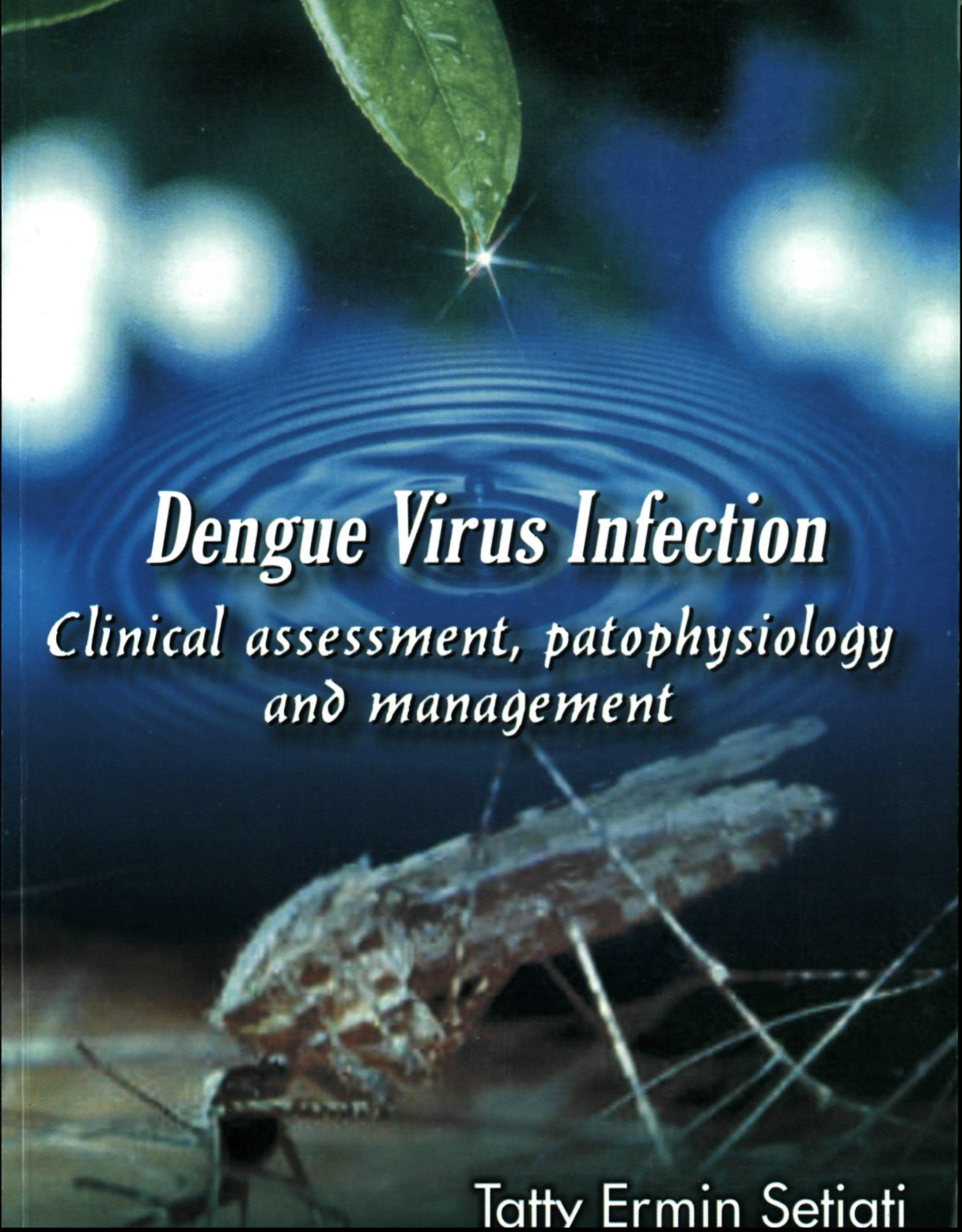
Dengue Virus Infection
Clinical assessment, patophysiology and management
Tatty Ermin Setiati

