. The levels of liver enzymes increase to a maximum 7-to-9 days after the onset of symptoms, and then often decrease to normal levels within 2 weeks. Pre-existing liver diseases such as chronic infection with virus hepatitis B or C, alcoholic liver disease, and cirrhosis may aggravate the liver impairment of dengue. It is likely that relatively more adult dengue patients have more liver impairment than children, especially during periods of epidemic.

Transaminase levels increase in virtually all dengue patients and have correlated with other markers of disease severity. Abnormal liver enzyme levels have been associated with a poor outcome in adults with vascular leakage and abnormal bleeding. Severe liver involvement may complicate the clinical picture of DF and DHF by causing liver failure and contributing directly to severe bleeding, as well as indirectly potentiating the severity of disseminated intravascular coagulopathy (DIC). Jaundice

and fulminant liver failure occur relatively late in the course of the disease usually without evidence of severe vascular leakage with shock (Innis *et al*, 1990, Ling *et al*, 2007). The association of severe liver disease and encephalopathy has been well described in both pediatric and adult patients with DF and DHF. Hence, dengue should be considered as a possible cause of acute liver failure in endemic areas if other viral markers are negative. The management of fulminant liver failure in dengue is primarily supportive. Splenomegaly is uncommonly observed in adult with dengue infection. Thickening of the gallbladder wall has been reported in conditions with hypoalbuminemia and ascites, as well as in several viral infections including DHF.

An increasing number of dengue infections have been related to other unusual manifestations. The unusual manifestations of dengue infection in adult include DF/DHF with severe internal hemorrhage, fulminant hepatic failure, encephalopathy, cardiomyopathy, cardiac arrhythmia, adult respiratory distress syndrome (ARDS), rhabdomyolysis, pancreatitis, appendicitis, co-infection with other tropical infectious diseases and neurological phenomena such as altered consciousness, convulsions, and coma resulting from encephalitis and encephalopathy (Thakane *et al*, 1996; Jusuf *et al*, 1998; Solomon *et al*, 2000; Garcia-Rivera and Rigue-Perez, 2002; Davis and Bouke, 2004; Promphan *et al,* 2004; Misra *et al*, 2006; Premaratna *et al*, 2007).

Neurological manifestations of dengue can include a wide range of neurological features in 0.5%-21% of hospitalized patients with dengue. These neurological manifestations were ascribed to non-specific complications such as myelitis, neuro-ophthalmic complications, polyradiculopathy, neuropathy, and neuromuscular complications secondary to dengue infection (Carod-Artal *et al*, 2013). Possible causes of dengue encephalopathy include hypotension, cerebral edema, focal hemorrhage, hyponatremia, and fulminant hepatic failure. However, a documented possibility is the invasion of the central nervous system (Lum *et al*, 1996; Chokephaibulkit *et al*, 2001).

Some studies have indicated that 5.5% of the patients with DHF/DSS also had dual infection (for example, urinary tract infection, diarrhea, or bacteremia) (Pancharoen and Thisyakorn, 1998; Tantawichien, 2012). Dual infection should be suspected in atypical presentation; for example, prolonged 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. The patient with dengue infection may have subsequent nosocomial infection after hospitalization. Failure in making a diagnosis of concurrent infection in patients with DHF may lead to otherwise preventable morbidity and mortality. A previous report revealed that prolonged fever and acute renal failure were independent predictive factors for dual infection (Lee *et al*, 2005).

In dengue endemic areas, obstetricians must be aware that dengue infection of pregnant women may occur and some history or laboratories consistent with dengue infection such as fever without coryza, facial flushing, petechiae hemorrhage, thrombocytopenia, and an increase of atypical lymphocytes must be identified. Many cases of DF/DHF among pregnant

women have been reported in Southeast Asia, highlighting the concept that some women in endemic area remain susceptible to dengue infection (Bunyavejchevin *et al*, 1997; Corles *et al*, 1999). Dengue during pregnancy is also particularly important in pregnant travelers from non-endemic countries to countries where dengue is endemic (Carroll *et al*, 2007).

Younger mothers would be more likely to become infected compared to older mothers, who are more likely to be seropositive and, therefore, less susceptible for dengue infections. Because surgical procedures performed on patients with dengue infection may unmask dengue–induced hemostatic defects, resulting in unexpected hemorrhage in post-operative period that is difficult to control. The route of delivery in such patients at the onset of labor should be considered under obstetric indications (Adam *et al*, 2010). It also has been reported that dengue infection was vertically transmitted to the fetus and that this led to a full-blown illness in the neonate similar to that seen in children and adults (Bunyavejchevin *et al*, 1997).

A low rate of fetal transmission is consistent with other studies that have reported no cases of congenital dengue infection in neonates born to mothers infected early in pregnancy. Accordingly, the gestation of maternal infection is an important factor related to fetal infection risk with symptomatic infection possibly unlikely with early gestational infection and increased with infection late in pregnancy (Waduge *et al*, 2006; Basurko *et al*, 2009; Pouliot *et al*, 2010; Chitra and Panicker, 2011). All reported cases of symptomatic congenital dengue infection have occurred in neonates born to mothers infected very late in pregnancy mainly when they were symptomatic at the time of delivery. Although the effects of dengue infection on pregnant women and their fetuses or newborns are unclear, recent studies have demonstrated that this infection did not cause any infant abnormalities but may have been responsible for fetus deaths and morbidity in pregnant women (Basurko *et al*, 2009).

The outcome of DHF and DSS depends largely on early diagnosis, the immediate replacement of fluid, and comorbidities. Adults have a higher prevalence of underlying diseases; for example, coronary artery disease, peptic ulcer, hypertension, diabetes mellitus, cirrhosis, or chronic kidney disease, which should be considered in management of adult dengue (Rigau-Perez and Laufer, 2006; Sam *et al*, 2013). With support through the critical period of illness, spontaneous resolution of vasculopathy and circulatory failure usually can be expected within 2 to 3 days with complete recovery afterward. At that time, the temperature decreases, and can increase slightly the day after. This pattern is known as the biphasic temperature curve. In the defervescence period, the patients usually have more appetite, bradycardia. Also, they develop a generalized, maculopapular rash with itching, sparing the palms and soles. This rash usually disappears in 1 to 5 days. The duration of dengue illness ranges from 7 to 10 days in most cases. Adults may have profound fatigue or mood disturbance for several weeks after recovery.

DIAGNOSIS

Early definite diagnosis of dengue infection can help clinicians in initiation of

early supportive care, adequate management, and identification of patients with severe dengue, who should be closely monitored for signs of plasma leakage, bleeding, and end organ damage. This information might promote early supportive therapies, prevent the use of potentially harmful drugs, encourage assessment of complications, ensure the adequate use of treatment guidelines, and lead to the effective control of dengue outbreaks. 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 human serum (Poerscha *et al*, 2005). The sensitivity of each approach is influenced by the duration and severity of the patient's illness. It should be stressed that in dengue endemic areas, while early accurate laboratory tests are not widely available, dengue infection should be considered in every patient presenting with an acute undifferentiated febrile illness.

However, monitoring all these patients for the development of warning signs of severity may impose a great burden on healthcare services. In the very early stage of illness when patients generally seek medical attention within the first 2-to-3 days of fever without specific symptoms, only RT-PCR or dengue virus NS1 Ag assay can reliably confirm the diagnosis of dengue. RT-PCR is definitely the most satisfactory test that might detect dengue viruses up to the seventh day after the onset of the symptoms, especially in severe cases (Yamada *et al*, 2002; Lanciotti, 2003). In addition, the presence of dengue virus in frozen and fixed tissues, saliva or urine can be determined by RT-PCR. As an alternative, the detection of viral antigens has been proposed, and more recently attention has been focused on NS1 of the dengue virus (Alcon *et al*, 2002; Vazqueza *et al*, 2010). A high circulating level of dengue virus NS1 was demonstrated in the early stage of dengue infection by different ELISA assays in the plasma and/or sera of dengue patients.

Hemagglutination-inhibition (HI), complement fixation (CF), neutralization test (NT), immunoglobulin M (IgM) capture enzyme linked immunosorbent assay (MAC-ELISA), and indirect immunoglobulin G ELISA have been used for the diagnosis of dengue infection. The dengue antibodies are better detected around the fifth day after the onset of the disease, making this technique unfeasible for rapid diagnosis. Until now, ELISA has been considered the most useful test for dengue diagnosis due to its high sensitivity and ease of use. ELISA has been used to detect acute phase (IgM) and convalescent phase (IgG) antibodies. The IgM antibody titers in primary infections are significantly earlier and higher than in secondary infections.

Some adult patients with primary infection such as travelers from Europe or North America have IgM detectable by the second to the fourth day after the beginning of the symptoms while patients with secondary infections mount rapid anamnestic antibody responses in which dengue virus–reactive IgG may predominate over IgM. There are several commercial kits of rapid tests of IgM and IgG detection for diagnosis of dengue infection (Kittigul and Suankeow, 2002; Blacksell *et al*, 2006). However, the sensitivity, specificity, and accuracy vary among these tests. In clinical settings where methods of RT-PCR are not avail-

able, combination tests for elevated levels of soluble NS1 or dengue virus–reactive IgM in serum is a pragmatic diagnostic approach in an adult patient in whom dengue infection is suspected.

TREATMENT AND PREVENTION

Currently, no specific therapeutic agent to treat dengue infection is available, and treatment remains supportive with particular emphasis on careful fluid management. The early recognition of dengue infection, bleeding tendency, signs of circulatory collapse, and complications would reduce mortality rates in adult patients with dengue infection. Mild dengue infections may be treated at home with oral hydration and antipyretics with instructions to return to the hospital immediately if bleeding or warning signs suggestive of severe disease develop. Oral rehydration is indicated to replace losses from vomiting and high fever. It is necessary to avoid the use of salicylates, NSAIDs, and traditional medicines that may contain hepatotoxic agents.

Development of any warning sign (*eg*, severe vomiting, gastrointestinal hemorrhage, hypotension, high liver transaminase, acute renal impairment, alteration of consciousness, severe thrombocytopenia, etc) indicates the need for hospitalization and close observation with appropriate use of parenteral fluids in patients with inadequate oral intake or a rapidly increasing hematocrit. Attentive clinical monitoring of patients with severe dengue or suspected DHF-DSS and anticipatory and supportive care are life saving and have reduced fatality rates. The critical activities for hospitalized dengue patients are monitoring of abnormal bleeding, circulation and vascular leakage by serial clinical assessments of pulse, blood pressure, skin perfusion, urine output, urine specific gravity, and hematocrit to trigger intravenous fluid or transfusion therapy (Nhan *et al*, 2001).

Patients with DHF need to be monitored closely for signs of shock until at least 24-48 hours after defervescence. The mainstay of treatment for DHF remains prompt fluid resuscitation to counteract massive plasma leakage. Timely and effective intravenous crystalloid replacement of plasma losses results in a favorable outcome in most adult cases. For patients suffering from DSS, the mainstay of therapy is early and effective replacement of plasma loss. To limit the risk of the development of fluid overload, parenteral fluid therapy should be kept to the minimum required to maintain cardiovascular stability until permeability reverts to a normal level. WHO recommends immediate volume replacement with Ringer's lactate, or physiologically normal saline solution, followed by fresh frozen plasma or colloid solutions such as albumin, or dextran in the event that shock persists. Crystalloid solutions should be used initially, and isotonic colloid solutions should be reserved for patients presenting with profound shock or those who do not have a response to initial crystalloid therapy. Recently, two randomized controlled trials evaluated therapeutic responses to colloid and crystalloid solutions (Dung *et al*, 1999; Wills *et al*, 2005; Akech *et al*, 2011).

Results indicate that Ringer's lactate performed the least well and that the more severely ill patients identified by a narrow pulse pressure (≤10 mmHg) would benefit more from initial resuscitation with colloid solution than with crystalloid solution.

Whole blood, platelet, and fresh-frozen plasma transfusions can be lifesaving for patients with severe bleeding that compromises cardiovascular function, but it should be undertaken with care because of the risk of fluid overload. The use of prophylactic blood or platelet transfusions may be harmful and should be avoided, and invasive procedures should be minimized to avoid hemorrhagic complications. Currently, there is no evidence to support the use of any adjunctive therapies such as corticosteroid, desmopressin, or carbazochrome sodium sulfonate (AC-17) for dengue infection.

Dengue prevention currently relies on public health and community-based *Aedes aegypti* control programs to remove and destroy mosquito-breeding sites. The most advanced approach is a potential vaccine consisting of a tetravalent combination of attenuated dengue strains, and other approaches are undergoing initial clinical evaluation. Dengue will continue to spread worldwide until a safe and effective vaccine is available alongside sustainable mosquito control practices. Furthermore, as the eventual implementation of a vaccine will shift the burden of disease, the age related differences in clinical manifestations and prognoses described here indicate the importance of comparing a wide range of ages in future clinical studies of dengue.

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LIVER COMPLICATIONS IN ADULT DENGUE AND CURRENT MANAGEMENT

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Abstract. Adult dengue patients have a lower prevalence of bleeding tendency and greater prevalence of abnormal liver function tests than children with dengue infections. At least two-thirds of adult dengue patients have abnormal liver function tests. Our article aims to detail the clinical findings of liver complications in adult patients with dengue infection. The predictors of liver complications and the associations with failure of other organs were reviewed; for example, high-level ALT during the febrile stage has been associated with shock. In addition, this review includes the current interventions for treatment of acute liver failure in adult dengue patients including N-acetylcysteine, and artificial liver dialysis.

Keywords: liver complication, adult dengue, treatment

INTRODUCTION

Dengue infection is the most common mosquito-borne viral disease in the world. Fifty million infections occur annually, with 500,000 cases of dengue hemorrhagic fever (DHF) and 22,000 deaths (CDC, 2014). The overall incidence rates of dengue infection in adult patients (aged ≥15 years) are up to one-third of the incidence rates in child patients. Clinical manifestations in adult patients may differ from child patients, and the data are still limited. Severe infection, DHF, or dengue shock syndrome (DSS) were more prevalent in adults than in children. Liver involvement is common in dengue infection, including hepatomegaly, jaundice, abnormal liver enzymes (60%), and acute severe hepatitis with an at least 10 times elevated level of transaminase (4%) (Wichmann *et al*, 2004).

Previous studies have shown more liver involvement in adult patients characterized by high rates of elevation in transaminase levels of which the average elevation of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were about two times that of the upper normal limit (AST 93 U/l and ALT 86 U/l) (Souza *et al*, 2004). This review article aims to detail the clinical findings of liver complications in adult patients with dengue infection. The predictors of liver complications and associations with the failure of other organs were reviewed; for example, high-level ALT during the febrile stage has been

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associated with shock. In addition, this review includes the current treatments of acute liver failure in dengue adult patients.

MATERIALS AND METHODS

We retrospectively reviewed data from a Medline and PubMed search that was performed to identify relevant literature using search terms "liver complications" and "dengue" during a 20-year period, from 1995 to 2014. All relevant literatures of adult dengue patients with liver complications were reviewed. Specific terms were defined: 1) adult was defined as age greater than or equal to 15 years; 2) abnormal AST (aspartate aminotransferase) and/or ALT (alanine aminotransferase) 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 WHO criteria with serological confirmation by ELISA test or rapid immunochromatographic test; and 4) the 1997 and 2009 WHO classifications were used to categorize dengue patients as dengue fever (DF) or DHF Grades I-IV (Treeprasertsuk *et al*, 2003).

RESULTS

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

Our study at the Hospital for Tropical Diseases in Bangkok, Thailand showed that the mean age of 127 adult dengue patients was 26.4±11.5 years, and 66% of them had a severe form of dengue infection. Most of them (96%) had no underlying liver disease. Abnormal transaminase levels, including AST and ALT, were commonly found. These accounted for 88% and 69% of patients respectively, with the average ratio of AST to ALT of 1.8:1 (Table 1). In addition, our study found that the dengue-infected patients with abnormal ALT had significantly older age and had a longer duration of fever of at least 7 days (Treeprasertsuk *et al*, 2003).

Those patients with significant or acute severe hepatitis with an at least 10 times elevated transaminase level accounted for 7% of adult dengue infected patients, which is consistent with previous reports of a prevalence of 4%-15% (Kuo *et al*, 1992; Treeprasertsuk *et al*, 2003; Souza *et al*, 2004; Parkash *et al*, 2010). A previous study showed that the level of AST was more prominent than that of ALT in the first week and the maximum transaminase levels occurred on the ninth day after the onset of fever (Treeprasertsuk *et al*, 2003), gradually decreasing to normal levels within two weeks. However, some reports have shown that about one-third of dengue infections had persistent symptoms of fatigue, and abnormal liver tests remained in 7.6% of patients with dengue infections at the end of the second month of follow-up (Tristao-Sa *et al*, 2012). Elevated AST levels tend to return to normal more rapidly than ALT levels possibly due to AST having a shorter half-life than ALT. Hyperbilirubinemia has been found in 0.7%-13.4% of adult dengue infections without significant association with severity (Kuo *et al*, 1992; Wahid *et al*, 2000; Trung *et al*, 2010).

Liver pathophysiology

The patients with dengue infection who had complications or severe acute hepatitis were usually infected with dengue serotype 3 or serotype 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). The possible pathophysiological factors associated with acute liver injury in dengue patients were the direct viral effect on liver cells or an adverse consequence of dysregulated host immune responses against the virus (Seneviratne *et al*, 2006) along with the severity of diseases including prolonged shock or ischemic hepatitis, drugs induced liver injury and preexisting liver damage (Ahmed *et al*, 2014).

Clinical findings of liver complications in adult patients with dengue infection

Hepatomegaly was significantly more commonly found in children with dengue infection than adults (43%-92% in children *vs* 28%-72% in adults) (Wichmann and Jelinek, 2004; Wichmann *et al*, 2004; Wang *et al*, 2009). An abnormal liver test can be used as one of the associated factors for dengue severity with an odds ratio of 1.9 (CI 0.97-0.99) (Khan *et al*, 2013). In addition, our study found an association between the patients with severe hepatitis during the febrile stage and clinical bleeding (Treeprasertsuk *et al*, 2003). Patients with at least a 10-fold elevation of ALT had a significantly greater proportion of hypotension than those with low-level ALT (25% *vs* 5%, respectively). Moreover, patients with high-level transaminases had a significantly longer duration of fever and a higher hematocrit level than those with low-level ALT.

In clinical practice, the common causes of abnormal liver tests in critical patients were ischemic hepatitis, sepsis, and drugs 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 (Behera *et al*, 2009; Parkash *et al*, 2010). One study of acute hepatitis severity in dengue fever and its outcomes reported that two-thirds of the deceased patients had severe acute hepatitis while one-third had mild to moderate acute hepatitis [elevated transaminase of at least 10 times had a higher mortality rate than those with low ALT level (HR 4.9; 95% CI 1.7-13.9; p=0.003) (Parkash *et al*, 2010)]. These findings are consistent with a previous report that the transaminase levels were associated with the severity of vascular leakage and the increased severity of bleeding (Trung *et al*, 2010).

Current treatment of acute liver failure in dengue adult patients

Principle management for hospitalized critically ill patients with abnormal liver tests (Berry *et al*, 2013):

A. Identify those patients with underlying chronic liver disease (Thomson *et al*, 2010). Due to the high risk to mortality in cirrhotic patients, the clinician has to identify the evidence of underlying chronic liver disease: for example, splenomegaly, low platelet count, or evidence of cirrhosis from abdominal ultrasonography (Agrawal *et al*, 2011).

B. Exclude treatable and/or emergency hepato-biliary diseases: for example, gallstone cholangitis or acute liver failure. The high-risk group for developing acute

Table 1
Laboratory findings of 127 adult dengue patients admitted to the Hospital for Tropical Diseases, Bangkok, Thailand.

Lab test	Days 1-7			Days 8-14		
	n	Mean±SD	Range	*n*	Mean±SD	Range
Hematocrit (%)	126	42.7±5.2	21.1-56.7	80	41.5±5.7	21.6-55.5
WBC (cell/mm³)	126	3,983±1,925	1,200-11,550	34	6,432±3,096	2,875-18,700
Lymphocyte (%)	125	31.3±0.9	3-56	34	41.1±11.6	10-63
Atypical lymphocyte (%)	125	5.8±7.9	0-40	34	8.0±6.9	0-32
Platelet (cell/mm³)	126	61,905±39,561	10,000-234,500	76	76,555±65,201	12,500-355,000
AST (IU/l)	127	243±372	14-2,215	18	651±1,463	52-6,468
ALT (IU/l)	127	152±215	5-1,580	18	512±1,030	80-4,586

Days 1-7, day 1-to -day 7 from onset of fever; days 8-14, day 8-to-day 14 from onset of fever; SD, standard deviation; WBC, white blood cell count; AST, aspartate aminotransferase; ALT, alanine aminotransferase.
Source: Treeprasertsuk *et al* (2003).

Table 2
The outcomes of treatment with N-acetylcysteine (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	Survive with normal liver tests in 2 weeks
Lim and Lee, 2012	6-year-old boy	Acute liver failure	No details	Survive
Kumarasena *et al*, 2010	*n*=7 cases; ages 6 months-12 yrs	Acute liver failure	Low GCS, prolonged shock	Survive

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

liver failure from dengue infection is those patients with a comorbidity, especially diabetes mellitus (Sam *et al*, 2013).