Dengue virus infection
Clinical assessment, pathophysiology and management

Een wetenschappelijke proeve
op het gebied van de Medische Wetenschappen

Proefschrift

ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de Rector Magnificus Prof. dr. C.W.P.M. Blom
volgens besluit van het College van Decanen
in het openbaar te verdedigen op donderdag 7 december 2006
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For my sons, daughter, grandson:
Tata, Endang, Dewi, Aby

Contents

I. Introduction and outline of the thesis

Chapter 1

INTRODUCTION AND OUTLINE OF THE THESIS

Tatty E. Setiati[1], Martijn D. de Kruif[2], Albert T.A. Mairuhu[2], Eric C.M. van Gorp[2], A. Soemantri[1]

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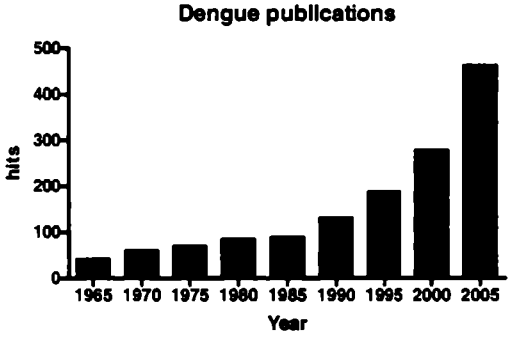
INTRODUCTION AND OUTLINE OF THE THESIS

Dengue virus infections are transmitted by mosquito bites and are particularly common in tropical and subtropical regions like South-East Asia and Latin America [1]. Due to factors like population and population density growth, increasing mobility and poor hygiene conditions in these areas, dengue virus infections are offering a growing public health problem. In South-East Asia with a total population of 1.5 billion people, approximately 1.3 billion live at risk of dengue virus infections. Infection may be fatal and nowadays it even is the leading cause of hospital admission and death among children in South-East Asia [2].

These figures emphasise that the search for appropriate treatment strategies needs to continue. The number of PubMed hits on dengue within one year has increased from 41 hits in 1965 to 463 hits in 2005 (Figure 1). The priorities of current research include the need to describe epidemiological patterns, to standardize methods of diagnosis and treatment, to unravel pathophysiological mechanisms and to develop new treatment strategies.

Figure 1. PubMed hits on 'dengue' by year.



In the current thesis, studies on dengue were carried out in Indonesia. This is by far the largest country of South-East Asia and it is particularly affected by dengue virus infections due to climate and demographic conditions that are further described in **chapter 2**. The studies were conducted in close collaboration with the Dr. Kariadi hospital in the city of Semarang in Java. This is a University based hospital serving a region with a population of over 1 million inhabitants. Our studies focussed in particular on epidemiological and clinical aspects of the disease and on the involvement of pathways of inflammation and coagulation in the disease.

CLINICAL ASPECTS AND MANAGEMENT

For clinical and scientific purposes, dengue virus infections are generally classified into Dengue Fever (DF), Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS) [2]. DF is a mild, uncomplicated and self limiting flu-like illness [3]. Symptoms usually occur after an incubation period of 4-7 days. When a case of DF is

suspected, one should consider the large differential diagnosis of DF, which includes malaria, leptospirosis, rickettsiosis, meningococcal infection, typhoid fever, hanta virus, measles, rubella and influenza virus infections including avian flu Dengue virus infections can be confirmed by ELISA based testing of specific IgM /IgG antibody titres [4] Other tests include commercial kits detecting IgM antibodies using a dipstick method, a Hemagglutination Inhibition test (HI), PCR testing and dengue virus isolation [1,2,5,6] Isolation is generally considered to represent the golden standard but unfortunately the test lacks sensitivity and requires a well equipped laboratory [6]