C. Identify the common causes of abnormal liver tests in critically ill patients, especially the causes from ischemic hepatitis and sepsis (Thomson *et al*, 2009).

D. Consider the risk of complication from the specific hosts: for example, pregnancy (Malhotra *et al*, 2006), AIDS, or elderly patients, all of whom may present with variable clinical courses. The few reports of HIV patients with dengue infection have shown that they usually present with mild symptoms (Mendes Wda *et al*, 2006; Siong *et al*, 2008). However, opportunistic infections in HIV patients must be excluded.

E. Exclude drug-induced liver injury (DILI). Recently, two reports have found evidence that acetaminophen overdose may play an important role in causing acute liver failure in dengue infection patients (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/day) were found in all child fulminant hepatitis patients compared with none in the control group (Ranganathan *et al*, 2006).

Recent suggestions for the treatment of dengue patients with acute liver failure

1. N-acetylcysteine (NAC).

2. Providing temporary liver support as a bridge to liver transplantation: artificial liver support. However, studies of both treatments have 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.

The rationale for N-acetylcysteine (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 acute liver failure, the following NAC dosage regimen has been used: an intravenous (IV) loading dose of 150 mg/kg/day 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/24 hours until an INR of <1.4 was achieved (Kortsalioudaki *et al*, 2008). Previous studies have shown that dengue patients with acute liver failure who were prescribed NAC had favorable outcomes as shown in Table 2 (Sklar and Subramaniam, 2004; Senanayake *et al*, 2013; 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/day infusion for 5 days in adult patients (Habaragamuwa and Dissanayaka, 2014);

2. IV 150 mg/kg bolus over 15 minutes followed by 12.5 mg/kg/hour for 4 hours and then 6.25 mg/kg/hour for 72 hours in children (Kumarasena *et al*, 2010);

3. Or, 100 mg/kg/day for 6 days in children (Lim and Lee, 2012).

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 proce-

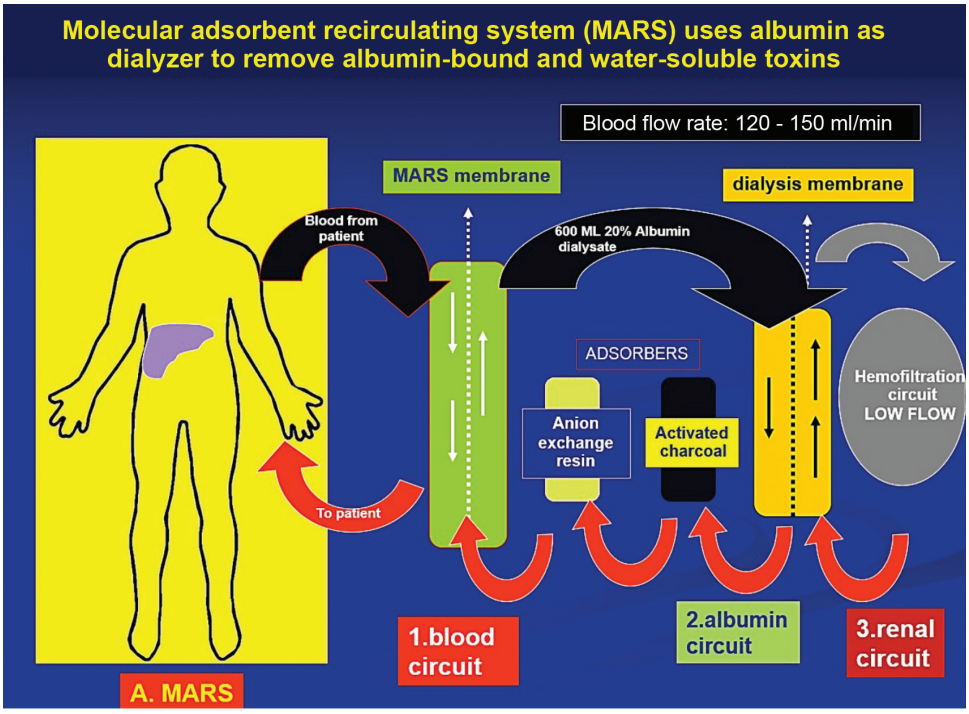

Fig 1–The Molecular Adsorbent Recirculating System:a nonbiological artificial liver support system.

dures, and it is different from the 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 nonbiological systems including the hemodiafiltration, albumin dialysis, and plasma exchange are available worldwide as follows (Carpentier *et al*, 2009):

Molecular Adsorbent Recirculating System (MARS, Gambro, Sweden), which was developed by Falkenhangen *et al* (1999).

Prometheus (Fresenius, Germany), which was developed by Falkenhagen *et al* (1999).

SPAD (Single pass albumin dialysis).

These nonbiological 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). Penafiel *et al* (2006) reported that supportive treatment of a dengue infected patient with fulminant liver failure by MARS (Fig 1) demonstrated a favorable outcome including rapid reversal of the biochemical profile and an improvement of encephalopathy; however, MARS has some limitations including a high cost and some technical difficulty in usage (Penafiel *et al,* 2006).

At King Chulalongkorn Memorial Hospital in Thailand, we use albumin dialysis more often than other modalities due to the availability of equipment, lower cost, and ease of usage (Boonsrirat *et al*, 2009). Albumin dialysis utilizes the scavenging function of albumin to remove toxins, and it can reduce hyperbilirubinemia as well as improve encephalopathy in liver failure patients (Sen *et al*, 2005). Our previous study of using SPAD in patients with acute liver failure 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% (Boonsrirat *et al*, 2009). We noted no significant changes in mean arterial pressure, and treatment modality was hemodynamically well tolerated. Recently, we have used SPAD in two dengue infected patients with liver failure, and these cases had good outcomes (local data).

CONCLUSION

At least two-thirds of adult dengue patients have shown abnormal liver function tests. Acute severe hepatitis with an at least 10 times elevation of transaminase levels has occurred in 4%-15% of adult dengue infected patients, and this should be a concern for the treating physician. Transaminases gradually decrease to normal levels within two 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 disease may play important role in causing acute liver failure in dengue infected patients. N-acetylcysteine and artificial liver support are 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 is needed to support their usage.

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ELECTROLYTE DISTURBANCE AND KIDNEY DYSFUNCTION IN DENGUE VIRAL INFECTION

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Abstract. Dengue virus infection (DVI) is endemic in tropical countries in both children and adults. The classical presentation includes fever, hepatomegaly, thrombocytopenia-related bleeding disorders, and plasma leakage. Multi-organ involvement, including kidneys is found in complex cases. Asymptomatic electrolyte disturbances, abnormal urinalysis, and more severe manifestation such as acute kidney injury (AKI) usually indicate kidney involvement. Such manifestations are not rare in DVI, but are often not recognized and can cause the physician to misread the real situation of the patient. The prevalence of electrolyte disturbances or kidney involvement reported in studies varies widely by country and mainly depends on the severity of DVI and age of the patients. The prevalence of DVI-induced AKI ranges from 0.2%-10.0% in children and 2.2%-35.7% in adults. The prevalence among all age groups appears to be increasing in the last decade. Dengue shock syndrome (DSS) has been reported to be an independent risk factor for AKI development. The mechanism of DVI-induced AKI is complex and the details are to date undetermined. Urinalysis, serum electrolytes and creatinine measurements should be performed to document renal involvement in DVI patients for early detection and initiation of appropriate fluid therapy with close monitoring. Renal replacement therapy may be required in some cases. The presence of AKI dramatically increases the mortality rate among both childhood and adulthood DVI from 12%-44% to more than 60%.

Keywords: acute kidney injury, dengue hemorrhagic fever, dengue shock syndrome, dengue viral infection, electrolyte disturbance, kidney involvement

INTRODUCTION

Dengue viral infection (DVI) has been a serious concern in terms of being widespread and incurring large expenses for many countries, especially tropical area, for several decades. Attempts to eradicate the DVI have not been successful despite the dramatic increase in the incidence of DVI worldwide. Additionally, the characteristics of dengue hemorrhagic fever and dengue shock syndrome (DHF/DSS) have become more complex in some cases. In DVI, the kidney is one of the major organs affected. The manifestations of kidney involvement vary from electrolyte disturbance, hematuria, proteinuria, and glomerulonephritis to severe acute kidney injury (AKI) (Futrakul *et al*, 1973; Hommel *et al*, 1999; Abboud, 2003; Mendez and Gonzalez, 2003; Nair *et al*, 2005; Vachvanichsanong *et al*, 2006;

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Lima *et al*, 2007; Khan *et al*, 2008; Kuo *et al*, 2008; Lima and Nogueira, 2008; Lee *et al*, 2009; Vasanwala *et al*, 2009; Laoprasopwattana *et al*, 2010; Lumpaopong *et al*, 2010; Basu *et al*, 2011; Bunnag and Kalayanarooj, 2011; Khalil *et al*, 2012; Mehra *et al*, 2012).

PREVALENCE

The prevalence of kidney dysfunction or electrolyte disturbance due to DVI may be under-recognized depending on the frequency of laboratory tests performed. The stage and severity of DVI are also important; if tests are performed too early, then the chance of detecting an abnormality is lower than when tests are performed at later stages. Evidences of kidney involvements in DVI may also be under-reported or ignored if the findings are mild or do not significantly affect the clinical condition or result in modification of therapy. Additionally, abnormal findings may not be a direct result of the DVI, but can result from a DVI-caused complication, such as hypotension, shock or associated septicemia.

ABNORMAL URINALYSIS

A general feature of kidney involvement is an abnormal urinalysis. To our knowledge, the first study in the literature that reported results of abnormal urinalysis in DVI was from a study of 24 DHF children in 1973 (Futrakul *et al*, 1973). Proteinuria, glycosuria, ketonuria, occult blood, microscopic hematuria and an abnormally high number of tubular cells were found in 71%, 19%, 38%, 38%, 80%, and 90% of the cases, respectively. Table 1 shows two recent studies from Thailand conducted in children with DVI, which demonstrated abnormal findings of the urine based on urinalysis (Lumpaopong *et al*, 2010; Vachvanichsanong *et al*, 2010). The rate of hematuria was significantly different between the two studies, which may have been due to various reasons, including stage of illness and timing of urine collection, particularly relative to when fluid therapy was initiated, all of which can affect the test results.

HEMATURIA

DVI generally causes bleeding disorders due to thrombocytopenia, and it is not surprising to find microscopic hematuria in DVI patients. However, the incidence of microscopic hematuria is often not reported. Macroscopic hematuria may be found in patients who have urinary catheterization.

In a cohort study of 154 adults and 147 children with DVI, the prevalence of a positive urine blood test was similar between the children and adults (55% *vs* 58%), although some symptoms were different (Hanafusa *et al*, 2008). A positive urine blood test can mean hemoglobinuria or myoglobinuria as well as hematuria.

PROTEINURIA

Proteinuria is one of the most common abnormalities found by urinalysis in DVI children (Vachvanichsanong *et al*, 2010). However, proteinuria is a non-specific finding that can result from many different conditions, including even mild conditions, such as fever (Loghman-Adham, 1998; Hogg *et al*, 2000). The significance of proteinuria usually depends on the severity and condition of the patient; it can be a warning sign of renal damage and further

Table 1
Prevalence of abnormal findings in urinalysis in dengue viral infection in two studies from Thailand.

	Lumpaopong *et al*, 2010		Vachvanichsanong *et al*, 2010
	DF (*n*=67)	DHF (*n*=73)	DF + DHF + DSS (*n*=1,342)
Abnormal UA	-	-	28.50%
Glycosurea	-	-	3.70%
Hematuria	18%	27%	6.30%
Proteinuria	15%	27%	22.10%

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

damage can occur if it persists. Vasanwala *et al* (2009) reported on two cases of adult DHF who had heavy proteinuria in the high nephrotic range, but neither had any other characteristics of nephrotic syndrome. Hutspardol *et al* (2011) reported the case of a child with nephrotic range proteinuria who also had hypoalbuminemia, right pleural effusion and azotemia. However, both studies showed that the proteinuria was eventually self-limiting.

GLOMERULONEPHRITIS

The classical glomerulonephritis characteristics of hematuria, edema, and hypertension are unlikely to be documentable in patients with DVI. Hematuria is often seen in DVI patients; therefore, physicians may not even consider glomerulonephritis as the possible cause. Glomerulonephritis in DVI patients can only be confirmed by histopathology. In one study of 20 patients with DHF, immune complex was found in half (Boonpucknavig *et al*, 1976). Interestingly, Rajadhyaksha and Mehra (2012) reported a case of a 22-year-old woman who developed systemic lupus erythematosus and lupus nephritis four weeks after diagnosis of DVI.

Other unusual findings have been reported, such as DF-induced hemolytic uremic syndrome in a 48-year-old patient (Wiersinga *et al*, 2006) and IgA nephropathy in a 15-year-old DF patient (Upadhaya *et al*, 2010).

ELECTROLYTE AND ACID-BASE DISTURBANCES

Table 2 shows electrolyte disturbance results from three studies in DVI children. Hyponatremia was found to be the most common electrolyte disturbance in these patients. In one study, hyponatremia was reported as high as 66% in 150 children with DF and DHF (Lumpaopong *et al*, 2010). However, half of these cases (50%) had mild hyponatremia (serum sodium 130-134 mEq/l), while 14.7% had serum sodium levels between 125-129 mEq/l, and only 1.3% had concentrations <125 mEq/l. In another study of 45 children with DSS, 46.7% had hyponatremia (Bunnag and Kalayanarooj, 2011), while from another study conducted in our institute, only 19.9%

Table 2
Electrolyte disturbances in dengue viral infection from three studies.

	Lumpaopong *et al*, 2010		Vachvanichsanong *et al*, 2010	Bunnag and Kalayanarooj, 2011
	DF (*n*=73)	DHF (*n*=77)	DF + DHF + DSS (*n*=1,249)	DSS (*n*=50)
Hyponatremia	61%	72%	19.90%	46.7% (*n*=45)
Hypernatremia	-	-	0.30%	-
Hypokalemia	14%	17%	11.60%	-
Hyperkalemia	-	-	5.00%	-
Hypocalcemia	-	-	-	68.3% (*n*=41)

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

of 1,249 DVI children had hyponatremia (Vachvanichsanong *et al*, 2010). Additionally, another study found that hyponatremia was 9.7 times more prevalent in 49 DVI children compared to 44 children with other acute febrile illnesses (Mekmullica *et al*, 2005). In an earlier study from our institution, abnormalities in urine and electrolytes significantly increased with increasing DVI severity (Vachvanichsanong *et al*, 2010).

Bunnag and Kalayanarooj (2011) reported a very high rate of hypocalcemia (68.3%) among 41 DSS children.

Metabolic acidosis is usually found in shock or AKI conditions. Bunnag and Kalayanarooj (2011) reported metabolic acidosis in 14% of 50 DSS children while Lumpaopong *et al* (2010) found metabolic acidosis in only 8.6% of 150 DF/DHF children.

In another study from the northeast of Thailand, in patients aged less than 18 years who died from DVI, electrolyte and acid-base disturbances were found in 7/8 (87.5%) and 51/91 (56.0%), DF and DHF patients, respectively (Lumbiganon *et al*, 2012).

ACUTE KIDNEY INJURY

Acute kidney injury (AKI), is the current medical term for what was formerly known as acute renal failure (ARF), and it describes a sudden onset of renal dysfunction, which primarily means a decrease in the glomerular filtration rate (GFR). This in turn, results in retention of waste products, leading to metabolic acidosis, hyperkalemia, hypocalcemia, hyponatremia or hypernatremia and water retention.

There was no consensus on the precise biochemical definition of ARF. Generally it was defined as a rise in serum creatinine greater than two times the baseline value or the upper limit of normal values for age and gender in children, and a serum creatinine greater than 2 mg/dl in adults (Chan *et al*, 2002; Kellum *et al*, 2002). Although serum creatinine levels can have a wide range, its effect on mortality is abrupt rather than gradual, and once a 'tipping point' is crossed, a small change in serum creatinine can have a high impact on a patient's mortality.

The Acute Dialysis Quality Initiative (ADQI) group introduced the RIFLE (Risk

Table 3
RIFLE criteria for acute kidney injury classification (Bellomo *et al*, 2004).

RIFLE stage	Creatinine/GFR criteria	Urine output criteria
Risk	Increased SCr × 1.5 or decreased GFR > 25%	< 0.5 ml/kg/h for 6 hr
Injury	Increased SCr × 2 or decreased GFR > 50%	< 0.5 ml/kg/h for 12 hr
Failure	Increased SCr × 3 or decreased GFR > 75% or SCr > 4 mg/dl	< 0.3 ml/kg/h for 24 hr or anuria for 12 hr
Loss	Persistent renal failure > 4 weeks	
End-stage kidney disease	End-stage kidney disease (> 3 months)	

GFR, glomerular filtration rate; SCr, serum creatinine.

Table 4
Modified RIFLE criteria for pediatric patients (pRIFLE) (Akcan-Arikan *et al*, 2007).

RIFLE stage	eCCr criteria	Urine output criteria
Risk	Decreased by 25%	< 0.5 ml/kg/h for 8 hr
Injury	Decreased by 50%	< 0.5 ml/kg/h for 16 hr
Failure	Decreased by 75% or eCCr < 35 ml/min/1.73 m^2	< 0.5 ml/kg/h for 24 hr or anuria for 12 hr
Loss	Persistent renal failure > 4 weeks	
End-stage kidney disease	Persistent renal failure > 4 months	

eCCr, Estimated creatinine clearance.

of renal dysfunction; Injury to the kidney; Failure of kidney function; Loss of kidney function; End-stage kidney disease) criteria to classify the stage and severity of renal dysfunction for early detection, treatment and prevention of further kidney damage (Bellomo *et al*, 2004). The criteria are listed in Table 3, with a modification of the RIFLE classification system for pediatric patients shown in Table 4 (Akcan-Arikan *et al*, 2007). The Risk, Injury and Failure stages refer to AKI, and increasing RIFLE stage indicates increasing risk of death.

The prevalence of DVI-related AKI seems to be low, because the number of reported cases in the literature is small (George *et al*, 1988; Gunasekera *et al*, 2000; Davis and Bourke, 2004; Lim and Goh, 2005; Vachvanichsanong *et al*, 2006; Wiersinga *et al*, 2006; Karakus *et al*, 2007; Lima *et al*, 2007; Laoprasopwattana *et al*, 2010; Wijesinghe *et al*, 2013). However, in recent decades the prevalence of DVI-related AKI has significantly increased (Table 5) from 0.9% to 35.7%, with the high variation attributable to the definition of AKI used, severity of DVI, age of the study patients, and capabilities of the hospital. AKI has a high impact on hospital stay, morbidity, and mortality in DVI patients

(Laoprasopwattana *et al*, 2010; Khalil *et al*, 2012).

In our institute during an 18-year period, 25 of 2,393 (0.9%) children admitted with DVI developed AKI. The mortality rate of those who developed AKI was high at 64%. The independent risk factors of AKI were DHF grade IV (odds ratio, OR: 16.9; 95% confidence interval, CI: 4.2-68.5) and obesity (OR: 6.3; 95% CI: 1.4-28.8). The indicators of mortality were dengue grade IV, oliguria AKI, respiratory failure and prolonged prothrombin or activated partial thromboplastin greater than twice the normal values. The serum creatinine of all patients who survived returned to normal within 1-48 days (Laoprasopwattana *et al*, 2010). In a similar study in Taiwanese adults hospitalized with DHF, 10 out of 304 (3.3%) developed AKI, with a mortality rate of 60%. DSS was the only independent risk factor for development of AKI (Lee *et al*, 2009).

In a study from Taiwan of 519 adults with DVI, the estimated glomerular filtration rates (eGFR) decreased by >25%, >50%, and >75% in 21.6%, 2.9%, and 2.6%, respectively, of the 273 cases that had at least two separate measurements for serum creatinine (Kuo *et al*, 2008). Compared to DF patients, patients with DHF/DSS were five times more likely to have a >50% decrease in their eGFR (15% *vs* 2.9%, $p = 0.001$). Additionally, 21 patients with chronic kidney disease (CKD), defined as eGFR <60 ml/min/1.73 m^2, were significantly more likely to have a >50% decrease in their eGFR (36.8% *vs* 3.1%, $p<0.001$) and had a significantly higher mortality rate (28.6% *vs* 1.2%, $p<0.001$). One study of 99 fatal DVI cases in 2010 found that AKI and acid-base disturbances were two of the most common complications (Lumbiganon *et al*, 2012).

MECHANISMS OF AKI IN DVI

The principle mechanism of AKI can be simply explained as hypoperfusion and hypoxia from shock (Laoprasopwattana *et al*, 2010). Moreover, DVI-induced AKI may result from various factors, such as the use of non-steroidal anti-inflammatory drugs for antipyrexia, acute tubular necrosis, immune complex mediated acute glomerulonephritis, or sepsis, any of which may require multiple drug therapy, particularly in patients with severe disease, thus making the patient more susceptible to nephrotoxicity as well (Laoprasopwattana *et al*, 2010). Lima *et al* (2007) reported a 48-year-old female with DHF-induced AKI in the absence of hypotension, rhabdomyolysis, hemolysis, or nephrotoxic drug usage, and postulated that the mechanism of AKI may be due to a direct kidney injury caused by the dengue virus.