In contrast to the relatively innocent nature of DF, the infection may also result in DHF or DSS DHF is a severe illness with bleeding complications and circulatory changes When in addition to these symptoms shock develops, it is called DSS DHF is defined according to strict WHO criteria [2] by the presence of 1) fever or a history of acute fever, 2) at least one haemorrhagic manifestation, 3) thrombocytopenia of less than 100 000 cells/mm^3 and 4) signs of vascular leakage such as haemoconcentration defined as a 20% increase in haematocrit (compared to the stabilized haematocrit at hospital discharge or a haematocrit of 20% above the normal value for age) or other signs of plasma leakage (pleural effusion or other effusions, hypoproteinemia, and hypoalbuminemia, ascites) DSS is diagnosed when all DHF criteria are met plus evidence of circulatory failure defined as hypotension for age (systolic pressure < 90mmHg for those ≥ 5 years of age and ≤ 80mmHg for those ≤5 years of age) or narrow pulse pressure (< 20 mmHg) If patients don't meet the criteria for DHF or DSS, they are considered to be suffering from DF Although the WHO classification system has been widely applied in research settings and publications, its use in everyday clinical practice has not proven to be easy or practical Studies revealed that a significant portion of the patients did not fulfil the WHO criteria for DSS, whereas they clinically presented with a clear picture of shock [7] In **chapter 3**, we will discuss and evaluate the system in detail

The treatment of dengue virus infections is primarily supportive and focuses on prevention and treatment of complications No specific treatment for dengue virus infection is available Bleeding and plasma leakage leading to shock are among the most dangerous complications Blood transfusions are indicated if overt bleeding is present Fluid replacement therapy is important for the prevention of shock Current WHO guidelines recommend replacement of plasma losses with crystalloid solutions like Ringer's lactate initially, followed by boluses of colloid fluids like dextran for patients who have recurrent or refractory shock In **chapter 4**, we investigated the use of this standard protocol in comparison to a newly developed protocol starting with an initial colloid fluid replacement strategy

COAGULATION AND ENDOTHELIUM

One of the major complications of DHF/DSS is the occurrence of severe bleedings [3] However, the pathophysiologic mechanism underlying these bleedings is not precisely clear Previous data showed that together with the bleeding complications extensive activation of the coagulation system occurs [8] After infection, the

endothelial cells change into a procoagulant state by expressing Tissue Factor [9]. These data suggest that disseminated intravascular coagulation is involved in the process, which is discussed in **chapter 5** and **6**. Furthermore, lysis of coagulation induced clotting is impaired due to increased levels of plasminogen acitvator inhibitor (PAI)-1 [10,11]. Polymorphisms in the promotor region of plasminogen activator inhibitor (PAI)-1 have been associated with a worse outcome after infection. The involvement of the fibrinolytic system is investigated in **chapter 7** and **8**.

VASCULAR LEAKAGE AND IMMUNOLOGY

Another major complication of dengue virus infection is shock due to excess plasma leakage. The pathophysiology of this so-called plasma leakage syndrome in DHF/DSS involves activation of inflammatory pathways [8,12-15]. This causes endothelial dysfunction with loss of barrier function, which leads to excessive losses of fluid to the extravascular compartment and may result in shock and death [16]. Cytokines are believed to be important mediators in this process. Previous studies showed elevated levels of numerous cytokines including interferon (IFN)-gamma, tumor necrosis factor (TNF)-a, interleukin (IL)-1b, IL-2, IL-6, IL-8, and IL-10 [12-15,17]. Together, a complex interaction between multiple inflammatory pathways is involved in the pathophysiology of DHF/DSS, characterized by excessive host damage due to its essentially protective immune system. In **chapter 9**, we describe the activation of inflammatory pathways during dengue infection in detail.

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Chapter 2

CHANGING EPIDEMIOLOGY OF DENGUE HAEMORRHAGIC FEVER IN INDONESIA

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Submitted

INTRODUCTION

Dengue fever (DF) and dengue haemorrhagic fever (DHF) are a growing public health problem in the (sub) tropics [1]. In South-East Asia with a total population of 1.5 billion people, approximately 1.3 billion live at risk of acquiring DF or DHF. Nowadays DHF is the leading cause of hospital admission and death among children in this region [2]

Major dengue epidemics date back too the late 17th century. However, the start of epidemics of severe dengue began in the Southeast Asia region following World War II, when conditions for mosquito-borne diseases were favourable [3]. Dengue virus infections during these newly described epidemics were accompanied by severe haemorrhage, shock and vascular leakage. The first recorded DHF epidemic occurred in Manila, the Philippines in 1953 [4]. Thereafter the epidemic spread quickly throughout Southeast Asia and further west via India, Sri Lanka, the Maldives and Pakistan and east to China [3]. Many factors are thought to be responsible for the global (re)emergence of DF and DHF. These include major global demographic chances, and worsening of healthcare systems and mosquito control programmes [1;1;5].