The actual mechanism of DVI-induced AKI is still undetermined. Some reports have indicated that the mechanism may involve rhabdomyolysis-induced acute tubular necrosis (Gunasekera *et al*, 2000; Davis and Bourke, 2004; Lim and Goh, 2005; Karakus *et al*, 2007; Wijesinghe *et al*, 2013; Repizo *et al*, 2014). DVI has also been connected with muscle injury, and although, as mentioned, the mechanism has not yet been determined, creatine phosphokinase should be monitored for muscle injury, especially in DVI patients.

Severe DHF/DSS patients usually develop multi-organ failure including hepatic failure, carditis, encephalopathy, respiratory failure, and bleeding disorders. However,

Table 5
Prevalence of dengue viral infection-induced acute kidney injury in various studies.

Author(s)	Country	Study year(s)	DVI	*N*	Age (yrs)	AKI (%)
Laoprasopwattana *et al*, 2010	Thailand	1989 - 2007	DF/DHF/DSS	2,893	< 15	0.9
Mendez and Gonzalez, 2003	Columbia	1992 - 2002	DHF	617	< 13	1.6
Khan *et al*, 2008	Saudi Arabia	2004	DHF	91	6-94	2.2
Lee *et al*, 2009	Taiwan	2002	DHF/DSS	304	> 18	3.3
Abboud, 2003	-	-	-	-	-	5.0
Kuo *et al*, 2008	Taiwan	2002	DF/DHF/DSS	273	48 ± 18	5.5[a] 27.1[b]
Bunnag and Kalayanarooj, 2011	Thailand	2008 - 2009	DSS	50	Children	10.0
Mehra *et al*, 2012	India	-	DF/DHF	223	26.2 ± 18.2	10.8
Khalil *et al*, 2012	Pakistan	2008 - 2010	DF/DHF/DSS	532	15 - 85	13.3
Lumbiganon *et al*, 2012	Thailand	2010	Fatal DF	8	< 18	25.0
			Fatal DHF	91		14.3
Basu *et al*, 2011	India	2007 - 2008	-	28	Adults	35.7

DVI, dengue virus infection; AKI, acute kidney injury; DF, dengue fever; DHF, dengue hemorrhagic fever; DSS, dengue shock syndrome.
[a]GFR decreased > 50%; [b]GFR decreased > 25%.

for patients without multi-organ failure, AKI is an unusual complication; when it does occur, it is usually associated with hypotension, rhabdomyolysis, or hemolysis (Lima and Nogueira, 2008).

Renal histopathology in DVI cases has rarely been reported in the literature due to the contraindication of performing a renal biopsy in patients with a hemorrhagic disease such as DVI. Nevertheless, some renal histopathological studies have been performed after the recovery phase or during post-mortem examinations. In one such study, the dengue virus was detected in the kidney in one third of renal necropsies performed on DHF/DSS patients, but not as high as found in the liver (5/10) (Guzman *et al*, 1999). The most important finding was generalized vascular injury.

TREATMENT

Optimizing fluid administration is the major treatment in DVI patients. Type of fluid prescribed (crystalloid or colloid), tonicity and rate of infusion are critical. Inappropriate fluid replacement can worsen the condition. A high prevalence of hyponatremia emphasizes that fluid therapy in DVI patients should be prescribed with high tonicity. Therapy is more complicated for patients who have abnormal electrolytes or impaired renal function.

Blood component transfusion may also be indicated. Renal replacement therapy (RRT) may be required in some patients who have severe metabolic acidosis, hyperkalemia, oliguric renal failure, or pulmonary edema. In two studies, 11/25 (44%) of childhood DHF/DSS cases with AKI and 3/10 (30%) adults had RRT (Lee *et al*, 2009; Laoprasopwattana *et al*, 2010).

PROGNOSIS

The mortality rate in DVI increases with severity of disease and presence of any complication. The mortality rate of DHF is less than 1%, while for DSS it is about 50 times higher (Anders *et al*, 2011). The mortality rate among DHF/DSS patients increases to more than 60% if AKI is also present (Lee *et al*, 2009; Laoprasopwattana *et al*, 2010).

SUMMARY

Asymptomatic electrolyte disturbance and deterioration of kidney function in DVI are highly prevalent in both children and adults. Transient abnormal urinalyses including heavy proteinuria are demonstrated. The number of DVI-induced AKI cases has been increasing. Kidney function impairment is one of the major potential fatal conditions, and when it occurs, accurate fluid therapy is mandatory with delicate adjustments and close monitoring required. Renal replacement therapy may be required in severe cases.

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DENGUE WITH CENTRAL NERVOUS SYSTEM INVOLVEMENT

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Abstract. Dengue has spread to new geographic areas affecting both children and adults, and it has become a global threat. Dengue with central nervous system involvement includes febrile seizures, encephalopathy, encephalitis, aseptic meningitis, intracranial hemorrhages, intracranial thrombosis, subdural effusions, mononeuropathies, polyneuropathies, Guillain-Barré syndrome, and transverse myelitis. These manifestations may be associated with co-infections, co-morbidities, or complications of prolonged shock. It is important to consider dengue as a cause for the above neurological presentations, particularly in endemic territories for dengue disease.

Keywords: dengue, nervous system

INTRODUCTION

Dengue, a mosquito-borne viral disease, is currently an expanding global problem. Dengue virus includes all four dengue serotypes DEN-1, DEN-2, DEN-3, and DEN-4, which belong to the genus *Flavivirus* in the family Flaviviridae. Dengue virus disease ranges from asymptomatic infection to undifferentiated fever, dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS) (Thisyakorn and Thisyakorn, 2015). Dengue with organopathy has been reported as unusual or atypical manifestations including the involvement of the following systems, sites, and manifestations: neurological, gastrointestinal, hepatic, renal, cardiac, respiratory, musculoskeletal, lymphoreticular, bone marrow, eye, post-infectious fatigue syndrome, depression, hallucination, psychosis, and alopecia (Thisyakorn and Thisyakorn, 1994a; Gulati and Maheshwari, 2007). These may be explained as complications of severe profound shock or associations with the hosts' underlying conditions/diseases or coinfections (Hemungkorn *et al*, 2007). The mentioned organopathies may be underreported, underecognized, or not related to dengue. However, it is essential that proper clinical assessment is carried out for appropriate management, and causal studies should be done (Thisyakorn and Thisyakorn, 1994a, b).

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CENTRAL NERVOUS SYSTEM INVOLVEMENT IN DENGUE PATIENTS

There have been reports of dengue infection with central nervous system involve-

ment from Southeast Asian countries since 1976. Sanguansermsri *et al* (1976) from Chiang Mai University, Thailand reported a DHF patient with encephalopathy, Wuler *et al* (1972) from Indonesia reported a girl with DHF and obvious signs and symptoms compatible with Reye's syndrome, (*ie*, high level of serum transaminases, ammonia, and characteristic histopathologic changes in the liver). Tin *et al* (1976) from Myanmar (formerly Burma) reported severe forms of DHF with encephalitic symptoms associated with loss of consciousness lasting from 2-to-5 days in nine children varying in ages from 8 months-to-9 years. Cerebrospinal fluid examinations in all cases were normal. DEN-2 was isolated from the acute sera of two cases while DEN-3 was isolated from the liver of another case. Sumarmo *et al* (1978) from Indonesia reported four virologically proven cases of encephalopathy in DHF and DSS patients with DEN-2 and DEN-3 viruses isolated from the sera. In 1981, Kho *et al* from Indonesia reported 41 cases of virologically proven DHF with neurological signs compatible with acute encephalopathy; all patients were seen in Jakarta from 1975 to 1977. Two of these children showed typical signs and symptoms of Reye's syndrome as confirmed by histopathology of their livers. Nimmannitya *et al* (1978) reported 18 cases of DHF with neurological manifestations seen at the former Children's Hospital, Thailand between 1972 and 1981; gross hemorrhage in the brain was noted in 6 of the 10 fatal cases while cerebral edema was noted in three cases. In 1988, George *et al* from Malaysia reported four cases of DF with cerebral and hepatic symptoms. Renal impairment was detected in two patients .

In 1987, Thisyakorn and Thisyakorn from Chulalongkorn University, Thailand conducted a prospective study in 505 serologically and/or virologically confirmed dengue patients. Fourteen patients had unusual manifestations, mainly involving central nervous system. All of their demographic data, unusual manifestations, and outcomes are shown in Tables 1 and 2. Lumbar puncture was done in six patients; all showed negative findings. None of the six patients had specific dengue IgM in their cerebrospinal fluid. Autopsies performed in two patients revealed massive centrolobular liver necrosis in both. Pathological examination of the brain revealed intracerebral hemorrhage at fronto-temporal area in one patient and viral encephalitis in the other. Comparison of all the data between dengue patients with usual and unusual manifestations is shown in Table 3, which shows that patients who had unusual manifestations tended to be in the younger age group, and those with unusual manifestations had higher mortality (Thisyakorn and Thisyakorn, 1994b).

Thisyakorn *et al* (1999) conducted a prospective study over a seven-year period from 1987 to 1994 to determine the clinical and laboratory findings of dengue patients with central nervous manifestations in two provinces, namely Bangkok, the capital city of Thailand and Songkhla, a province in the southern region of Thailand. Thirty serologically confirmed dengue patients with central nervous system manifestations were seen during the period of study. Their ages ranged between 3 months and 14 years with a mean age of 6.2 years. Seventeen were boys, and 13 were girls. The central nervous system manifestations included alteration of consciousness (76.7%), seizures (63.3%), pyramidal tract

Table 1
Clinical data of 14 patients with dengue infections and unusual manifestations.

No	Age (yr)	Sex	Severity of diseases	Prior medications	Nutritional status
1	7	Female	DSS	Yes	Normal
2	8/12	Female	DHF grade III	Yes	1st degree malnutrition
3	5	Male	DSS	Yes	Normal
4	12	Female	DF	No	Normal
5	7	Female	DHF grade III	Yes	Normal
6	4	Female	DHF grade III	Yes	Normal
7	14	Male	DF	Yes	Normal
8	5	Female	DSS	Yes	Normal
9	1^6/12	Female	DHF grade II	Yes	1st degree malnutrition
10	3^8/12	Female	DHF grade III	Yes	Normal
11	2^4/12	Male	DSS	Yes	Normal
12	7	Female	DHF grade II	Yes	Normal
13	1	Female	DSS	Yes	Normal
14	6	Male	DHF grade III	Yes	Normal

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

Table 2
Clinical manifestations and outcomes of 14 patients with dengue infection and unusual manifestations.

No	Unusual manifestations	Outcome
1	Alteration of consciousness, jaundice	Expired
2	Alteration of consciousness, convulsion, jaundice	Expired
3	Alteration of consciousness, convulsion, jaundice	Survived
4	Alteration of consciousness	Survived
5	Alteration of consciousness	Survived
6	Alteration of consciousness, convulsion, adult respiratory distress syndrome	Survived
7	Alteration of consciousness, convulsion	Survived
8	Reye-like syndrome	Expired
9	Reye-like syndrome	Survived
10	Jaundice	Survived
11	Alteration of consciousness, convulsion, jaundice	Expired
12	Alteration of consciousness, convulsion	Survived
13	Alteration of consciousness, jaundice	Survived
14	Adult respiratory distress syndrome	Survived

signs (36.7%), meningeal signs (30%), and headache (26.7%). Eleven patients had primary while 19 had secondary dengue infection. Cerebrospinal fluid examination showed lymphocytic pleocytosis in 6 out of 28 patients while presence of anti-dengue IgM antibodies was detected in 2 out of 19 specimens of cerebrospinal fluid tested. Two patients died; autopsy was done on one patient and the result of the brain

Table 3
Clinical data of patients with dengue infections at Chulalongkorn Hospital, 1987.

Clinical data	Usual manifestations	Unusual manifestations
Cases, *n*	491	14
Age, *n* (5)		
< 5 yrs	126 (25.7)	8 (57.1)
5-10 yrs	241 (49.1)	4 (28.6)
>10 yrs	124 (25.3)	2 (14.3)
Girls, *n* (%)	252 (51.3)	10 (71.4)
DSS, *n* (%)	255 (51.9)	10 (71.4)
With prior medications, *n* (%)	389 (79.2)	13 (92.9)
With malnutrition, *n* (%)	60 (12.2)	2 (14.3)
Death, *n* (%)	1 (0.2)	4 (28.6)

DSS, dengue shock syndrome.

examination was compatible with viral encephalitis (Thisyakorn *et al*, 1999).

With the geographical expansion of dengue illness, there have been increasing reports of dengue patients with neurological manifestations. A diverse range of central nervous system manifestations in dengue patients include febrile seizures, encephalopathy, encephalitis, aseptic meningitis, intracranial hemorrhages, intracranial thrombosis, subdural effusions, mononeuropathies, polyneuropathies, Guillain-Barré syndrome, and transverse myelitis. Exhaustive investigations should be done in these cases to exclude concurrent infections. It is essential that proper clinical assessment is carried out for appropriate management, and causal studies should be done. Unlike encephalitis caused by other viruses, most dengue patients with encephalopathy and encephalitis had uneventful recoveries. Long-term neurological sequelae in these patients were rare (Thisyakorn and Thisyakorn, 1994a, b; Thisyakorn *et al*, 1999; Gulati and Maheshwari, 2007).

CONCLUSION

Neurological manifestations in dengue patients are diverse, including febrile seizures, encephalopathy, encephalitis, aseptic meningitis, intracranial hemorrhages, intracranial thrombosis, subdural effusions, mononeuropathies, polyneuropathies, Guillain-Barré syndrome, and transverse myelitis. Exhaustive investigations should be done in these cases to exclude concurrent infections. It is essential that proper clinical assessment is carried out for appropriate management.

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FLUID AND HEMODYNAMIC MANAGEMENT IN SEVERE DENGUE

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Abstract. In the critical phase of dengue fever, the leakage of intravascular fluid into interstitial space and 3rd space can cause hemoconcentration and severe complications such as dengue shock syndrome (DSS), and it can lead to multiple organ failure, followed by death. Close monitoring, early detection and prompt management are the keys in successful treatment. In a hemodynamically unstable patient, crystalloid is the fluid of choice in initial management. However, if they are not responsive despite adequate resuscitation, a careful search for others causes is mandatory and fluids should be switched from crystalloid to colloid. If the leakage leads to restriction of the use of fluids (pulmonary edema), the addition of a vasopressor such as norepinephrine needs to be considered. After stabilizing the hemodynamics and clinical improvement, the physician has to know when to reduce and discontinue the fluid to avoid congestion and others complications.

Keywords: fluid management, hemodynamic, severe dengue

INTRODUCTION

In the critical phase of dengue fever, a severe complication is dengue shock syndrome (DSS), which may be caused by the leakage of intravascular fluid into interstitial space and 3rd space as well as from bleeding. This complication can lead to multiple organ failure and death.

In the World Health Organization guidelines on dengue (WHO, 2012), it is suggested that when frontline physicians encounter a severe dengue patient with signs of shock (cold, clammy extremities, prolonged capillary refill time and a weak pulse), or signs of severe bleeding or impaired consciousness, they should give intravenous fluid immediately. This should start with isotonic crystalloid solutions at 5-10 ml/kg/hour over one hour. Then the physician must urgently arrange to refer the patient to hospital.

During the critical phase, the main pathophysiology is increased capillary permeability, leading to intravascular volume loss. The clinical management in this phase needs close observation of plasma leakage (Fig 1) (hemodynamically stable), and if patients have hypotension, they should receive the treatment (Fig 2).

FLUID MANAGEMENT IN PATIENT WITH PLASMA LEAKAGE

We can classify patients into 4 groups (Table 1) (modified from WHO, 2012).

1. Normal blood pressure and pulse pressure >20 mmHg.

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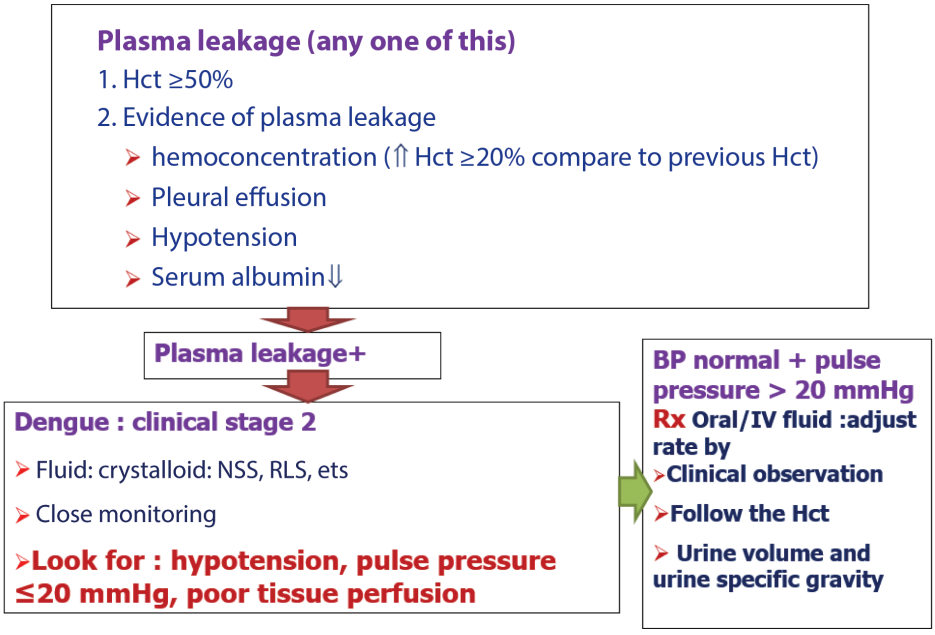

Fig 1–Hemodynamically stable patient.

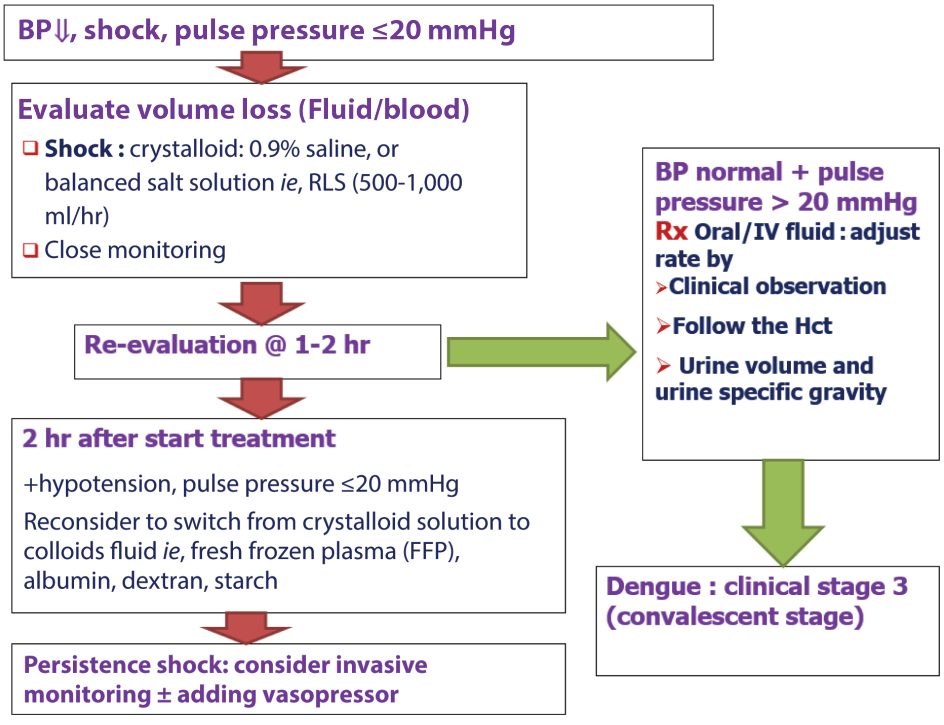

Fig 2–Hypotensive patient.

Table 1
Hemodynamics management.

Normal blood pressure and pulse pressure >20 mmHg	Hypotension and/or pulse pressure ≤20 mmHg	Shock	Persistent shock despite adequate crystalloid replacement
Target end point Normal blood pressure and pulse pressure >20 mmHg Urine sp.gr. 1010-1020 and urine output 0.5-1.0 ml/kg/hr Hct ~40%-45%			
Limitation Fluid resuscitation can increase amount of plasma leakage into the 3rd space that increases the possibility of pleural effusion, ascites, and pulmonary edema.			
Treatment	Treatment	Treatment	Treatment
Oral fluid intake in most of case. IV fluid (5%D/saline, 0.9% saline) in patients who are unable to have adequate oral intake. Amount of fluid: 40-80 ml/hr and adjust according to vital signs, urine parameter, and Hct.	IV isotonic crystalloid *eg*, 0.9% saline or RLS 5-7 ml/kg/hr for 1-2 hr. If the clinical status & parameters are improved, decrease the rate to 3-5 ml/kg/hr for 2-4 hr, and then 2-3 ml/kg/hr until stable vital signs. If clinical status and parameters are worsened or not improved, increase the rate to 7-10 ml/kg/hr for 1-2 hr and re-evaluate. If not improved, the patient should be managed as "shock".	IV isotonic crystalloid; *eg*, 0.9% saline or RLS 10-20 ml/kg/hr (500-1,000 ml) for 1-2 hr. If clinical status & parameters are improved, decrease the rate to 7-5 ml/kg/hr for 1-2 hr and then gradually decrease the rate. If clinical status and parameters are worsened or not improved, change to colloid solution: 5% albumin, Dextran, FFP 10 ml/kg/hr for 1 hr. If not improved, the patient should be managed as "persistent shock despite adequate crystalloid replacement".	Evaluate for other comorbidities, *eg*, condition: severe bleeding, severe sepsis, metabolic acidosis, or pneumothorax. Start vasopressor, *eg*, norepinephrine 0.1-0.2 µg/kg/min. Adjusted dosage every 10-15 min (max dose 1-2 µg/kg/min). Decrease the dose when clinical status & parameters are improved. Note: Patients with shock should have their vital signs and parameters closely monitored until resolution of shock.

2. Hypotension and/or pulse pressure ≤20 mmHg.

3. Shock.

4. Shock despite of fluid resuscitation with crystalloid solution.

The end point of treatment is to bring their hemodynamics back to normal (that is, normalized blood pressure, pulse pressure >20 mmHg, urine specific gravity 1010-1020, urine output 0.5-1 ml/kg/hr, and Hct 40-45 vol%).

For groups 1-3, the main treatment is

crystalloid solution, which has some limitation because of increasing the volume of leakage into the 3rd space, which can lead to pleural effusion, ascites, or pulmonary edema.

MONITORING THE PATIENTS DURING MANAGEMENT

1. Vital signs, peripheral perfusion and clinical status should be assessed every 15-30 minutes until the shock is resolved, and then assessed every 1-4 hr.

2. Hematocrit should be monitored 1-4 times per day according to clinical status.

3. Urine output and specific gravity should be monitored every 1 hr during unstable hemodynamics, and then monitored every 4-6 hr when the clinical status is stable.

4. Monitoring of other parameters such as oxygen saturation, acid-base balance, liver function, and renal function depends on each clinical situation.

TYPE OF FLUID

A study in Vietnamese children (Wills *et al*, 2005) found that moderately severe shock should be treated with fluid therapy using Ringer's lactate solution, and severe shock should be treated with starch over dextran. However, there is no clinical study in adult dengue infection to compare fluid management between crystalloid and colloid in volume resuscitation. Evidences comparing crystalloid and colloid for volume resuscitation in critically ill patient from other causes exist. A report for the WHO secretariat by Pablo Perel found that there is lack of effectiveness of colloid compared to crystalloid (Perel *et al*, 2013).

Also, a study of Dextran 70 in adults with septic shock showed a higher rate of severe bleeding in patients who received Dextran 70 compared with crystalloids (Hvidt and Perner, 2012). Other studies have found no significant differences in the effectiveness of crystalloid or colloid. A study in critical patients compared hydroxyl starch to saline for fluid resuscitation, and found no significant differences in mortality outcome between these 2 types of fluid (Myburgh *et al*, 2012). A recent study comparing albumin to saline in severe sepsis and septic shock also had the same result (Caironi *et al*, 2014). These recent evidences suggest that fluid resuscitation in adult dengue patients should be crystalloid. If the patient has limitation in crystalloid therapy such as developing pulmonary edema then physician should consider using colloid. Because hydroxyl starch has an increased risk of renal complications (Myburgh *et al*, 2012), and Dextran is associated with major bleeding (Hvidt and Perner, 2012), albumin or fresh frozen plasma might be considered as the colloids of choice.

VASOPRESSOR

Administration of vasopressor should be evaluated individually because they may raise blood pressure despite inadequate intravascular volume, and this leads to inadequate tissue perfusion, which can increase morbidities. However, in situation of prolonged shock in a patient who has adequate volume resuscitation or a patient who develops signs and symptoms of fluid overload such as pulmonary edema, vasopressor has a role in maintaining their perfusion pressure.

Currently, there are not any direct studies of the efficacy of vasopressors in DSS. According to the international guidelines for management of septic shock: 2012 (Surviving Sepsis Campaign), vasopressors could be applied in this situation (Dellinger *et al*, 2013). Norepinephrine should be considered as the first line agent, and vasopressin or epinephrine should be added if the patient does not respond to norepinephrine. Dopamine should be avoided because it increases risk of arrhythmia (De Backer *et al*, 2010).

WHEN TO STOP THE FLUID

During fluid therapy in the critical phase, the concern of treatment is plasma leakage, which can cause death. Then the fluid therapy should be reduced or discontinued when:

1. Patient has signs of cessation of plasma leakage.
2. Hemodynamics are stable (BP, pulse, and peripheral perfusion).
3. Hct decreases in the presence of a good pulse volume.
4. Apyrexia (without the use of antipyretics) for more than 24-48 hours.
5. Resolving bowel/abdominal symptoms.
6. Improving urine output.

Continuing intravenous fluid therapy beyond the 48 hours of the critical phase will put the patient at risk of pulmonary edema and other complications such as thrombophlebitis.

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Chapter 5.

Dengue prevention and control

INTERIM ANALYSIS OF THE CONTRIBUTION OF HIGH-LEVEL EVIDENCE FOR DENGUE VECTOR CONTROL

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Abstract. This interim analysis reviews the available systematic literature for dengue vector control on three levels: 1) single and combined vector control methods, with existing work on peridomestic space spraying and on *Bacillus thuringiensis israelensis*; further work is available soon on the use of Temephos, Copepods and larvivorous fish; 2) or for a specific purpose, like outbreak control, and 3) on a strategic level, as for example decentralization *vs* centralization, with a systematic review on vector control organization. Clear best practice guidelines for methodology of entomological studies are needed. There is a need to include measuring dengue transmission data. The following recommendations emerge: Although vector control can be effective, implementation remains an issue; Single interventions are probably not useful; Combinations of interventions have mixed results; Careful implementation of vector control measures may be most important; Outbreak interventions are often applied with questionable effectiveness.

Keywords: dengue, evidence, systematic review, vector control

INTRODUCTION

Dengue guidelines

Since the publication of the 2009 WHO dengue guidelines (WHO, 2009), new developments in dengue include further evidence for clinical management (WHO, 2013), evidence supporting the use of the 2009 WHO dengue case classification (Horstick *et al*, 2012, 2014), new epidemiological estimates (Bhatt *et al*, 2013), further developments in vector control methods and last but not least, the first clinical phase 3 trials of a dengue vaccine (Capeding *et al*, 2014; Villar *et al*, 2015) warranted an update of the guideline, which was scheduled for 2014.

During the development of the 2009 WHO dengue guidelines, the organization aimed for higher standards for guidelines, with the establishment of the "guidelines review committee" (WHO, 2014). Following the WHO handbook for development of guidelines (WHO, 2012), specifically high-level evidence is needed, including systematic reviews.

This need for systematic reviews arises from developing public health policy based on available research, including implementation and operational research; therefore, linking research and practice. Especially in the context of Neglected

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Tropical Diseases filling this gap of high-level evidence is needed (Nagpal *et al*, 2013). In dengue, however, many topics have been addressed in the last years with systematic reviews, leading often to policy recommendations, as in the case of the 2009 WHO dengue case classification, but often highlighting research gaps.

Vector control

Regarding vector control in dengue, a meta-analysis analysing all available vector control methods highlighted the efficacies of each method (Erlanger *et al*, 2008). However, for a technical meta-analysis, many studies need to be excluded from the analysis, to achieve the necessary comparability of data. Further to this, no technical standards exist for establishing efficacy and community-effectiveness of dengue vector control studies, which results in a large variability of existing studies and their respective designs; therefore, a meta-analysis will only look at a very limited amount of published studies.

The question arises, whether careful analysis of all available vector control methods, with systematic reviews, can further contribute to public health decisions on 1) the efficacy and community effectiveness of each vector control method, 2) and combinations of vector control methods, 3) identifying research gaps, and 4) practical recommendations concerning the use of vector control to reduce dengue transmission.

METHODS

This article reviews the evidence for dengue vector control, including the published and unpublished evidence of systematic reviews, considering the existing meta-analysis on dengue vector control (Erlanger *et al*, 2008). A literature search was performed on existing systematic reviews dealing with dengue vector control, also asking experts in the field for relevant high level (summary) evidence. Studies have been included if relevant to the topic (dengue vector control) and are summarized according to predefined categories, systematic reviews analysing 1) single vector control interventions with efficacy and or community-effectiveness measurements; 2) a service orientated purpose, such as outbreak control; and 3) strategic levels, such as service organization. Furthermore, implementation aspects derived from the individual systematic reviews were analysed, with a view towards practical public health recommendations.

RESULTS

Systematic reviews of single-method vector control

For single vector control interventions, there is existing work on peridomestic space spraying (Ekpereonne *et al*, 2010) and on *Bacillus thuringiensis israelensis* (Boyce *et al*, 2013). Further work is available as university master's theses, and scientific articles will soon be published on the use of Temephos, Copepods, and larvivorous fish.

For Copepods (Lazaro *et al*, 2014, submitted) 11 articles were included in the systematic review, focusing on efficacy and community effectiveness. There is limited evidence that Copepods (*Mesocyclops* spp) could potentially be an effective vector control option, as shown in 5 community effectiveness studies in Vietnam. This includes long-term effectiveness on larval

and adult control of *Aedes aegypti*, as well as human disease parameters. However, this success has so far not been replicated elsewhere (6 further studies). With this limited evidence for the use of Copepods as a single intervention, further implementation studies in other communities/environments are needed.

For larvivorous fish (Han *et al*, submited), considering 13 eligible articles, elimination of *Aedes* larvae in treated containers was shown in three efficacy studies. Further nine of the ten community effectiveness studies reported a considerable reduction in immature forms of vectors and a continuous decline over two years was observed in three studies. Two studies showed also reductions in adult mosquitoes and a fall in dengue cases after the intervention was mentioned in another two studies. The systematic review showed that the use of larvivorous fish as a single agent or in combination with other control measures produced a considerable reduction in the immature vector forms. However, the evidence to suggest community effectiveness of larvivorous fish as a single agent is limited, especially when considering study design. Further studies utilizing cluster-randomized controlled designs and incorporating the assessment of impact on dengue are recommended.

Peridomestic space spraying is one of the most commonly used dengue vector control methods, using different insecticides. The systematic review (Ekpereonne *et al*, 2010), which included fifteen studies, 13 studies showed reductions in immature entomological indices that were not sustained for long periods. The remainder showed space spray interventions to be ineffective at reducing adult and/or immature entomological indices. Only one study measured human disease indicators, but its outcomes could not be directly attributed to space sprays alone. Although peridomestic space spraying is commonly applied by national dengue control programs, there are very few studies evaluating the effectiveness of this intervention and there is no clear evidence for recommending peridomestic space spraying as a single, effective control intervention.

Twenty-nine studies were included in the systematic review on temephos (George *et al*, 2014, submitted), including 12 single intervention studies and 17 studies using temephos with other interventions (multiple interventions). All 12 single intervention studies showed consistently that using Temephos lead to a reduction of entomological indices. All 17 multiple intervention studies showed that Temephos application together with other chemical vector control methods was either not sustainable or failed to reduce the immature stages. The analysis of this study is on going, especially regarding the implementation implications of the use of multiple interventions.

Fourteen studies were included in the systematic review of Bti (Boyce *et al*, 2013); 12 reported a reduction in entomological indices with an average duration of control of between 2-to-4 weeks. One of the studies linked the reduction of entomological indices with epidemiological data, with one dengue case in the treated area compared to 15 dengue cases in the untreated area during the observed study period. With this, Bti is effective in reducing the number of immature *Aedes* in treated containers, and there is very limited evidence that dengue morbidity can be reduced through the use

of Bti alone. There is currently insufficient evidence to recommend the use of Bti as a single agent for the long-term control of dengue vectors and prevention of dengue fever.

Systematic reviews for a service orientated purpose

For the interventions focusing on a particular service delivery in the context of vector control, there is existing work on outbreak response (Pilger *et al*, 2010). Twenty-four studies showed different strategies in the organization of outbreak response emphasizing an intersectoral approach. Studies that managed the outbreak response by creating multidisciplinary response teams, including vector control teams working on a door-to-door basis, and studies that monitored and evaluated their activities, showed successful outbreak control. Combining interventions that use 1) vector control (elimination of larval habitats with community involvement; appropriate use of insecticides in and around houses), and 2) capacity training of medical personnel in combination with laboratory support were crucial for the successful control of outbreaks. Spatial spraying of insecticides alone proved ineffective in achieving outbreak control and its usefulness in combination with other interventions remains doubtful. The available evidence recommends that in order to achieve rapid control, the outbreak response must employ a multidisciplinary approach combined with monitoring and evaluation.

Systematic review of the organizational context of vector control

A systematic review on vector control service delivery (Horstick *et al*, 2010) highlighted many shortcomings about how vector control is being delivered globally. Three of nine studies on vector control services indicated that there was little change of control operations over time. The studies showed that there were however attempts towards strategic changes in decentralization and intersectoral collaboration. Staffing levels, appropriate capacity building, management and organization, sustained funding, and mechanisms for achieving community engagement were insufficient and weak, and remained key problem areas.

This systematic literature review used a mixed methods approach; also interviewing stakeholders, and case studies in four countries confirmed most of the information from the systematic review. With the stated limitations, doubts of key public health stakeholders about the effectiveness of services in reducing vector densities and significantly reducing virus transmission were widespread. But the stakeholders believed that the interventions could be effective, if the necessary resources were available.

The analysis of existing vector control services underlined the need for: 1) the development of operational standards for vector control services, including minimum financial and personnel requirements in accordance with the geographical area(s) to be covered, their demography and the vector control methods to be implemented; 2) evidence based selection and delivery of different interventions or combinations of interventions, adapted to different settings; 3) development and application of monitoring and evaluation tools for vector control service delivery; and 4) needs driven capacity building, especially in public health entomology and communication.

Crosscutting issues in all systematic literature reviews

As a crosscutting issue in this series of systematic reviews, it emerged that clear best practice guidelines for methodology of entomological studies need to be developed. Without standardization of the methodology future studies will continue producing low quality studies and non-comparable data.

Further to this, there is a need to include measuring dengue transmission data; most analysed studies do not provide such measurements.

CONCLUSIONS AND RECOMMENDATIONS

Based on the extensive work of systematic literature reviews, a list of recommendations can be drawn. These need to be seen firstly in the light of the limitations—limitations for each of the systematic literature reviews include publication bias, especially as studies not presenting positive results are often not reported. The substantial experience of dengue vector control globally from on-going national vector control programs is often not documented. However, these limitations have been addressed in each systematic literature review including a search of grey literature and including a thorough reference check of included literature. The authors have also identified further studies recommended by prominent dengue entomologists.

Until recently, not all dengue vector control methods, as they are applied in practice, have been analysed with systematic literature reviews. However, the consistency of the data emerging from the currently available studies may be a good indicator that the few remaining studies will not change substantially the main messages of this review. Furthermore, this review is not systematic in its approach, identifying all available systematic literature reviews for dengue vector control. This limitation will be addressed with a future study, once all available dengue vector control methods have been looked at with systematic literature reviews. However, looking at the seven systematic literature reviews on dengue vector control, analysed in this study, the following practical recommendations emerge:

- Although vector control can be effective, implementation remains an issue. No clear evidence exists for delivery structures of vector control services (Horstick *et al*, 2010).
- Single interventions are probably not useful, efficacy varies between the different interventions, but sustained community-effectiveness can almost never been shown (Ekpereonne *et al*, 2010; Boyce *et al*, 2013; Han *et al*, submitted; George *et al*, submitted; Lazaro *et al*, submitted).
- Combinations of interventions have mixed results related to the complexity of implementing multiple interventions (George *et al*, submitted).
- In order to be efficacious and community-effective careful implementation of vector control measures may be more important than the actual choice of the combinations of vector control methods.
- In reality, interventions are often applied in outbreaks (compared to routine vector control) although the effectiveness is also 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 measures, including health promotional campaigns.

- The development for standards for vector control studies is urgently needed
- Studies should attempt to include measuring dengue transmission for an ultimate proof of efficacy and community-effectiveness of dengue vector control.

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DENGUE VACCINES

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Abstract. The uniqueness of the dengue viruses (DENVs) and the spectrum of disease resulting from infection have made dengue vaccine development difficult. Several vaccine candidates are currently being evaluated in clinical studies. The candidate currently at the most advanced clinical development stage, a live-attenuated tetravalent vaccine based on the chimeric yellow fever-dengue virus (CYD-TDV), has progressed to Phase 3 efficacy studies. Several other live-attenuated vaccines, as well as subunit, DNA, and purified inactivated vaccine candidates are at earlier stages of clinical development. Additional technological approaches, such as virus-vectored and Virus-Like Particles (VLP)-based vaccines are under evaluation in preclinical studies.

Keywords: dengue, vaccine

INTRODUCTION

Dengue is a mosquito-borne flavivirus disease, which is currently an expanding global health problem. Four closely related viruses, the dengue viruses 1-4, cause the disease. In the Asia-Pacific region, dengue is a serious and growing threat to public health, and this region bears nearly 75% of the current global dengue burden. Specific antiviral medications are not available for dengue. Prevention using vector control has had only limited success. There is no specific dengue therapeutics, and prevention is currently limited to vector control measures. Despite dengue control programs, case management guidelines and surveillance efforts, dengue virus transmission remains high, and prevention remains a public health priority. Development of an effective dengue vaccine would, therefore, represent a major advance in the control of the disease and is considered a high public health priority. While a licensed dengue vaccine is not yet available, the scope and intensity of dengue vaccine development has increased dramatically in the last decade, with the lead candidate currently in Phase III clinical trials. Dengue vaccine may be the major means to effectively control dengue with the high feasibility of a dengue vaccine (Thisyakorn, 2014).

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DEVELOPMENT OF DENGUE VACCINES

The first dengue vaccines were evaluated in 1929 (Thisyakorn and Thisyakorn, 2014). Development of safe and effective dengue vaccines faces many challenges.

The pathogeneses of dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) are not clearly understood. One debated hypothesis concerning virus virulence is the immune enhancement hypothesis (Prommalikit *et al*, 2004). Although evidence suggests that dengue disease severity may be associated with genetic differences in dengue strains, virus virulence has been difficult to measure because of the lack of *in vivo* and *in vitro* models of disease (Prommalikit and Thisyakorn, 2015). Infection by one of the four dengue virus serotypes has been shown to confer lasting protection against homotypic re-infection, but only transient protection against a secondary heterotypic infection, which may lead to an increased risk of severe disease. Due to these dengue-specific complexities, vaccine development focuses on the generation of a tetravalent vaccine aimed at providing long-term protection against all 4 dengue virus serotypes. Additional challenges are posed by the lack of an adequate animal disease model and the resulting uncertainty around correlates of protection. In spite of these challenges, vaccine development has made remarkable progress in recent years, and the current dengue vaccine development is advanced, diverse, and overall promising (Thisyakorn and Thisyakorn, 2014).

A tetravalent dengue vaccine must be developed without the benefit of a full understanding of the pathogenesis of severe dengue disease or an adequate animal disease model. The concerns of disease enhancement and limited research funding for a disease that primarily affects the developing world limit dengue vaccine development. However, in the last decade, the efforts to develop dengue vaccines have increased dramatically due to an increased awareness of the dengue pandemic and the development of new molecular techniques. At present, several dengue vaccines have been tested in human clinical trials, and a single candidate is now in phase III clinical trial (Table 1).

DIFFERENT APPROACHES IN DENGUE VACCINE DEVELOPMENT

Live attenuated virus

The first major effort for live attenuated dengue vaccine development began at the University of Hawaii using the traditional method of serial passage of virus in non-human host, and then it was transferred to Mahidol University in Bangkok, Thailand for further passage and development of candidate vaccines and testing (Thisyakorn and Thisyakorn, 2014). The candidate vaccine was used for Phases I and II clinical trials in Thai adults and children. Not all of the volunteers seroconverted to all four dengue serotypes, and some showed unacceptable reactogenicity. Consequently, further clinical testing was stopped. Although the development of this candidate vaccine was not successful, the initiative was responsible for the subsequent progress that has been made in developing a tetravalent dengue vaccine.

The second tissue-culture-passaged dengue vaccine was developed at the Walter Reed Army Institute of Research (WRAIR). The WRAIR-produced tetravalent dengue vaccine formulation also showed problems of unbalanced immunogenicity and reactogenicity. New formulations seemed to be safe and immunogenic in a phase II study; however, the protective efficacy needs to be further

Table 1
Development of dengue vaccines.

Type of vaccines	Dengue virus genes (N)	Stage of development
Live attenuated virus (traditional)	10	Phase II tetravalent (WRAIR & GSK)
Live attenuated virus (molecular)	10	Phase II monovalent (USNIH) Phase I tetravalent (USNIH) Protects nonhuman primates (USFDA)
Yellow fever chimera	Chimera 2+8 yellow fever virus	Phase III tetravalent (SP)
Dengue chimera	Chimera 2+8 DEN-2	Phase II tetravalent (Inviragen)
Purified inactivated	3	Protects monkeys (GSK, WRAIR)
Recombinant subunit	< 1	Phase I DENV1 (Hawaii Biotechnology)
DNA	2 +	Protects monkeys (Naval Medical-Research Center and Maxygen)

Source: Halstead and Thomas (2013).

evaluated. Further testing has been delayed by manufacturing complexities and the determination of the optimal dose and schedule of the vaccine.

The US National Institutes of Health introduced a new era of dengue vaccine research with direct mutagenesis technology. Dengue and other flavivirus genomes were readily altered genetically, resulting in attenuated variants while US Food and Drug Administration created a molecularly attenuated dengue vaccine. Both techniques provide an alternative approach to constructing a live attenuated tetravalent dengue vaccine.