Indonesia is the largest country in the region and counts approximately 245 million inhabitants. Almost sixty percent of the Indonesians live on the island Java, which is most severely afflicted by periodic outbreaks of dengue disease [6]. However, the disease is endemic in many large cities and small towns throughout Indonesia and has also spread to certain smaller villages, where population movement and population density is high [7]. Epidemic DF has been described in all 27 Indonesian provinces, whereas in 1968 only 2 provinces reported dengue cases [7;8]. This article will address the epidemiology of dengue in Indonesia, by means of a chronological overview.

METHODS

Literature Search and Data Sources

A systematic review was performed to identify eligible articles. The MEDLINE database from 1966 through 2005 was searched by two reviewers (JFPW and MDK). This was performed, by combining the Medical Subject Headings (MeSH terms) and text words *Dengue, Dengue Virus, Outbreak, Epidemiology* and *Indonesia*. Cross-references cited in selected articles and reviews were hand-searched for relevant articles. In addition, the Indonesian Ministry of Health and experts in the field were contacted for data on epidemics occurring from 1966 through 2005. This was performed by two different reviewers (TES and AS).

Study Selection and Data Extraction

All identified epidemiological studies were selected. Inclusion was restricted to studies in which primary data describing morbidity or mortality could be extracted. Selections of studies and data extraction were independently performed by two of the authors (ATAM and JFPW). Disagreement was resolved by discussion and if necessary by adjudication of a third reviewer (ECMG).

RESULTS

The epidemiology of dengue in Indonesia

The first initial dengue cases in Indonesia were reported in the year 1968 in the cities of Jakarta (West Java) and Surabaya (East Java) [7;9-11]. These reports were followed by reports from Bandung (West Java) and Yogyakarta (Central Java) [12;13]. During the first year of recognition, a total of 58 cases (including 24 deaths) were recorded [7]. In 1970, a total number of 34 dengue cases were identified among 48 suspected paediatric patients in Yogyakarta [12]. Subsequently, eight were classified with DHF and two cases progressed to dengue shock syndrome (DSS). The first isolations of dengue virus took place during the 70s in Jakarta, Medan (North Sumatra) and Semarang [12;14-17]. During the 1973 outbreak, a total number of 10.189 cases were reported, 6.225 of these cases were recorded in Semarang (Central Java) [7]. Limited data suggest that Dengue virus serotype 2 (DEN-2) was the predominant virus in that time [18] and indeed DEN-2 was isolated most frequently from the Semarang epidemic [14]. In 1974 an outbreak was reported outside the island of Java, in Manado, North Sulawesi [19]. Of the 195 cases assessed, 125 suffered from DHF, the other 70 of DSS. Of the 125 serological identifications of the etiological agent, Dengue virus serotype 1 (DEN-1) was found predominantly in both non-shock and shock cases. Early 1976, Dengue virus serotype 3 (DEN-3) was increasingly associated with fatal cases of DHF in Jakarta [20]. Epidemics of DHF associated with DEN-3 continued to spread into 1977 in Java and West Kalimantan [20]. It was suggested that the virulence of DEN-3 had chanced.

A very explosive epidemic of DHF characterized by severe disease and high viremia occurred in Bantul, a rural area in Central Java, during the latter part of 1976 and early 1977 [14;21]. The case fatality rate (CFR) based on reported cases was 2.5%. Three dengue serotypes were isolated of which DEN-3 was the predominant one. If al shock cases were considered, no relationship between dengue serotype and disease severity were found, however all three fatal cases were associated with DEN-3. A year later a less explosive dengue type 3 outbreak occurred in Sleman, 40 km north of Bantul, characterized by mild disease [22]. During the first 3 months of that year, 166 cases and two deaths were reported. The age distribution was similar to that of the Bantul outbreak, however, the contrast between disease severity and the viremia levels were noteworthy.