There are several important safety issues for live dengue vaccines. Principal among these concerns is the theoretical risk of enhanced disease following dengue vaccination. The rationale for a tetravalent vaccine is the perceived requirement to simultaneously induce primary-type immune responses to all four dengue viruses. The simultaneous production of neutralizing antibodies specific to each of the four dengue viruses is predicted to minimize the risk of disease enhancement following natural infection. However, antibody-dependent enhancement appears to occur with neutralizing antibodies at sub-neutralizing concentrations, so a vaccine that induces protection for a period of time might later increases the risk for enhanced disease.

This is particularly a concern for vaccines that induce low levels of neutralizing antibodies, but enhanced disease might occur with any vaccine given enough time. To adequately assess this risk, the risk of incomplete immunization, and waning antibody titers, dengue vaccine clinical development plans must include flavivirus-primed and flavivirus naïve volunteers and sufficiently long-term follow-up to make statistically powered conclusions regarding the safety of dengue vaccination in flavivirus-endemic areas. However, neither live attenuated nor live chimeric dengue vaccines inoculated into dengue-immune

children or adults have resulted in enhanced disease caused by a vaccine virus.

Other safety concerns with live attenuated virus vaccines include cell-culture-derived adventitious agents, community spread of the vaccine virus by resident vector mosquitoes, vaccine virus neurovirulence, and the effects of vaccine administration to immunocompromised hosts. Numerous dengue vaccine developers have also performed risk assessments of vector transmission by vaccine recipients. Results of published studies indicate a very low likelihood that a vaccine could transmit vaccine-derived dengue viruses to a mosquito.

Molecular clone-based strategies for a tetravalent dengue vaccine offer important advantages over traditional attenuation in cell culture. These include a reduced risk of adventitious agents, which will also reduce product quality assurance costs, and a molecular explanation for attenuation. Interference observed when mixtures of four dengue viruses are inoculated in susceptible human volunteers must also be studied in genetically modified vaccine viruses.

Chimeric virus

Chimeric dengue vaccine viruses can be derived by inserting serotype-specific dengue antigen genes into a single attenuated dengue genomic construct. A different approach was taken to insert dengue structural genes into the infectious cDNA backbone of the well-established yellow fever vaccine virus strain 17D. This was started at Washington and St Louis University Medical Schools. These yellow fever chimeras are being further developed commercially by Acambis, Inc and are licensed for manufacture to Sanofi Pasteur. Vero cells serve as the substrate for vaccine virus production.

The first Phase III efficacy trial for a recombinant, live, attenuated tetravalent dengue vaccine (CYD-TDV) in highly dengue-endemic area in 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 contribution of each of the 4 serotypes, and more than 80% of severe dengue episodes were avoided with a two-thirds reduction in hospitalization. Higher efficacy was observed in the immunogenicity subset seropositive at baseline.

The safety profile was consistent with the good safety profile observed in previous studies. Over the 25-month follow-up period, no evidence of antibody dependent enhancement in partially or completely vaccinated individuals was observed. The interesting finding of this trial was 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; Wilder-Smith, 2014).

A second Phase III clinical trial done in Latin American countries in 20,875 children and adolescents aged 9-16 years demonstrated a 60.8% (95% CI: 52.0-68.0) overall efficacy with the contribution of each of the 4 serotypes. Additional observation of the results showed a significant reduction of the risk of hospitalization by 80.3%. Higher efficacy was observed in the immu-

nogenicity subset seropositive at baseline. The safety profile was consistent with the good safety profile observed in previous studies, showing no evidence of antibody dependent enhancement in partially or completely vaccinated individuals. Results confirm the potential public health impact of the vaccine and support the vaccine's potential to reduce the public health burden of dengue. It should be recognized as the dawn of a new era of dengue control, because the potential use of this vaccine could be a major turning point for global dengue control. The interesting finding of this trial was that vaccine efficacy was higher for participants who were seropositive for dengue than for those who were seronegative (Dengue Vaccine Initiative, 2014). The results from Latin America complement those in Asia and provide a more global picture of the vaccine's potential to contribute to reaching the 2020 WHO target of reducing the global burden of dengue by decreasing morbidity by 25% and mortality by 50% (WHO, 2012; Thisyakorn *et al*, submitted).

Inactivated virus

Inactivated whole virus vaccines have two advantages: they cannot revert to a more pathogenic phenotype, and they are unlikely to interfere with each other in combination. Moreover, induction of cell-mediated and humoral immune responses has been demonstrated with inactivated flavivirus vaccines. Conversely, inactivated vaccines express only the part of the viral genome that encodes structural proteins. In the context of dengue immunity and immunopathology, raising antibodies that are not fully protective may lead to breakthrough infections or enhance infections with wild-type dengue viruses. Other potential disadvantages of these vaccines are the cost per dose and their usual requirement for multiple immunizations.

Newer complex adjuvant systems have been shown to mediate long-lasting antibody response. Two, AS03 and AS04, have been incorporated into vaccines licensed for human use and are being explored by the WRAIR/GSK/Oswald Cruz Foundation Killed Dengue Vaccine Initiative.

Subunit vaccines

Recombination subunit approaches offer the advantages of anticipated minimal reactogenicity, freedom from adventitious agents, and low cost. However, incomplete post-translational processing of proteins can lead to proteins that differ from native proteins and antibody responses. Production in mammalian cells may reduce some of these concerns. A Phase I study to assess the safety and tolerability of a DEN-1 candidate in healthy US adults has been completed and the publication of the results is pending. Risk of enhanced disease upon exposure to wild-type viruses post-vaccination would need to be assessed as for all other approaches to dengue vaccines. Vaccines that elicit a cytotoxic T-cell memory response may lower this risk.

DNA vaccines

Dengue DNA vaccines offer a possible method to raise protective immunity, which bypasses the problem of interference seen with multivalent live virus vaccines. DNA vaccines are composed of a plasmid or plasmids containing dengue genes. These are reproduced to a high copy number in bacteria such as *E. coli.* The plasmid contains a eukaryotic promoter and termination

sequence to drive transcription in cells after being inoculated into a vaccine recipient. The transcribed RNA is translated to produce proteins that are processed and presented to the immune system in the context of the host's own MHC molecules. New genes such as intracellular trafficking and immunostimulatory sequences can be added to the plasmid, producing expressed antigens, which lead to B- and T-cell responses.

In theory, DNA vaccines afford numerous advantages over conventional vaccines, including ease of production, stability and transport at room temperature, the ability to add new genes to the vaccine, and the possibility of vaccinating against multiple pathogens in a single vaccination with reduced reactogenicity.

Tetravalent DNA vaccines have been created by shuffling the envelope genes from the four dengue serotypes and transfecting the resulting chimeric genes into human cells by DNA plasmids. The transfected human cells were then incubated with type-specific dengue antibodies and subjected to flow cytometry. Antibody markers permitted rapid screening of libraries and identification of novel expression of C-terminal truncated antigens that combined envelope and pre-membrane epitopes from all four dengue serotypes when inoculated in mice and monkeys that had successfully raised neutralization antibodies. Monkeys resisted challenge with DEN-1 but not DEN-2.

A DENV-1 DNA vaccine was evaluated in flavivirus-negative volunteers with a three-dose series at day 0, and at 1 and 5 months. None of the low-dosage recipients and half of the high dosage recipients developed neutralizing antibodies. More recently, protection has been achieved in a rhesus monkey model by boosting tetravalent DNA vaccination with a tetravalent live attenuated dengue vaccine.

The DNA approach also carries unique risks. The first is the theoretical risk of nucleic acid integration into the host's chromosomal DNA to potentially inactivate tumor suppressor genes or activate oncogenes. This risk appears to be well below the spontaneous mutation frequency for mammalian cells. However, if a mutation due to DNA integration is a part of a multiple hit phenomenon leading to carcinogenesis, it could take many years for this problem to become evident. Another concern is that foreign DNA may induce anti-DNA antibodies leading to autoimmune diseases such as systemic lupus erythematosus. However, to date, studies on lupus-prone mice, normal mice, rabbits, and people have not validated this concern (Thisyakorn and Thisyakorn, 2014).

Although no licensed dengue vaccine is yet available, the increasing knowledge of dengue vaccine development is providing more insights into improved vaccine design. Several promising dengue vaccine candidates are in preclinical and clinical development, and one is moving into Phase III testing. If the safety concerns can be surmounted, economic forces and technologic advances should soon bring one or more dengue vaccines into the market. It remains for the vaccine community to develop and implement plans for the strategic use of dengue vaccines by developing evidence-based policies to target high risk groups and decrease virus transmission.

Early preparation and understanding

of the true burden of disease will be essential for successful vaccine introduction and, with this in mind, the ASEAN Member States Dengue Vaccination Advocacy Steering Committee (ADVASC) convened a Regional Workshop to review the current status of dengue surveillance and diagnostics in the ASEAN Region (Thisyakorn, 2012). The ADVASC have recommended an evidence-based approach to strengthening and harmonizing key attributes of dengue surveillance, including case classification, data collection, data analysis and laboratory testing. Strengthening vaccination policy will require further investment in existing health systems, and recommendations for research and advocacy are also outlined here (Thisyakorn *et al*, submitted).

CONCLUSION

Dengue virus is the causative agent of a wide spectrum of clinical manifestations, ranging from mild acute febrile illness to classical dengue fever, DHF, and DSS. DHF and DSS are the potentially fatal forms of dengue virus infection, which has become an intractable global health problem.

Vector control has achieved only limited success in reducing the transmission of dengue and there are currently no licensed antivirals to treat dengue. The most effective way to control dengue diseases in the future will be through the use of a safe and effective vaccine. Dengue is a unique and complex disease; developing a dengue vaccine has proven equally complex. Although no licensed dengue vaccine is yet available, several vaccine candidates are under development, including live attenuated virus vaccines, live chimeric virus vaccines, inactivated virus vaccines, and live recombinant, DNA and subunit vaccines. The CYD-TYD live attenuated dengue vaccine, being developed by Sanofi Pasteur, has demonstrated clinical efficacy and a good safety profile.

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Chapter 6. Adult Dengue

Adult dengue cases

FATAL RHABDOMYOLYSIS IN DENGUE HEMORRHAGIC FEVER: A CASE REPORT

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Abstract. Dengue hemorrhagic fever is caused by dengue virus infection. The classical manifestations consist of fever, thrombocytopenia, and hemoconcentration. However, its unusual complications may be fatal, such as prolong shock, massive bleeding, volume overload, and unusual manifestations, for example, severe rhabdomyolysis. Here we report a case of 17-year old Thai man who was referred to our hospital because of 7-day fever with thrombocytopenia, hemoconcentration and right pleural effusion. The serology tests confirmed to be dengue infection. He developed various complications: severe hepatitis, coagulopathy, and heavy proteinuria; encephalopathy that needed a respiratory ventilator. On day 12 of fever, he had myalgia and passed dark urine. Serum creatinine and serum creatinine phosphokinase (CPK) were found abnormally high. He was diagnosed as severe rhabdomyolysis with acute kidney injury, and immediate hemodialysis was performed. He did not respond to treatment and expired within three hours. Although the mechanism of severe rhabdomyolysis in dengue fever is not clearly known, it may theoretically be proposed such as direct muscle cell injury leading to myositis by dengue virus, myotoxic cytokines which are produced in response to viral infection, dehydration or hypophosphatemia.

Keywords: dengue hemorrhagic fever, fatal rhabdomyolysis

INTRODUCTION

Dengue infection is the very common viral infection in tropical areas particularly in rainy season. Mosquitoes, mainly *Aedes aegypti*, transmit it. Its clinical manifestations include dengue fever, dengue hemorrhagic fever, and dengue shock syndrome. In the case of classical dengue hemorrhagic fever, there are three phases: febrile, toxic, and recovery, as follows:after having acute fever for 3-5 days, the patients will develop thrombocytopenia due to immune complex including platelet destruction and hemoconcentration due to the leakage of the intravascular volume during the toxic phase. Before accessing the recovery phase within 1-3 days, the minority of the patients may develop the unusual or severe complications such as renal failure, acalculous cholecystitis, conduction abnormalities (Nimmagadda *et al*, 2014), dengue shock syndrome, dengue encephalopathy (Hendarto and Hadinergoro, 1992), acute hepatic failure, severe pancreatitis (Jain *et al*, 2014) or fatal rhabdomyolysis (Karakus *et al*, 2007).

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Rhabdomyolysis or lysis of the striated muscle cells is characterized by the dark urine due to myoglobinuria, weakness of muscle, myalgia, high serum creatine phosphokinase (CPK), and possibly renal failure. It can result from various factors, such as drugs, severe strenuous exercise, or viral infections, for example, influenza, HIV, coxsackievirus, and CMV. The report of rhabdomyolysis in patient with dengue fever has been occasionally reported (Sargeant *et al*, 2013). Herein, we report a case of proven dengue hemorrhagic fever complicated by fatal rhabdomyolysis.

CASE REPORT

A 17-year old Thai man was referred to Maharat Nakhon Ratchasima Hospital because of confusion for six hours. Seven days before the referral, he had high-grade fever with poor appetite, and two days later, he was found to have low platelet (100,000/mm^3); the diagnosis of dengue fever was proposed, and fluid therapy was cautiously initiated at the community hospital. The platelet was followed daily and found to decrease until it finally reached 27,000/mm^3. The hematocrit rose from 45% to 55%, and the patient started becoming confused on the day of referral. The physical examination on the day of admission, day 7 of fever was as follows: blood pressure, 126/79 mmHg; pulse 89, beats/min; body temperature, 38.0^oC; and respiratory rate, 30 breaths/min. He looked drowsy with poor compliance, and he had mild jaundice, decreased breath sounds with dullness on percussion at the RLL field, stiffness of neck, no hepatosplenomegaly, and no petechiae.

Investigations on the 1st day of admission, day 7 of fever: a hematocrit of 55.3%, a white blood cell count of 10,000 cells/mm^3 with 52% neutrophils, 23% lymphocytes, 12% monocytes, 13% atypical lymphocytes and a platelet count of 9,000/mm^3. Blood chemistry revealed 21.1 mg% of BUN, 1.16 mg% of creatinine, 2.6 mg% of total bilirubin, 1.5 mg% of direct bilirubin, 3,823 IU/l of AST, 1,657 IU/l of ALT, 69 IU/l of ALP, 2.9 g% of albumin, 2.3 g% of globulin, 7.2 mg% of Ca, 1.0 mg% of P (normal 2.7-4.5).

Further investigation results were: negative for dengue virus antigen (immunochromatography),weakly positive for dengue IgM, positive for dengue IgG, negative for leptospira IgG and IgM, less than 1:50 for scrub and murine typhus IgG and IgM, negative for *O. tsutsugamushi*, HBsAg, anti-HCV or anti-HIV, no growth for hemoculture.

Urinalysis found a specific gravity of 1.025, protein 4+, sugar-negative, blood (RBC, Hb or myoglobin) 3+, ketone-negative. Coagulogram showed: PT 21.4 seconds (normal 8.9-15.1), INR 1.82, aPTT 79.1 seconds (normal 21.9-34.5), and TT >120 seconds.

A chest x-ray film showed moderate amount of right pleural effusion, no cardiomegaly, or pulmonary congestion. The computerized tomography of the brain showed diffuse brain swelling.

He was definitely diagnosed as dengue hemorrhagic fever with right pleural effusion. Other possible diagnoses included dengue or hepatic encephalopathy, coagulopathy, hypocalcemia with hypophosphatemia, and heavy proteinuria with hypoalbuminemia.

The treatment was immediately started

with fresh frozen plasma 100-200 ml/hr, platelet concentrate transfusion, parenteral fluid, and meropenem 2g every 8 hours. The hypocalcemia and hypophosphatemia were corrected. The blood pressure and urine output could be maintained, but his impaired consciousness was not improved; finally, his respiration was supported with ventilator.

On days 9-11 of fever, with fully supportive treatments, he still had high-grade fever, confusion, occasionally shiver, and urine output was adequate, although the urine color was dark. Hematocrit and platelets could be kept between 36%-45% and 9,000-40,000/mm^3, respectively.

On day 12, he developed more confusion with concurrent fever, creatinine level rose from 1.16 to 3.93 mg%, and CPK 151,760 U/L. The chest film showed pulmonary congestion without cardiomegaly. The diagnosis of acute renal failure as well as severe rhabdomyolysis was established, and emergency hemodialysis on double lumen at the right femoral vein was immediately performed. Three hours after dialysis, he became more comatose, developed tachypnea, hypotension, and ventricular tachycardia, and did not respond to inotropic drugs, and finally expired. The autopsy was not allowed.

DISCUSSION

Our case was diagnosed as secondary dengue infection, based on the clinical syndrome of acute fever for a few days, with subsequent thrombocytopenia, hemoconcentration and right pleural effusion and immunologically confirmed by the positive tests of dengue IgM and IgG antibodies (CDC, nd). In addition, he developed unusual but severe complications, for example, severe hepatitis (ALT >300 U/L) (Parkash *et al*, 2010), coagulopathy without clinical bleeding symptom, acute encephalopathy, heavy proteinuria, severe rhabdomyolysis, acute kidney function impairment, and finally death. All of these derangements may possibly be due to the direct involvement of the virus, because the dengue virus antigen can be demonstrated in the kidney, liver, heart, lung, and spleen in cases of fatal dengue infection (Póvoa *et al*, 2014).

Rhabdomyolysis has been occasionally reported in cases of dengue fever. Although, the exact mechanism is not known, many theories are proposed, for instance, rhabdomyolysis is the direct effect of the dengue virus itself, because it shares several features with other viruses that are well-known causes of severe myositis and finally rhabdomyolysis, such as influenza A and B, coxsackievirus, Ebstein barr virus (EBV), and HIV, although the dengue virus has not been demonstrated in muscle cell, thus far. Other possibilities may be myotoxic cytokines, especially the tumor necrosis factor and the interferon alpha (Sargeant *et al*, 2013) that generally respond to viral infection.

Rhabdomyolysis can happen in case of dehydration, our case runs quite longer course than usual. In general, acute febrile phase of dengue fever lasts for two to seven days (Chaturvedi and Nagar, 2008), and toxic phase usually takes 1-3 days. During toxic phase, the main pathogenesis is the fluid leakage leading to hemoconcentration and right pleural effusion and the fluid is needed to keep enough intravascular volume and urine output.

In case of hypophosphatemia (serum

phosphate < 2.0 mg%), rhabdomyolysis can also commonly occur. although it has been corrected, but severe hypophosphatemia can be masked because of the ongoing rhabdomyolysis (Singhal *et al*, 1992).

Most patients with dengue fever complicated by rhabdomyolysis always have myalgia and dark urine as the warning symptoms. And, they may subsequently develop renal failure due to concentrated myoglobinuria interacting with Tamm-Horsfall protein, leading to the high mortality rate (around 29%) (Sargeant *et al*, 2013). If a dengue fever patient is complicated by azotemia and heavy proteinuria without rhabdomyolysis, he should generally have self-resolution (Hutspardol *et al*, 2011).

In summary, a 17-year old man presented with a high-grade fever for a few days; he developed subsequent thrombocytopenia, hemoconcentration, and right pleural effusion. The diagnosis of dengue infection was confirmed with serology tests. During the toxic phase, he developed severe hepatitis, coagulopathy, heavy proteinuria, and encephalopathy followed by severe rhabdomyolysis with acute kidney injury. The immediate hemodialysis was performed, but we could not save the patient's life. The only warning symptom of this fatal complication was dark urine.

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DENGUE INFECTION IN PREGNANCY

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Abstract. While dengue infection is still on the increase in adults in Thailand, it also affects pregnant women, especially pregnant teenagers. This study was designed to investigate dengue infection during pregnancy**.** Seven cases of dengue infection in pregnant women were admitted to Ban Pong Hospital, Ratchaburi, Thailand, between 2008 and 2012. Dengue infection presented in all pregnancy trimesters. There were two severe cases: one was dengue hemorrhagic fever in the first trimester, and the second was at a critical stage of the infection during labor. There were three cases of abortion. These three cases included one complete, one incomplete, and one threatened abortion, with rising hematocrits of 22.8%, 17.1%, and 14.7%, respectively. Two out of the three teenage pregnancies experienced complete and threatened abortions, while the third abortion case was a threatened abortion pregnancy at the critical stage of infection during intrapartum. Leukopenia was identified in six out of seven women. Low baseline hematocrit and low maximum hematocrit were laboratory findings. Clinical management involved administration of intravenous fluids and antipyretics. Favorable outcomes can be obtained through early diagnosis and supportive treatment. The morbidity profile can be more serious in teenage pregnancies. Additional studies should be conducted to establish whether low baseline hematocrit, low percentages of rising hematocrit in pregnant women with dengue infection, and abortions (with a high degree of increasing hematocrit during the critical stage of the disease) are typical clinical signs.

Keywords: dengue infection, HELLP, pregnancy, teenage, Thailand

INTRODUCTION

Dengue infection is a major public health problem in tropical and subtropical countries. This mosquito-borne viral disease is a potential threat to 50-100 million people each year. Of those infected, about 500,000 develop dengue hemorrhagic fever (DHF), and 22,000 die of the disease (Thisyakorn and Pengsaa, 2012). Dengue infection severity is variable, ranging from mild, non-specific febrile illnesses to classic dengue fever (DF) or DHF and dengue shock syndrome (DSS), which are more severe forms of the disease. DF, an acute febrile disease, frequently presents with headache, bone, joint and muscle pain, rash, and leukopenia. In contrast, DHF is characterized by four major clinical manifestations: high fever, hemorrhagic

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phenomena (often with hepatomegaly), and signs of circulatory failure in severe cases. DHF patients may develop hypovolemic shock (known as DSS) resulting from plasma leakage; this can be fatal (WHO, 1997; CDC, 2013). Currently, neither a vaccine nor specific antiviral therapy exists.