A five month long outbreak emerged in September 1993 trough February 1994 in the city of Jayapura, the provincial capital of Papua, easternmost Indonesia. Although the first case of DHF was reported in 1979, the disease had long been gone from this region [8]. A total of 217 cases were reported and 72 suspected DHF cases were enrolled into an outbreak investigation [8]. Virus types 1, 2 and 3 were isolated, all from seronon reactive cases. Of these, DEN-3 was the predominant virus found. The majority (68.7%) did not have significant levels of specific IgM at time of their admission, 11.4% had a primary infection and 17.1% a secondary infection. These results indicate that over time more than one dengue virus has been introduced or reintroduced in this area.

A prospective study initiated in 1995, showed that the total dengue infection rate among 4-9 year old children in Yogyakarta was 29.2% [23]. All four dengue viruses

were transmitted, of which DEN-2 slightly predominated. An average of 56.2% had antibodies against one or more virus types at the start of the study, of these 34.3% were immune to a single dengue virus. Six of the 1.837 included children were hospitalized during follow up, all of them appeared to have a secondary or tertiary dengue infection. In the whole cohort, 6.5% of the observed secondary infections were of the sequence DEN-2 — DEN-1, an unexpected finding because DEN-1 — DEN-2 was found highly pathogenic in previous studies.

During the period November 1997 till May 1998, again DHF outbreaks were reported throughout the Indonesian archipelago [24]. Eleven provinces were involved and all the outbreaks took place in urban areas. The outbreak started during November 1997 in Jambi, South Sumatra, and Lampung (western part of Indonesia). From here the endemic spread involving all provinces in Sulawesi and the provinces of West Nusatenggara and East Timor, continuing towards Ambon, the capital of Maluku province and finally it arrived in Jakarta in March 1998. The average attack rate was 1.2 per 1000 population whereas the highest attack rate occurred in Palembang, a city in south Sumatra (1.6 per 1000 population).

Trend analysis of the 1998 Palembang outbreak demonstrated a 3-fold increase of dengue cases between January and April compared to historical records [25]. Serum samples, obtained during acute illness from 221 hospitalized patients, showed in 59% evidence of a dengue infection. All serotypes were identified, predominantly serotype 3. Serotype 1 was associated with less severe disease. Concerning age distribution it has to be noted that a relatively large proportion of adolescents and young adults were infected.

A prospective cohort study was initiated among adult textile factory workers in Bandung, West Java from 2000 till 2002 [26]. All 2.536 recruited subjects were adults. The incidence of symptomatic dengue disease was 18 cases/1000 persons-years and an estimated asymptomatic/mild infection rate of 56 cases/1000 persons-years. All four serotypes were detected, with DEN-2 predominating. Four cases of DHF were identified; three of them were due to DEN-3 virus. Only one subject developed DSS, due to a secondary infection of the sequence DEN-2 — DEN-1.

In April 2001, a second outbreak in Papua occurred [27]. In Merauke, a town located in the southeastern corner of the province, a retrospective case control study was performed. Fifteen acute cases, 37 convalescing subjects and 32 comparable controls were enrolled. Dengue virus IgM antibodies were detected in 27% of the acute clinical cases, 30% in the convalescing cases and in only 3% of the controls. Dengue 3 was the only dengue virus serotype detected by RT-PCR, all in the acute samples. By reviewing hospital records, a total number of 172 suspected cases were identified. The estimated CFR among all suspected dengue cases was 1.2%.

To study natural history of dengue virus infection in West Jakarta, 785 volunteers were included using a cluster investigation method in the period from October 2001 to October 2003. All subjects were family members or neighbours of 53 index cases, which had previously been hospitalized [28]. Among the index cases the predominant virus type was DEN-1, followed by DEN-2. Seventeen new infections (2.2%) were identified post enrolment, most of them caused by DEN-2. Nine were symptomatic of which one case progressed to DHF. The calculated incidence rate of dengue infection

was 567 cases/1000 persons-years of follow up, the calculated DHF incidence rate was 33 DHF cases/1000 persons-years of follow up.