Dengue infection is most often encountered in children. However, more recently, dengue infection in Thailand has started to affect people over 10 years of age as well as adults. In Thailand, the modal age group affected by dengue has shifted from <10 years of age to 10-34 years of age. In 2012, 79,593 cases of dengue infection were reported in 76 Thai provinces. The highest proportion of cases by age group was found in 15-24 year olds (23.79%, *n*=18,923), followed by 10-14 year olds (21.59%, *n*=17,182), 7-9 year olds (11.85%, *n*=9,432), and 25-34 year olds (9.86%, *n*=7,853) (Department of Disease Control, 2012).

Dengue infection can appear in pregnant women at any time as well as in the immediate post-partum period (Waduge *et al,* 2006). These infections can have various complications, including maternal mortality, preterm delivery, fetal death, low birth weight, neonatal admissions, fetal anomalies, and spontaneous abortion. In East Sudan, dengue infection during pregnancy has poor maternal and prenatal outcomes (Ishag *et al,* 2010). In this Sudanese region (2008-2009), 17 (21.7%) maternal deaths, 14 (17.9%) of the 78 women with preterm deliveries, and 19 (24.3%) admissions of neonates to neonatal intensive care units were reported. Additionally, 19 (24.3%) women gave birth to low birth weight babies, and there were seven (8.9%) perinatal deaths. Furthermore, because of the presence of various obstetrical indicators, eight (10.2%) patients were delivered by Caesarean section (Ishag *et al,* 2010).

During normal pregnancy, physiological changes to the cardiovascular system and blood increase the cardiac output, heart rate, and stroke volume while slightly decreasing the diastolic blood pressure (Ouzounian and Elkayam, 2012). These normal signs could be misinterpreted as plasma leakage and hypotensive shock caused by dengue infection. Some of the overlapping clinical and/or laboratory features of dengue infection in pregnancy can be confused with a diagnosis of pre-eclampsia, eclampsia, or HELLP syndrome (hemolysis, elevated liver enzymes, and low platelet count).

Despite the high prevalence of dengue infection in Thailand, there have been few reported cases of this infection in pregnant women. This study was conducted to investigate pregnancy-related dengue infection in an area of high prevalence.

MATERIALS AND METHODS

This descriptive retrospective study was conducted at Ban Pong Hospital, Ratchaburi, Thailand, which is a dengue endemic area. All hospitalized pregnant women with dengue infection were included in the study, which took place from 2008 Jan 1 to 2012 Dec 30. Diagnoses and classification of dengue infection as DF, DHF, and DSS were made according to WHO (1997) criteria. Maternal clinical signs, laboratory findings, pregnancy outcomes, and information concerning complications in mothers and fetuses were examined. The study was approved by

the Ethics Committee for research involving human subjects at Ban Pong Hospital (Registered Nº R 2014-001). The patients provided written informed consent.

RESULTS

There were 11,690 deliveries and 2,829 dengue infections at Ban Pong Hospital during the five-year study period. The seven pregnancy-related dengue infection cases had no associated mortality. Three cases of dengue infection were teenagers (14, 16, and 19 years old), and four were adult pregnant women (27, 32, 33, and 34 years old). The women's ages, gestational ages, clinical manifestations, laboratory data and pregnancy outcomes for expectant mothers with dengue infection are shown in Table 1. Infection occurred in three women in the first trimester at 5, 10, and 12 weeks of gestational age, two women in the second trimester at 14 and 26 weeks, and two women in the third trimester at 33 and 38 weeks. There were two severe dengue cases: the first was DHF in the first trimester, and the second was when labor occurred at the critical stage of the infection. Both cases were teenaged women.

The clinical manifestations in all cases were fever (duration 3-5 days) and myalgia whereas bleeding episodes such as epistaxis and petechia were less common. Tourniquet tests were done in four cases, of which three cases were positive.

Complete blood counts in all seven cases revealed low white blood cell counts of 2,100, 4,900, 3,110, 6,700, 4,980, 1,680, and 3,500/µl of blood while the thrombocytopenia values were 74,100, 84,400, 95,800, 73,200, 26,100, 83,200, and 39,000 / µl of blood. Hematocrits were low baseline (35%, 35%, 29%, 35%, 30%, 35%, and 34%) and low maximum (43%, 41%, 33%, 37%, 32%, 37%, and 39%). The maximal percentages of rising hematocrit during the critical stage of the disease were 22.8%, 17.1%, 13.8%, 5.7%, 6.7%, 5.7%, and 14.7%. The first and the seventh cases had dengue virus-positive IgM rapid test results; the other cases were not tested.

Of the pregnancy outcomes of the four women who carried infants of 20 weeks or less gestation, two out of four had complete and incomplete spontaneous abortions, while one had a threatened abortion. All three abortion cases had high maximal rising hematocrit percentages during the critical stage of the disease: 22.8% (for the complete abortion), 17.1% (for the incomplete abortion), and 14.7% (for the threatened abortion). The times from onset of fever to abortion in the three cases were 8 weeks (for the complete abortion), 3 weeks (for the incomplete abortion) and 1 day (for the threatened abortion). Four out of seven cases, including the threatened abortion, progressed to normal deliveries with healthy full-term neonates.

Two of the three teenaged pregnant women with dengue infections had complete and threatened abortions; whereas, the other infection was intrapartum at the critical stage of infection, so a referral to a tertiary care hospital was necessary.

The clinical management of the pregnant women with dengue infection included supportive care, rest, intravenous fluids, and antipyretic medication. None of patients required platelets or other blood components except for one patient whose critical stage of infection occurred during intrapartum. Because she may have needed

a blood transfusion, she was referred to a tertiary care hospital.

DISCUSSION

From 2008 to 2012, dengue infection was present in both teenaged and adult pregnant women admitted to Ban Pong Hospital, Thailand. Three out of seven cases were teenaged pregnant women. Over the last decade, dengue infection has started to affect people over 10 years of age (Department of Disease Control, 2012). Concurrently, the number of teenage pregnancies in Thailand has increased (Prohmmo, 2007). At delivery, 94.7% of the Thai pregnant women had dengue HAI antibodies, and a mother's age was the only risk factor associated with dengue infection, because older mothers were significantly more likely to be seropositive than younger ones (Perret *et al,* 2005). The seropositivity rate for dengue infection increases with advancing maternal age, indicating that younger women are more at risk of contracting dengue infection during pregnancy. These three factors (older susceptible age group, higher numbers of teenage pregnancies, and low antibody seropositivity in younger women) are likely to have increased the numbers of pregnant women with dengue infection although teenagers tend to experience more severe effects than older women do.

Dengue infection can appear at any time during pregnancy and the intrapartum period. In Sri Lanka (2000-2004), 26 patients were reported with dengue-associated pregnancies. One (3.8%), 2 (7.7%), and 20 (77%) presented in the first, second, and third trimesters, respectively, as well as 3 (11.5%) in the immediate postpartum period (Waduge *et al*, 2006). In the present study, the dengue infections occurred mainly during the first trimester. The clinical signs and symptoms experienced by the seven pregnant women with dengue infection (fever of 3-5 days duration, myalgia, nausea and vomiting, epistaxis, and petechiae) were similar to those of non-pregnant women with this infection.

Leukopenia was identified in all the cases except for Case 4, who was 38 weeks pregnant and had a normal white blood cell count during the intrapartum period (Table 1). During the febrile phase of dengue fever in non-pregnant women, a leukopenia (white blood cell count below 5,000/µl) indicates that the fever will likely dissipate within the next 24 hours and that the patient is entering into the critical stage of the disease (CDC, 2013); however, in normal pregnancy, a modest leukocytosis is observed. The normal white blood cell count ranges during the first, second, and third pregnancy trimesters and in non-pregnant adults were 5.7-13.6, 5.6-14.8, 5.9-16.9, and 3.5-9.1 ($\times 10^3$/µl), respectively (Abbassi-Ghanavati *et al*, 2009). Therefore, pregnant women presenting with febrile illness after travelling to or living within a dengue-endemic area who have significantly decreased white blood cell counts (leukopenia) compared with those of normal pregnancies warrant further investigation. Careful monitoring of infection indicators in pregnant women suspected of having dengue infections is essential.

Thrombocytopenia was present in all the case studies. Assuming that the earliest abnormality found in a complete blood count is a progressive decrease in the total white cell count followed by progressive thrombocytopenia, an obstetrician should

Table 1
Dengue infection in pregnant women.

Case number	1	2	3	4	5	6	7
Diagnosis (WHO, 1997)	DHF	DF	DF	DF	DF	DF	DF
Age (years)	14	32	33	16	34	27	19
Gestational age (weeks)	12	14	33	38	26	5	10
Fever	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Duration of fever (days)	5	3	3	3	3	3	5
Tourniquet test	No data	Positive	Positive	No data	No data	Positive	Negative
Petechiae	No	No	No	Yes	Yes	Yes	No
Epistaxis	No	No	Yes	No	Yes	No	No
Nausea/Vomiting	Yes	No	Yes	Yes	No	Yes	Yes
Myalgia	Yes	Yes	Yes	Yes	Yes	Yes	Yes
White blood cell (/μl) min	2,100	4,900	3,110	6,700	4,980	1,680	3,500
Platelet count (/μl) min	74,100	84,400	95,800	73,200	26,100	83,200	39,000
Hematocrit (%)							
max	43	41	33	37	32	37	39
min (baseline)	35	35	29	35	30	35	34
Rising hematocrit (%)	22.8	17.1	13.8	5.7	6.7	5.7	14.7
Serological IgM rapid test	Positive	No data	No data	No data	No data	No data	Positive
Blood component transfusion	None	None	None	No data	None	None	None
Outcome of pregnancy	Complete abortion	Incomplete abortion	Normal labor: healthy baby	Referral	Normal labor: healthy baby	Normal Labor: healthy baby	Threatened abortion/ Normal labor: healthy baby
Duration from diagnosis to outcome of pregnancy (weeks)	8	3	7	Referral	12	35	1 day/ 30 weeks

DF, Dengue fever; DHF, Dengue hemorrhagic fever.

be alert to the likelihood of dengue infection.

Among the seven reported cases, low baseline hematocrit and low maximum hematocrit were present. Three out of four cases with pregnancies of 20 weeks or less had complete (*n*=1), incomplete (*n*=1) spontaneous abortions, and a threatened abortion (*n*=1); these cases had high maximal rising hematocrit values during the critical stage of the disease (22.8%, 17.1%, and 14.7%, respectively). By comparison, the other cases (with no abortion) all had low maximal rising hematocrit percentages during the critical stage of the infection. However, access to a larger data set is necessary to confirm whether these findings are generally applicable to pregnancies of 20 weeks or less.

Low baseline hematocrit and rising hematocrits during pregnancy may be affected by plasma leakage or normal physiological changes in the cardiovascular system. For example, an increase of 40%-50% in plasma volume, which is relatively greater than the accompanying 20%-30% increase in red blood cell mass, results in hemodilution and decreased hematocrit (Ouzounian and Elkayam, 2012). Hence, the criterion for a diagnosis of anemia in pregnancy is a hematocrit of <30%, 33%, and 30% in the first, second, and third trimester, respectively; these values are lower than those of normal adult females (Abbassi-Ghanavati *et al*, 2009).

Having an enlarged gravid uterus makes it difficult to evaluate the clinical signs of plasma leakage such as pleural effusion and ascites. Therefore, routine abdominal and chest ultrasound examinations to detect free fluid in abdominal or thoracic cavities should be considered for pregnant women with dengue infection.

All cases in the present study except the one who was intrapartum and at the critical stage of the disease were managed by supportive care, rest, intravenous fluids and antipyretic medication similar to recent reports in which good clinical outcomes were obtained (Malhotra *et al*, 2006; Carroll *et al*, 2007; Ishag *et al*, 2010). Three cases of teenaged pregnant women with dengue infection had poor outcomes; two experienced spontaneous abortions, and one full-term pregnancy experienced labor during the critical stage of the disease. While the four pregnant women (>20 years old) with dengue infection had three normal deliveries and full term healthy neonates, there was only one case of incomplete abortion. This is not the first report of dengue infection associated with spontaneous abortion, or of the increased risk of such abortions occurring during the first trimester (Waduge *et al*, 2006; Tan *et al,* 2012).

Fever with thrombocytopenia during pregnancy, especially during the intrapartum period, can cause massive bleeding; therefore, obstetricians have to be extremely careful with cases presenting with these clinical signs. DHF and DSS are associated with fatality rates ranging from 2.9%-22% (Morta *et al,* 2012; Marchado *et al*, 2013). These fatality rate differences probably result from differences in the ways these diseases are managed (Ishag *et al*, 2010; Pouliot *et al,* 2010). Hence, accurate and rapid diagnosis of DF, DHF, and DSS in pregnant women is very important; however, hemodilution in normal pregnancy can conceal the classical features of hemoconcentration associated plasma leakage in DHF. Ultrasound detection of free fluid in the chest or abdomen may precede clinical detection of DHF.

Pregnant women with severe dengue infection must be differentiated from those with HELLP syndrome, preeclampsia or eclampsia. Laboratory findings for severe dengue infection and HELLP syndrome overlap with thrombocytopenia and elevated liver enzymes whereas laboratory findings supporting a diagnosis of HELLP syndrome include hemolysis of peripheral blood smears, serum lactate dehydrogenase ≥ 600 IU/l and proteinuria. However, in pregnancy, proteinuria is the only one criterion used to diagnose preeclampsia. In 2013, the American College of Obstetricians and Gynecologists' Task Force on Hypertension in Pregnancy modified (in recognition of the syndromic nature of preeclampsia) the dependence of the diagnosis on proteinuria. In the absence of proteinuria, preeclampsia should be diagnosed as hypertension in association with thrombocytopenia (platelet count less than 100,000/µl), impaired liver function (elevated blood levels of liver transaminases to twice the normal concentration), the new development of renal insufficiency (elevated serum creatinine greater than 1.1 mg/dl or a doubling of serum creatinine in the absence of other renal disease), pulmonary edema, or new-onset cerebral or visual disturbances (American College of Obstetricians and Gynecologists, 2013). In cases where proteinuria is absent, similar clinical and laboratory findings of thrombocytopenia, impaired liver function, renal insufficiency, pulmonary edema, and new-onset cerebral or visual disturbances in severe preeclampsia and severe dengue infection in pregnant women may be found. Therefore, a history of fever and hypertension that predates pregnancy may be helpful for arriving at a provisional diagnosis while the definite diagnosis of dengue infection should be confirmed serologically.

In the cases described herein, the dengue infections in the pregnant women were managed by supportive care, rest, administration of intravenous fluids, and antipyretic medication. None of the patients required platelets or other blood components except one patient who had the critical stage of the infection during intrapartum and was, therefore, referred to a tertiary care hospital because of the possibility of needing a blood transfusion. The handbook for clinical management of dengue (WHO and TDR, 2012) advises that clinicians need to maintain a high index of suspicion when dealing with pregnant women who present with febrile illness after travelling to or living in dengue-endemic areas. Early admission to hospital for close monitoring is desirable, particularly for pregnant women close to full-term and labor while the treatment of choice is medically conservative with obstetrical management of dengue disease (WHO and TDR, 2012).

Dengue infection occurs during all trimesters and the morbidity levels associated with it can be more serious in teenage pregnancies. Favorable outcomes can be obtained by early diagnosis and supportive treatment. Leukopenia was observed in most of the pregnant women with dengue infection. Further studies should be conducted to determine whether the low baseline hematocrits and low rising hematocrits (or abortions associated with increasing hematocrits during the critical stage of the disease) that we observed in pregnant women with dengue infection are typical clinical signs.

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CASE REPORTS

SEVERE DENGUE IN PREGNANT WOMEN

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Abstract. Over half of the world's population lives in areas at risk of dengue infection with 70% of overall disease burden in Asia. A shifting in age group of dengue patients towards adulthood has been widely seen in Asia. This will affect an increase in incidence of dengue infection in childbearing age and pregnant women. Two cases of severe dengue in pregnant women admitted to Photharam Hospital, Ratchaburi, Thailand were described. Both of them had dengue shock syndrome with organopathy involving the central nervous system with alteration of consciousness in one. They had uneventful recoveries following intensive care. This report emphasizes the hazards of dengue infection in pregnant women, which needs special consideration. Early recognition with careful monitoring and symptomatic management are the key factors in a favorable outcome for a dengue patient.

Keywords: HELLP, pregnancy, severe dengue

INTRODUCTION

Dengue is a mosquito-borne viral disease caused by four closely related dengue serotypes, and it ranges from asymptomatic infection to undifferentiated fever, dengue fever (DF), and dengue hemorrhagic fever (DHF). DHF is characterized by fever, bleeding diathesis, and a tendency to develop a potentially fatal shock syndrome (dengue shock syndrome, DSS). Dengue infection with organ impairment mainly involves the central nervous system and liver. Consistent hematological findings include vasculopathy, coagulopathy, and thrombocytopenia. Laboratory diagnosis includes virus isolation, serology, and detection of dengue ribonucleic acid. Successful treatment, which is mainly supportive, depends on early recognition of the disease and careful monitoring for shock. A revised severity-based dengue classification for medical interventions has been developed and validated in many countries. Currently, no specific dengue therapeutics exist, and prevention is limited to vector control measures.

At present, dengue is a growing global health concern with over half of the world's population living in areas at risk of dengue infection, and 70% of overall disease burden is in Asia. In Thailand, dengue is the most important arbovirus infection of the 21st century, and dengue disease is prevalent in all provinces. In Asia, there has been a general shift in age group predominance of dengue disease over the past decades

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to adulthood. Hence, awareness of the increased incidence of dengue infection in women of childbearing age is needed (Thisyakorn and Thisyakorn, 2015). Dengue infection complicated by severe hemorrhage and vertical transmission in a parturient woman has been described. That report emphasized the hazards of surgical intervention in patients with acute dengue infection and the mother-to-child transmission of dengue during the perinatal period (Thaithumyanon *et al*, 1994). This report highlights the higher risk of severe dengue disease in pregnant patients.

CASE REPORTS

Two cases of severe dengue in pregnant women admitted to Photharam Hospital, Ratchaburi, Thailand were described. 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; WHO, 2009). Analyses of the cases of two dengue patients were done after the approval of an ethics review committee.

Case 1

A 19-year-old, gravida 2, para 1 woman was hospitalized in Photharam Hospital at 20 weeks of gestation with a history of fever for 3 days, anorexia, vomiting, abdominal pain, headache, and drowsiness. Physical examination revealed a temperature of 39.7ºC, a blood pressure of 100/70 mmHg, a pulse rate of 110/min, and respiratory rate of 26/min. She was drowsy, and petechiae were seen on her legs. Laboratory findings included a hemoglobin of 12.3 g/dl, a hematocrit of 36.1%, a white blood cell count of 7,980/mm^3 (neutrophils 86%, lymphocytes 10%, and monocytes 4%), and a platelet count of 147,000/mm^3. She was admitted as having dengue infection. She received crystalloid intravenous fluid replacement, and her vital signs were closely monitored. Despite the crystalloid replacement, her blood pressure dropped to 80/50 with a rapid and weak pulse with a rate of 104/min. Her hematocrit increased to 45% with the platelet count decreasing to 11,000/mm^3. Colloid (dextran, albumin) replacement and dopamine infusion were then given. At recovery, her chest roentgenogram showed mild pulmonary congestion and cardiomegaly, which became normal later on. She had uneventful recovery after 6 days of hospitalization. She eventually delivered a normal baby boy weighing 3,090 grams at 39 weeks of gestation.

Case 2

A 22-year-old, gravida 1, para 0 woman was hospitalized in Photharam Hospital at 16 weeks of gestation with a history of fever for 4 days, nausea, and abdominal pain. Physical examination revealed a temperature of 38.8ºC, a blood pressure of 100/70 mmHg, a pulse rate of 90/min, and a respiratory rate of 24/min. Laboratory findings included a hemoglobin of 10.7 g/dl, a hematocrit of 33.4%, a white blood cell count of 7,800/mm^3 (neutrophils 62%, lymphocytes 33%, monocytes 3%, and eosinophils 2%), and a platelet count of 112,400/mm^3. She was then admitted as having dengue infection. Crystalloid intravenous fluid was given with close monitoring of vital signs. During the course, her hematocrit rose to 38% with a drop in platelet count to 24,000/mm^3. She finally had an uneventful recovery after 5 days of hospitalization. Subsequently, she delivered a normal baby boy weighing

2,370 grams at 37 weeks of gestation.

DISCUSSION

Dengue epidemics are known to have occurred regularly during the past decades in Asia, causing a heavy burden on the healthcare system. A shift in the age group of dengue patients towards adulthood has been widely seen**,** which will have the consequence of an increased incidence of dengue infection in childbearing age and pregnant women (Thisyakorn and Thisyakorn, 2015). The cases in this report illustrate severe dengue in pregnant women. Both of them had DSS, and organopathy involving the central nervous system occurred in one patient. Dengue infections with organopathy mainly involving the central nervous system and liver have been reported in patients with dengue infection. These manifestations may be associated with co-infections, co-morbidities, or complications of prolonged shock. Exhaustive investigations should be done in these cases (Innis *et al*, 1990; Thisyakorn and Thisyakorn, 1994a,b; Thisyakorn *et al*, 1999; Hemungkorn *et al*, 2007).

Both patients had uneventful recoveries due to early diagnosis, careful monitoring, and intensive management. Evidence-based data on the management of dengue specific for pregnancy are sparse but are needed because pregnant women with dengue infection have a higher risk of severe disease than non-pregnant patients. The following are special considerations in pregnant women with dengue (Royal College Physician of Thailand, 2013):

- Physiologic hemodilution in pregnancy may obscure hemoconcentration in DHF as seen in Case Report 2.

- Dengue infection should be a differential diagnosis of pregnancy-related conditions, especially HELLP (Hemolysis, Elevated Liver enzymes, Low Platelet count) syndrome.

- Platelet transfusion is indicated in in-labor pregnancy, when the platelet count is <50,000/mm^3.

- There are increased risks of abortion, premature uterine contraction, intra- and post-partum hemorrhage, maternal death, fetal distress, low birth weight, or death of the fetus *in utero*, which is associated with disease severity and gestational age.

- With a vertical transmission rate of 1.6%-10.5%, dengue infection is a cause of low platelets in the newborn (usually occurring in pregnant women who have had fever for 1 week before delivery).

The WHO's "Global Strategy for Dengue Prevention and Control, 2012-2020" targets reducing the global burden of dengue by decreasing its morbidity by 25% and its mortality by 50% (WHO, 2012). Two of the key technical elements are diagnosis and case management. Mortality from dengue can be reduced to almost zero by implementing timely, appropriate clinical management, which involves early clinical and laboratory diagnoses, intravenous rehydration, staff training, and hospital reorganization.

Despite the dengue control programs, case management guidelines, and surveillance efforts, rates of dengue virus transmission remain high, and prevention remains a public health priority. Ultimately, an effective and long lasting vaccine needs to be used (Thisyakorn and Thisyakorn, 2014).

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DENGUE INFECTION IN ELDERLY PATIENTS

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Abstract. From 2005 to 2013, there were 15 dengue patients aged over 60 years old who were admitted to Photharam Hospital, Ratchaburi, Thailand. Ten were females and five were males. Nine had dengue fever (DF), and 6 had dengue hemorrhagic fever (DHF). A trending shift in age group towards adults has been seen during the past decades. No deaths were seen in these elderly patients with dengue disease, indicating early recognition and effective management of these dengue patients. The trend towards higher age in dengue patients is a problem of concern, which needs further elaboration.

Keywords: dengue, elderly, Photharam Hospital, Thailand

INTRODUCTION

Dengue infection is one of the major public health problems in Asia and Latin America. The etiological agents include four dengue serotypes, and the principal vector is the *Aedes aegypti* mosquito (Thisyakorn and Thisyakorn, 2014). In past decades, a trend of increasing age in dengue towards adulthood has been evident in Asia (Tanayapong *et al*, 2013). Moreover, clinical manifestations and severity of dengue infection varied with age (Panpitpat *et al*, 2007). With co-morbidities, elderly dengue patients have a higher risk of severe dengue and mortality (Tantawichien, 2012).

In Thailand, a dengue patient was first seen in Bangkok in 1958, after which further cases appeared in other parts of the country (Thisyakorn, 2014). Dengue epidemics are known to have occurred regularly during the past decades in Photharam District, Ratchaburi Province, Thailand causing a heavy burden on the healthcare system. Despite dengue control programs, case management guidelines, and surveillance efforts, dengue virus transmission rates remain high, and prevention remains a public health priority (Capeding *et al*, 2013).

Complex disease presentation and sudden development of hemorrhagic symptoms in seemingly stable patients can cause a fatal outcome even in well-prepared hospitals. There are currently neither an approved preventive vaccine nor a specific anti-viral treatment against dengue. Main public health preventive interventions consist of mosquito control, which is currently used in endemic countries, and the use of vector repellents. Both these measures have produced generally only limited results. The development of a dengue vaccine is regarded as the best hope to fight this disease (Thisyakorn *et al*, 2014).

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Table 1
The clinical manifestations in 15 elderly patients with dengue diseases.

Clinical manifestations	Patients *n,* (%)
Fever	15 (100)
Headache	5 (33)
Upper respiratory symptoms	5 (33)
Nausea	4 (27)
Abdominal pain	4 (27)
Vomiting	2 (13)
Petechiae	2 (13)
Diarrhea	1 (7)
Alteration of consciousness	1 (7)
Confluent petechial rash (during convalescence)	1 (7)

MATERIALS AND METHODS

Analysis of the data of the elderly dengue patients admitted to Photharam Hospital, a provincial hospital in Ratchaburi Province, Thailand from January 2005 to December 2013 was done after the approval of an ethics review committee. Photharam Hospital is among the ten clinical trial sites of a potential dengue vaccine (Capeding *et al*, 2014). The hospital is in Ratchaburi Province, which is approximately 100 kilometers west of Bangkok. 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).

RESULTS

From 2005 to 2013, there were 15 dengue patients, aged over 60 years old, who were admitted to Photharam Hospital, Ratchaburi, Thailand. Ten were female and 5 were male. The age range was between 60-to-87 years old, with a mean age of 68.7 years and a median age of 66 years.

The clinical characteristics of the 15 elderly patients with dengue disease are summarized in Table 1. All patients had fever with an average of 3 days in duration and with a range of 1-5 days prior to admission. The symptoms and signs included headache (33%), upper respiratory symptoms (33%), nausea (27%), abdominal pain (27%), vomiting (13%), petechial hemorrhage (13%), diarrhea (7%), alteration of consciousness (7%), and confluent petechial rash occurring at both legs during the convalescent stage of the disease (7%).

According to 1997 WHO case classification of dengue, 9 patients had dengue fever (DF), and 6 had dengue hemorrhagic fever (DHF).

The mean maximal hematocrit value was 38.9% with a range of 32%-45%. The mean minimal value of white blood cell count was 3,061 cells/mm^3 with a range of 1,540-5,940 cells/mm^3. The mean minimal value of platelet count was 72,467/mm^3 with a range of 8,000-158,000/mm^3.

Increased liver enzymes (alanine aminotransferase, ALT and aspartate ami-

notransferase, AST) were found, and AST was higher than ALT.

All patients recovered uneventfully. There was no mortality.

DISCUSSION

During the past decades, dengue epidemics are known to have occurred regularly in Ratchaburi Province, Thailand causing a heavy burden on the healthcare system. Population growth together with a remarkable degree of urbanization has allowed dramatic expansion of the mosquito numbers through an increase in urban breeding sites, which explains the explosive increase of reported cases of dengue infection. A greater awareness and high reporting behavior could have contributed to some of the increase over time. A trend of increasing age in dengue towards adulthood has been evident in Asia including the area of Ratchaburi Province, Thailand (Tanayapong *et al*, 2013).

This study showed that the full range of severity of dengue manifestations could happen in elderly patients. DF is usually self-limiting, and death is uncommon. However, age-related differences in dengue severity are poorly understood, and data on clinical features in elderly patients are limited. Older age has previously been reported to be a risk factor for mortality in patients with DF or DHF because the co-morbidities associated with ageing and waning immunity pose a substantial risk for fatality in elderly patients with active infection. Although shock and plasma leakage seem to be more prevalent in younger patients, the frequency of internal hemorrhage increases with age. Furthermore, complications of dengue infection observed in adults, including DF with unusual bleeding and DHF, have been increasing (Tantawichien, 2012).

Increased liver enzymes as seen in our patients have been found in children and adults during dengue infection, indicating liver involvement. Unlike conventional viral hepatitis, AST level is higher than ALT in dengue infection as seen in our patients. Pre-existing liver disease such as chronic hepatitis is more likely to be present in adults than in children with dengue and may exacerbate the liver impairment. Liver injury is often self-limiting, but fulminant hepatitis and death have been reported. Alteration of consciousness in dengue patient as seen in one of our patients has been described as dengue encephalopathy. Possible causes of dengue encephalopathy include hypotension, cerebral edema, focal hemorrhage, hyponatremia, and fulminant hepatic failure (Tantawichien, 2012). However, a documented possibility is dengue invasion of the central nervous system. (Thisyakorn and Thisyakorn, 1994).

All patients had an uneventful recovery due to early diagnosis, careful monitoring, and effective management. Evidence-based data on the management of dengue specific for elderly patients are scant but are needed because the disease poses a substantial risk for fatality. The following are special considerations in adults with dengue (Royal College Physician of Thailand, 2014):

- Adults have higher co-morbidities and underlying diseases.

- More than 90% of adult dengue patients have elevated liver enzymes. Therefore, administration of hepatotoxic medications should be avoided.

- If jaundice presents, other diseases should be suspected.

- Dual infections should be suspected in a patient with atypical presentation.

- Internal hemorrhage should be suspected in a patient with a rapidly decreasing hematocrit.

Prevention of dengue depends on the control of the mosquito vector by limiting its breeding places and treatment of stored water with larvicide. These measures against dengue are effective only with a high level of government commitment, education, and community participation (Thisyakorn, 2014). Ultimately, an effective and long lasting vaccine needs to be utilized. Due to the unique challenges of dengue, including the need to provide protection against the four antigenically-distinct serotypes of the viruses (Thisyakorn *et al*, 2014); no vaccine is yet licensed to protect against this disease.

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ANNEX I

PRACTICAL GUIDELINE FOR MANAGEMENT OF DENGUE IN ADULTS: 2014

Royal College Physician of Thailand

Dengue fever (DF) and dengue hemorrhagic fever (DHF) are mosquito-borne infections from dengue viruses of the flavivirus group. The four dengue virus serotypes are called DEN-1, DEN-2, DEN-3, and DEN-4. The insect vectors*, Aedes aegypti, Ae. albopictus, Ae. polynesiensis*, can transmit dengue viruses to humans and increases the risk of global pandemic dengue transmission, especially in Southeast Asia, the Western Pacific and the Americas. In Thailand, epidemic DF and DHF have expanded geographically and increased in incidence. These changes have been caused by the modern dynamics of climate change, globalization and the geographic spread of the virus and its vectors due to travel. Epidemics of DF and DHF occur throughout the year and usually peak during the rainy season, which may be between June and September. DF and DHF in Thailand usually outbreak every 3 years; however, at present, the timing of epidemics of DF and DHF in Thailand is unpredictable.

DF and DHF cases have increased over time whereas the fatality rate has decreased significantly to 0.15%. This decrease in fatality rate may have been caused by good treatment practices. DF and DHF morbidity and mortality rates have been highest in children, especially in the 5-9 year age group. While dengue infection has traditionally been considered a pediatric disease, the distribution of dengue has been rising and more cases have been observed in adolescents and adults.

In Thailand, 20%-40% of DF/DHF cases have been reported in adults, and the rate of DF/DHF reported has increased over time in adults. The clinical features of DF/DHF in adults are similar to that of children, but some adults with dengue may present with a high grade fever, myalgia, arthralgia, bone pain, bleeding, and signs of circulatory failure or development of dengue shock syndrome (DSS). The range of clinical manifestations of dengue infection includes asymptomatic disease, undifferentiated febrile disease, self-limiting febrile illness associated with myalgia, classic dengue fever, dengue hemorrhagic fever or DDS.

The incidence of dengue among Thai adults is underestimated due to under-detection of the acute febrile phase of DF, especially in older ages. Older adults usually have clinical manifestation as DF; whereas, DHF is a common manifestation of adolescents and younger adults that is similar with children. Being an adult is also a risk factor for mortality in DF/DHF because of delayed diagnosis and treatment, comorbidity associated with older age and the increasing frequency of internal hemorrhage with age.

Dengue fever: DF (WHO, 1997)

Clinical manifestations of dengue fever range from asymptomatic to dengue fever with

unusual hemorrhage, which makes dengue fever difficult to detect. A definitive diagnosis can only be made in the laboratory by detecting viral antigen, NS1, PCR (polymerase chain reaction) and/or antibodies in serum.

Criteria for diagnosis of dengue fever

Probable case: an acute febrile illness has two or more of the following manifestations;

- Headache
- Retro-orbital pain
- Myalgia
- Arthralgia
- Rash
- Hemorrhagic manifestations: petechiae, epistaxis, a positive tourniquet test
- Leukopenia, neutropenia, and atypical lymphocyte findings;

and

- Supportive serology: positive IgM/IgG antibody test by immunochromatographic test or rapid ELISA test on a late acute phase of the disease; or patients who live in an endemic area at the same time as other confirmed cases of dengue fever with negative laboratory findings of other causes of febrile illness.

Confirmed case: a case is confirmed by the following laboratory criteria:

- Detection of dengue virus genomic sequences by PCR, demonstration of dengue virus antigen by non-specific protein of dengue virus (dengue NS1) and/or a seropositive of IgM by MAC-ELISA test (anti DEN IgM ≥40 units and more than anti JE IgM, or a rise of anti DEN IgG titer of ≥2 times and convalescent IgG ≥100 unit)

Dengue hemorrhagic fever: DHF (WHO, 1997)

The following must be present:

1. Fever, or history of acute fever, lasting 2-7 days.
2. Hemorrhagic tendencies: a positive tourniquet test with bleeding.
3. Thrombocytopenia [≤100,000 cells per mm^3 (≤$100x10^9$/l) or platelet in blood smear ≤6 cells/ oil field].
4. Evidence of plasma leakage, for example, a rise in the hematocrit (Hct) ≥20% of baseline hematocrit, signs of plasma leakage such as pleural effusion, ascites or hypoalbuminemia.

Note: The positive predictive value of tourniquet test with clinical of pleural effusion/ascites is 96%.

Criteria for diagnosis of dengue hemorrhagic fever: DHF (WHO, 1997)

Diagnosis of DHF is made by recognizing the clinical manifestations and hemodynamic change. Platelet count and sign of plasma leakage may help clinicians to establish an early diagnosis before the onset of shock.

Clinical manifestation as the following lists indicators of DHF/DSS

1. Fever, or history of acute fever, lasting 2-7 days.
2. Hemorrhagic tendencies: a positive tourniquet test with bleeding.

3. Hepatomegaly with tenderness.
4. Hemodynamic change or shock.

Laboratory findings

1. Platelet count ≤100,000 cells per mm^3 (≤$100x10^9$/l).
2. A rising of hematocrit ≥20% of baseline hematocrit (hemoconcentration) or signs of plasma leakage such as pleural effusion, ascites or hypoalbuminemia.
3. Leukopenia, neutropenia, and atypical lymphocyte findings.

Note: Platelet count can be calculated by slide smear. If platelets are less than 6 cells/oil field; it can be predicted that the platelet count is less than 100,000 cell per mm^3 (≤$100x10^9$/l).

The stages of DF/DHF disease can be divided into 3 stages:

Stage I (acute febrile stage): All patients have acute high grade of fever. This phase usually lasts 2-7 days. Some patients may have myalgia, facial flushing, hemorrhagic spots, or an erythematous maculopapular rash over the body and limbs. Other non-specific constitutional symptoms such as nausea, vomiting, abdominal pain or hepatomegaly may be present, especially at the end of this phase.

Stage II (critical stage): The patients go on to develop plasma leakage usually 24-48 hours after the fever begins to subside. Some patients also may have early signs of shock/circulatory failure including restlessness, hemorrhagic manifestations or severe abdominal pain.

Stage III (convalescence stage): Sudden arrest of plasma leak with clinical improvement occurs within 2-3 days. The patients usually have good appetite, normal blood pressure, a full and slow pulse, and a hematocrit that returns to baseline. Some cases also have a convalescence rash as a macular confluent rash over the face, thorax, and flexor surfaces.

Grading severity of dengue hemorrhagic fever (DHF)

DHF is classified into 4 grades of severity:

Grade I Unspecified fever, the only hemorrhagic manifestation is a positive tourniquet test and/or easy bruising without hypotension.

Grade I Spontaneous bleeding usually in the forms of skin or other hemorrhages without hypotension.

Grade III Circulatory failure manifested by a rapid, weak pulse and narrowing of pulse pressure; or hypotension with the presence of cold, clammy skin and restlessness.

Grade IV Profound shock with undetectable blood pressure or pulse.

Note: The presence of hemoconcentration and platelet count is less than 100,000 cell per mm^3 (≤$100x10^9$/l) differentiates DHF grade I and II from DF. Dengue hemorrhage fever (DHF) grade III and IV are considered to be dengue shock syndrome (DSS).

Severe dengue and warning signs in dengue infection (WHO, 2009)

Severe dengue is classified as patients with dengue infection with one or more of the

following manifestations:

1. Severe plasma leakage *eg* hypotension or poor capillary perfusion.
2. Severe bleeding.
3. Severe organ impairment: liver failure, AST/ALT >1,000 unit/ml, kidney injury, respiratory failure, alteration of consciousness.

Clinicians should evaluate signs, symptoms and laboratory tests as the warning signs of severe dengue infection, for example frequent vomiting, abdominal pain, liver tenderness, respiratory distress, irritability, spontaneous bleeding (epistaxis, bleeding per gum), retinal hemorrhage, plasma leakage, oliguria or hemoconcentration with decreased platelet count.

Descriptions

1. Dengue infection should be suspected if the patients have the following clinical presentations:

1.1. Fever of 10 days or less with myalgia, arthralgia, bone pain, headache, peri-orbital pain, flushing, nausea or vomiting.
1.2 No obvious respiratory tract symptoms or signs.
1.3 No organ specific symptoms of other infectious diseases.

Despite dengue infection, other infectious diseases (*eg*, malaria, gram-negative bacteremia) should be suspected.

2. Presumptive diagnosis of dengue infection should be considered in patients with the following signs or symptoms.

2.1 Fever of 3 days or less with positive tourniquet test or white blood cell count (WBC) less than 10,000/mm^3 (<$10x10^9$/l).
2.2 Fever of 4-10 days with WBC less than 5,000/mm^3 (<$5x10^9$/l), platelet count less than 140,000/mm^3 (<$140x10^9$/l), or hematocrit (Hct) of 45% or more.

In these patients, the laboratory diagnosis of acute dengue infection can be performed for the confirmation.

The tourniquet test is performed by using the optimal size of the blood pressure cuff. The cuff is inflated at the mid-point between systolic and diastolic for 5 minutes. The interpretation is done at 1 minute after cuff deflation. The petechiae count per 1 square inch should be recorded. A count of 10 or more petechiae per square inch is considered as a positive test.

3. The laboratory test for definite diagnosis of acute dengue infection.

3.1 Fever of 1-3 days, NS1 or PCR from serum or plasma (diagnostic yield of 80-90%, but the yield decreases after 3 days of fever) and/or collection of the first serum for antibody should be considered.
3.2 Fever of 4 days or more, antibody tests, for example, ELISA or rapid immunochromatography test (rapid test IgM has a 10-20% rate of false positive and false negatives) should be considered.

Investigation for acute dengue infection:

- Viral isolation and identification, NS-1, or PCR;
- Antibody capture EIA.

For single serum: anti DEN IgM of ≥ 40 units and greater than anti-JE IgM is considered as positive of dengue infection.

For paired sera: anti-DEN IgM of the first serum of <15 units and ≥30 units from the second serum is considered as positive of dengue infection.

a. Primary infection: Anti-DEN IgM/IgG ratio ≥1.8:1 is considered as primary infection.

b. Secondary infection:

i. Anti-DEN IgM/IgG ratio <1.8:1 is considered as secondary infection.

ii. Anti-DEN IgG (convalescence serum):IgG (acute serum) rise of ≥2 times and Anti-DEN IgG (convalescent serum) ≥100 units.

There are several commercial kits of rapid tests for dengue infection. However, the sensitivity, specificity, and accuracy vary among these tests. Therefore, these tests are suitable for screening, and should be confirmed by the above tests.

4. Physicians should be aware of warning signs (signs, symptoms, and hematocrit) in patients with dengue infections before the patients develop severe infections (shock from plasma leakage, abnormal hemorrhage or organ failure) (Fig 1).

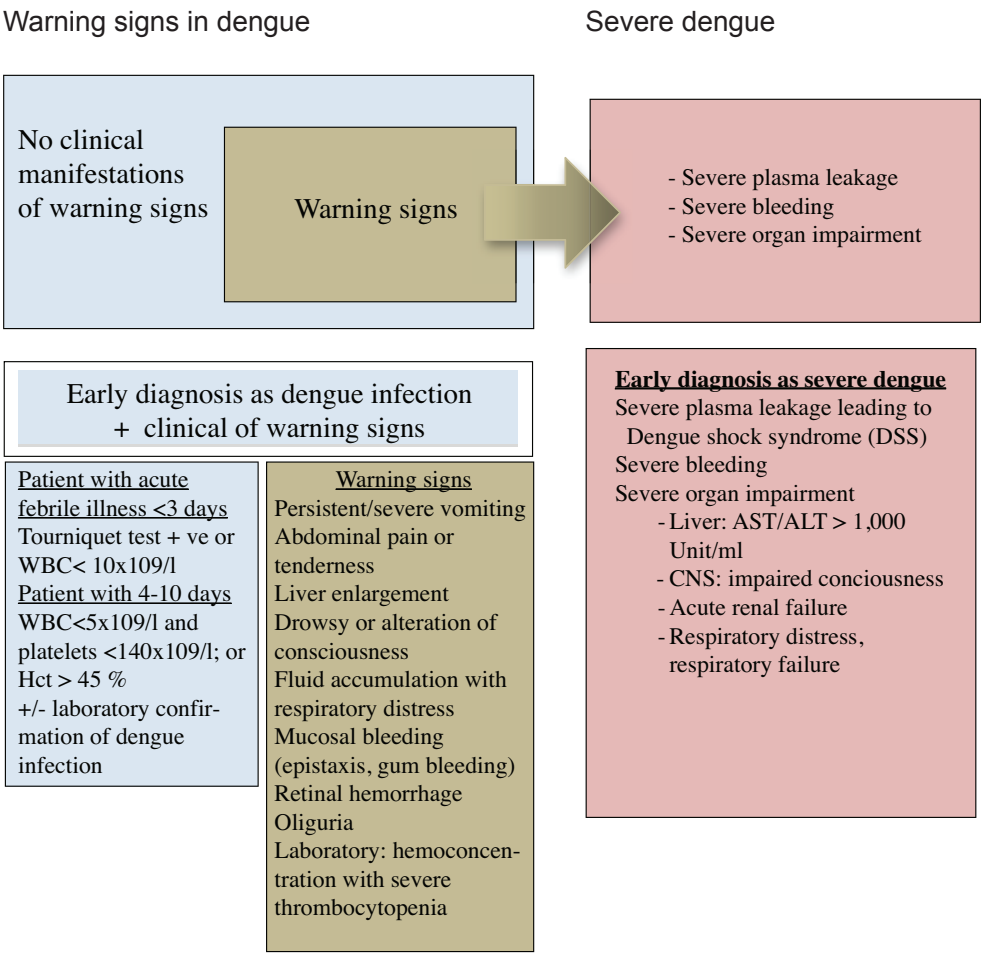

Fig 1–Clinical manifestation in severe dengue and the warning signs in dengue infection.

Guidelines for outpatient/inpatient management of dengue infections and adults (Figs 2 and 3)

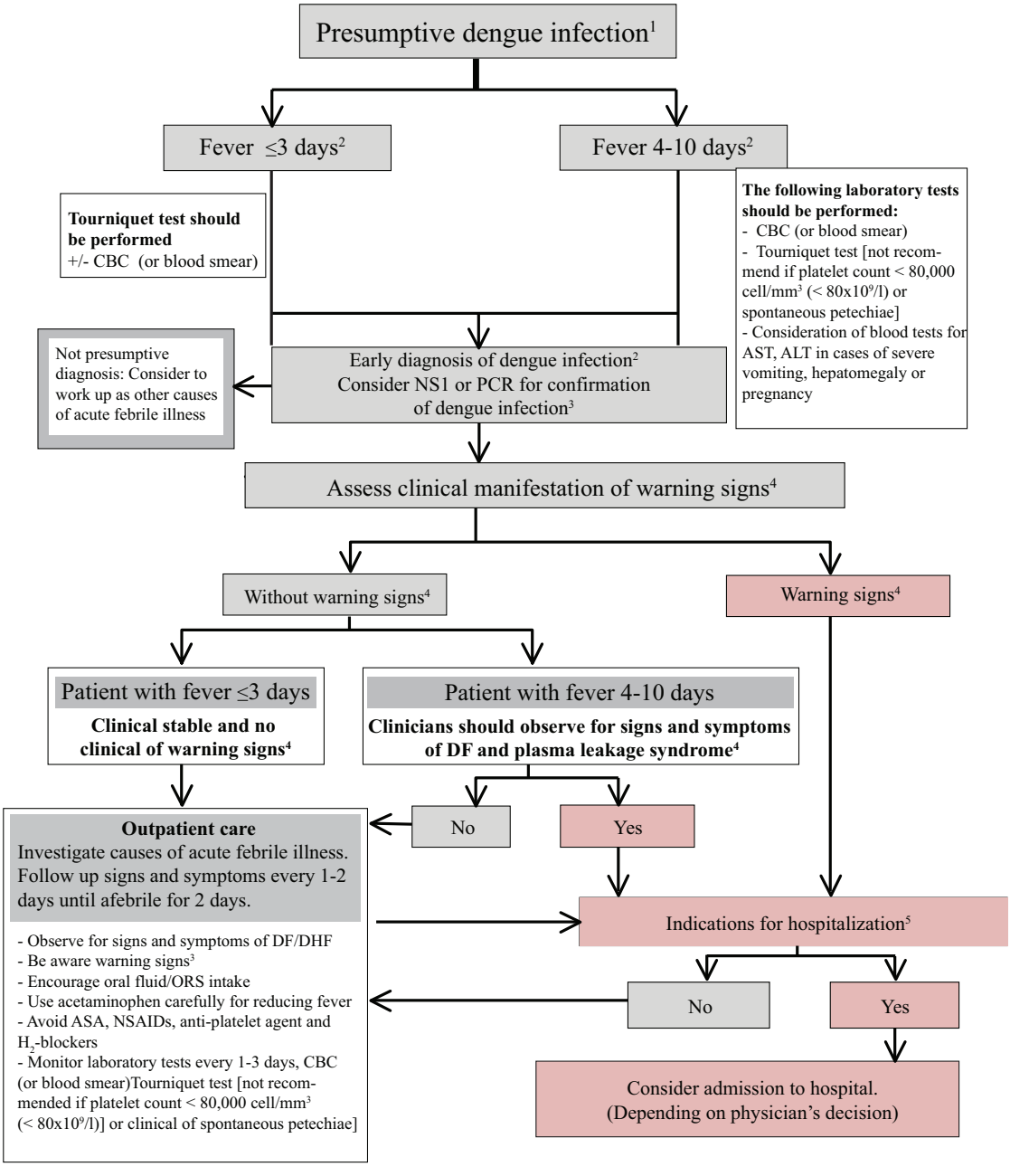

Fig 2–Guidelines for management of dengue infections in adults.

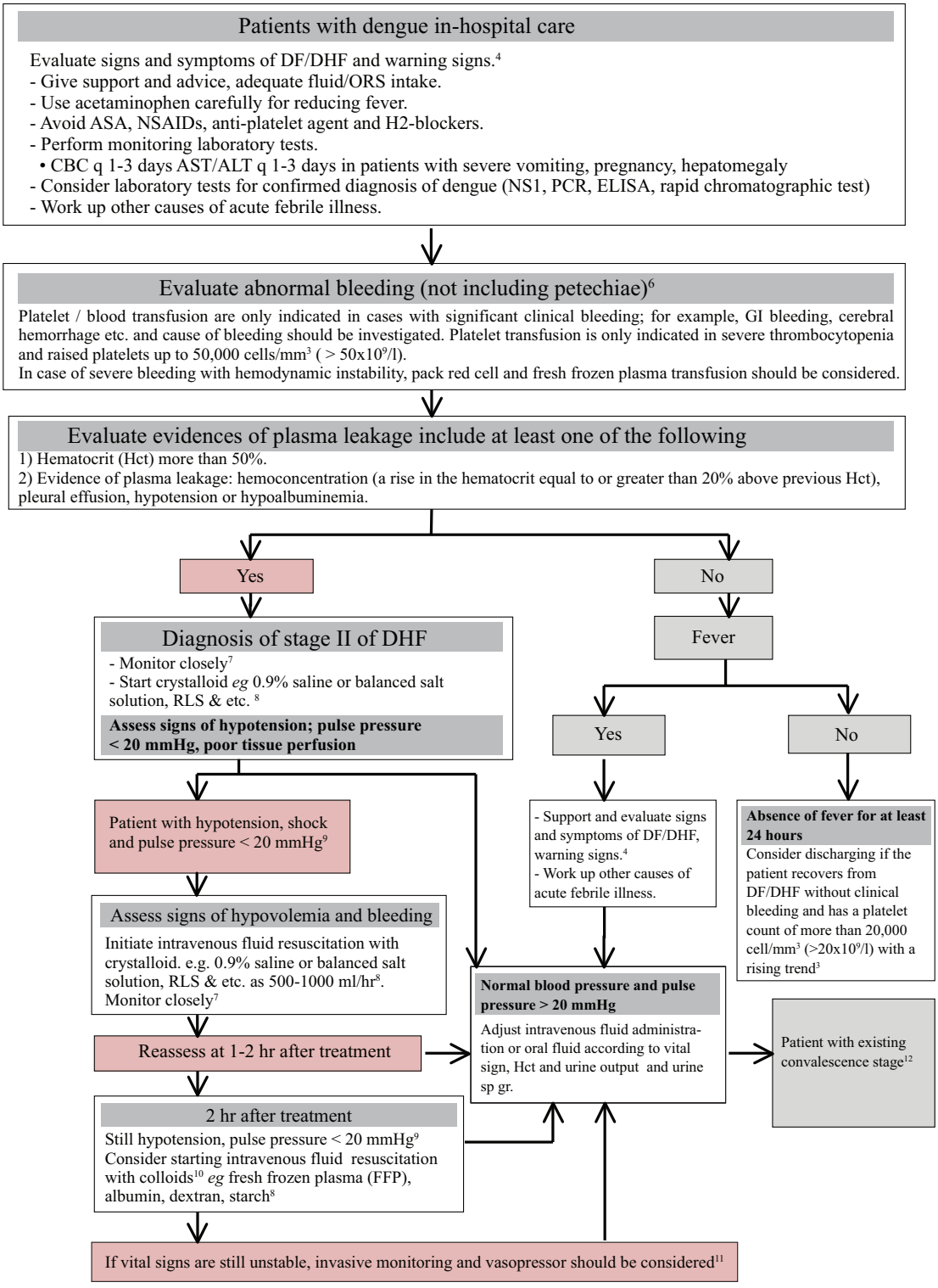

Fig 3–Guidelines for the management of dengue infections in adults.

5. Indications for admission of patients with dengue infection:

5.1 Signs/symptoms that the physician considers make the admission of the patient necessary, for example, nausea/vomiting;

5.2 Severe hemorrhage, for example, hematemesis, hematochezia or abnormal vaginal bleeding;

5.3 Dengue shock syndrome, or hypotension;

5.4 Hct > 50%;

5.5 Platelet ≤20,000/mm^3 (≤20x10^9/l);

5.6 AST or ALT >500 U/ml;

5.7 Renal failure, liver failure, heart failure, drowsiness, or severe hypoxemia;

5.8 Pregnant women;

5.9 Morbid obesity;

5.10 Patients could not follow up as out-patient setting.

6. Bleeding complications usually occur in 5-8 days after onset in dengue infection. The abnormal bleeding is associated with low numbers of platelets and abnormalities of walls of vessels. Risk factors of bleeding are platelets ≤20,000/mm^3 (≤20x10^9/l), increased AST or ALT, prolonged PT, severe dengue hemorrhagic fever, patients with DIC or liver failure. Patients with coagulopathy usually have GI bleeding.

6.1 Platelet transfusion is not necessary in patients with only petechiae or minor bleeding despite low platelets. The indications for platelet transfusions are bleeding associated with active peptic ulcer, trauma, liver failure, receiving antiplatelet, or platelets <10,000/mm^3 (<10x10^9/l).

6.2 In patients with severe GI bleeding, other conditions such as peptic ulcers or gastritis should be suspected. In some GI bleeding, there may be only melena without hematemesis.

6.3 In women, uterine bleeding can be found in 5-25% of dengue infections. Most uterine bleeding is not severe and does not require blood or platelet transfusion. However, hormonal therapy might be indicated in some cases.

6.4 In patients receiving anti-platelet, anti-coagulant or heparin, the bleeding complications may be more severe.

7. Close monitoring:

7.1 Vital signs, peripheral perfusion, and clinical assessment should be assessed every 15-30 minutes until resolution of shock, and every 1-4 hours thereafter.

7.2 Hematocrit should be monitored 1-4 times per day according to clinical presentations and platelet count should be achieved as indicated.

7.3 Fluid intake and output should be assessed every 1-4 hours. An adequate urine output of 0.5-1 ml/kg/hour and a urine specific gravity of 1.010-1.020 should be achieved. Massive pleural effusion or ascites may cause breathing difficulty and physicians should be wary of this.

7.4 In severe cases or cases with co-morbidity, pulse oximetry, ECG, arterial blood gas, blood sugar, serum electrolyte, lactate, BUN/Cr, liver function test, coagulogram should be measured as indicated.

8. Guidelines for fluid replacement and resuscitation in dengue patients with plasma leakage syndrome.

Stage II of dengue hemorrhage fever (plasma leakage syndrome)			
Normal blood pressure and pulse pressure >20 mmHg	**Hypotension and/or pulse pressure ≤20 mmHg**	**Shock**	**Persistent shock despite adequate crystalloid replacement**
End point	**End point**	**End point**	**End point**
Target: Normal blood pressure and pulse pressure >20 mmHg. Urine sp gr 1010-1020. Keep urine output 0.5-1.0 ml/kg/hr, Hct~40%-45%. **Limitation:** Leakage syndrome, *eg*, pleural effusion, ascites, crepitation.	**Target:** Normal blood pressure and pulse pressure >20 mmHg. Urine sp gr 1010-1020. Keep urine output 0.5-1.0 ml/kg/hr, Hct~40%-45%. **Limitation:** Leakage syndrome, *eg*, pleural effusion, ascites, crepitation.	**Target:** Normal blood pressure and pulse pressure >20 mmHg. Urine sp gr 1010-1020. Keep urine output 0.5-1.0 ml/kg/hr, Hct~40%-45%. **Limitation:** Leakage syndrome, *eg*, pleural effusion, ascites, crepitation.	**Target:** Normal blood pressure and pulse pressure >20 mmHg. Urine sp gr 1010-1020. Keep urine output 0.5-1.0 ml/kg/hr, Hct~40%-45%. **Limitation:** Leakage syndrome, *eg*, pleural effusion, ascites, crepitation.
Methods	**Methods**	**Methods**	**Methods**
IV 5%D saline, NSS for patients without shock, (intravenous fluid replacement only in patients with vomiting, or cannot tolerate oral diet or ORS), with rate of 40-80 ml/hr and adjust according to vital signs, Hct, urine output, urine sp.gr. If patients turn to critical phase, the rate of fluid replacement should be adjusted as indicated by vital signs, Hct, urine output.	IV isotonic crystalloid, *eg*, 0.9% saline or RLS 5-7 ml/kg/hr for 1-2 hr. • If clinical setting and parameters are improved, decrease the rate to 3-5 ml/kg/hr for 2-4 hr, and then 2-3 ml/kg/hr until stable vital signs. • If clinical setting and parameters are worsened or not improved, increase the rate to 7-10 ml/kg/hr for 1-2 hr and re-evaluate within 2-4 hr. If not improved, patients should be treated as "shock."	IV isotonic crystalloid *eg*, 0.9% saline or RLS 10-20 ml/kg/hr (500-1000 ml) for 1-2 hr. • If clinical setting and parameters are improved, decrease the rate to 5-7 ml/kg/hr for 1-2 hr and then gradually decrease the rate. • If clinical setting and parameters are worsened or not improved, change solution to colloid, *eg*, 5% albumin, dextran, or FFP 10 ml/kg/hr for 1 hr. If not improved, patients should be treated as‘persistent shock despite adequate crystalloid replacement.'	Evaluate for other co-morbidities, *eg,* severe bleeding, metabolic acidosis, severe sepsis, pneumothorax. Start vasopressors, *eg*, norepinephrine 0.1-0.2 mcg/kg/min. Adjust dosage every 10-15 min (max dose 1-2 mcg/kg/min), and decrease dosage when clinical setting and parameter are improved. **Note** Patients with shock should have their vital signs and parameters closely monitored until resolution of shock.

9. Blood pressure and pulse rate are essential for evaluation of patients with dengue. In some instances, patients with circulatory failure might be fully conscious with only fatigue. In patients with underlying hypertension who develop inadequate tissue perfusion, the blood pressure might be within normal range, so the administration of antihypertensive agents should be done careful.

10. If shock does not response to crystalloid, the colloid solutions (such as FFP, NSS plus albumin, or dextran) should be used instead. However, dextran should be used with caution because it can cause platelet dysfunction. The admiration of starch does not reduce mortality in non-dengue shock and might cause acute renal failure.

11. Administration of vasopressors should be evaluated individually because they might raise blood pressure in spite of inadequate intravascular volume. In dengue hemorrhagic fever, the plasma leaks profusely and continuously from the blood vessels, so the priority of treatment is intravascular volume replacement. However, in situations of prolonged shock despite adequate intravascular replacement, or of development of the signs/symptoms of volume overload in the patient such as pulmonary edema, vasopressors should have a role in rising of blood pressure. At the time of writing, no studies of the efficacy of vasopressors in dengue shock syndrome exist; however, the Surviving Sepsis Guidelines, 2012 should be applied in this situation. According to these guidelines, norepinephrine should be considered, as the first agent and vasopressin or adrenaline should be added if the patient does not respond to norepinephrine. Dopamine should be avoided in this situation because of the increased risk of arrhythmia.

12. Signs and symptoms of the convalescence stage should be assessed:

- Improved well-being and appetite.
- Absence of fever, normal blood pressure, bradycardia, convalescence rash at legs or arms which is associated with pruritus.
- Hct <50% and stable, increased WBC with a percentage of lymphocytes greater than the percentage of neutrophils, increased platelet count.

If patient is in the convalescence stage, the rate of intravenous fluid should be decreased to prevent volume overload from re-accumulation of fluid from the third space. If fever is absent for more than 1 day with no clinical bleeding with an increasing platelet count of more than 20,000/mm^3 (> $20x10^9$/l), the patient can be discharged.

Guidelines for the management of dengue fever and dengue hemorrhagic fever in pregnancy

The diagnosis and treatment of dengue fever and dengue hemorrhagic fever in pregnancy are the same as non-pregnant patients. In an epidemic of dengue infection, dengue infection should be considered in a pregnant woman who has fever.

Special consideration in pregnancy

Diagnosis:

- Physiologic hemodilution in pregnancy may obscure hemoconcentration in DHF.
- Dengue infection should be a differential diagnosis of pregnancy-related condi-

tions, especially HELLP (hemolysis, elevated liver enzymes, thrombocytopenia) syndrome.

- Treatments are anti-pyretic drug, hydration, rest and supportive care. Platelet transfusion is indicated in in-labor pregnancy when the platelet count is <50,000/mm^3 (< $50x10^9$/l).

Effects of dengue infection on pregnancy:

- There are increased risks of abortion, premature uterine contraction, intra-partum and post-partum hemorrhage, maternal death, fetal distress, low birth weight, or death fetus *in utero* which is associated with disease severity and gestational age.
- With a vertical transmission rate of 1.6-10.5%, dengue infection is a cause of low platelets in the new born (usually occurs in pregnant women who have had fever for 1 week before delivery).

Effect of pregnancy on dengue infection:

- Pregnant patients have higher risk of severe disease than non-pregnant patients.

Special consideration in adult patients

1. Co-morbidity/underlying diseases

Adults have a higher prevalence of underlying diseases, for example, coronary artery disease, peptic ulcer, hypertension, diabetes mellitus, cirrhosis, or chronic kidney disease, which should be considered in dengue management.

2. Elevation of transaminase level

More than 90% of cases of DF/DHF in adults have elevated transaminase levels, of which ALT is usually elevated more than AST. In nearly all patients, the elevated AST and ALT are found within 48 hours before defervescence. AST and ALT will reach their peak in 7-9 days after onset and subside within 2-3 weeks after defervescence. Acute liver failure is rarely observed; however, administration of hepatotoxic medications such as antipyretics or antiemetics should be careful and avoided in patients who have elevated transaminase.

3. Jaundice

Jaundice presents infrequently in dengue infection. Therefore, if jaundice presents, other diseases should be suspected such as acute cholangitis, hepatotrophic viral hepatitis, drug allergy, malaria, acute pancreatitis, or secondary bacterial infection. In unconjugated hyperbilirubinemia, the degree of jaundice is mild and may be caused by acute hemolysis in patients with Thalassemia, hemoglobinopathy. In case of dengue infection with severe jaundice, the severe complications such as liver failure, pancreatitis, severe bacterial sepsis or dual infection should be suspected.

4. Dual infection

Dual infection should be suspected in atypical presentation, for example, fever for more than 10 days, diarrhea, jaundice, persistent abdominal pain, recurrent fever, WBC >10,000/mm^3 (>$10x10^9$/l) with neutrophilia or presence of the band form of PMN. The patient with dengue infection may have subsequent nosocomial infection after hospitalization.

5. Internal hemorrhage in dengue infection

In a rapid decrease of Hct, internal hemorrhage should be suspected. Blood components such as PRC, FFP, and platelet concentration should be replaced as soon as possible after the patient has not responded to intravenous fluid.

Conclusions of guideline for the management of dengue infection in adults

1. Dengue infection should be suspected and monitored in adult patients who present with fever, because misdiagnosis and delayed treatment will worsen the disease progression and prognosis.

2. Complications of dengue infection should be closely monitored, for example, abnormal bleeding according to thrombocytopenia, shock (grade III or IV). Clinical assessment, hematocrit level, urine output, and urine specific gravity are used to adjust the rate of intravenous fluid.

3. Liver transaminase should be measured in adult patient especially when hepatitis is suspected or a history of paracetamol use of more than 2 gram per day has been noted. When AST and ALT are elevated, hepatotoxic medications such as paracetamol should be avoided.

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