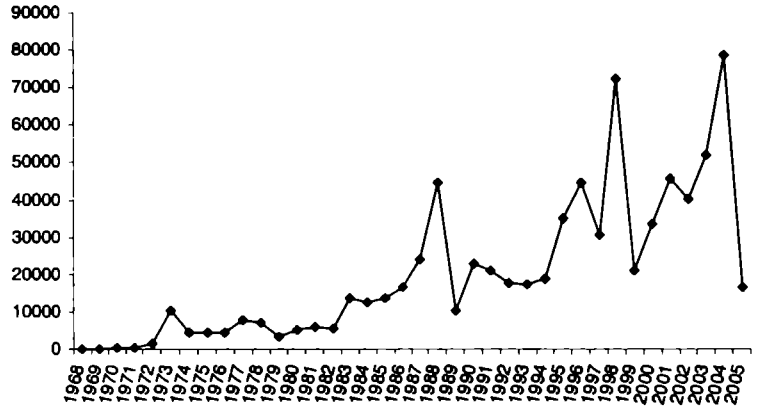
In 2004 another outbreak began to spread throughout Indonesia, with Jakarta being the most affected area [29]. According to the WHO, 78.690 cases and over 900 deaths were reported in Indonesia that year. Ten hospitals, all located in Jakarta, included a cohort of 272 hospitalized patients to confirm disease aetiology [6]. Dengue infection was determined in 66.2% of all cases, 55.6%, 17.2% and 27.2% had DF, DF with hemorrhagic manifestations and DHF respectively. Of the confirmed DHF cases, 82.5% had evidence of a secondary infection. Viral isolation identified all 4 serotypes. Serotype 3 was recovered most frequently. There was a greater extend of infections among those of 15 years and older.

Morbidity and Case Fatality Rates

Over time the total number of reported dengue cases in Indonesia is still increasing (Figure 1). The epidemic has spread over all 29 Indonesian provinces (Figure 2). During the years 1998 and 2004 a record number of 72133 and 78690 cases were documented. The inter-epidemic background lies between 10.000 and 25.000 cases annually. The Indonesian health care authorities reported a dengue morbidity rate of 15.28 per 100.000 persons, 30 per 100.000 persons and 13.7 per 100.000 persons in the years 1997, 2004 and 2005 respectively (Unpublished data, Indonesian Ministry of Health).

The last decade, dengue disease caused every year more then 400 deaths in Indonesia and in 1998 this number was even 1414 (Figure 3). When appropriate supportive therapy is given the CFR in Asian countries lies approximately between 0.5 and 3.5% [30]. In Indonesia, CFR have steadily declined over time from 41% in 1968 [11] to less than 2% since 2000 and a lowest rate of 1.21% in 2004 (Figure 4) [31]. In 2005 the mean case fatality rate was 1.34%, but this number varies between the Indonesian provinces (Figure 5) [31].

Figure 1. DHF in Indonesia: 1968-2005

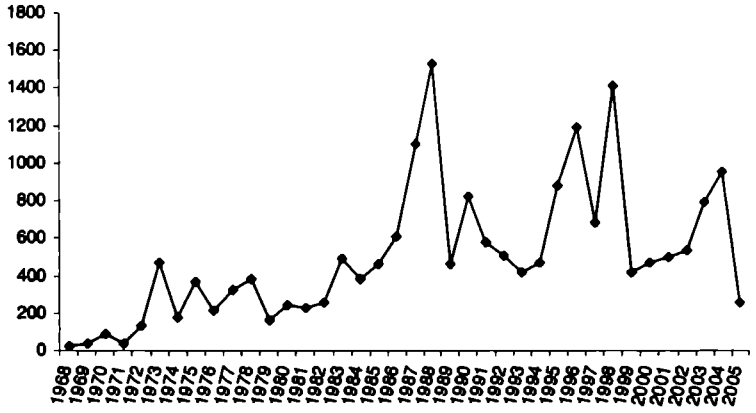


The Y axis represents the total number of DHF cases officially reported during the years 1968-2005. (source: WHO, Sumarno 1987)

Figure 2. The incidence rate of DHF

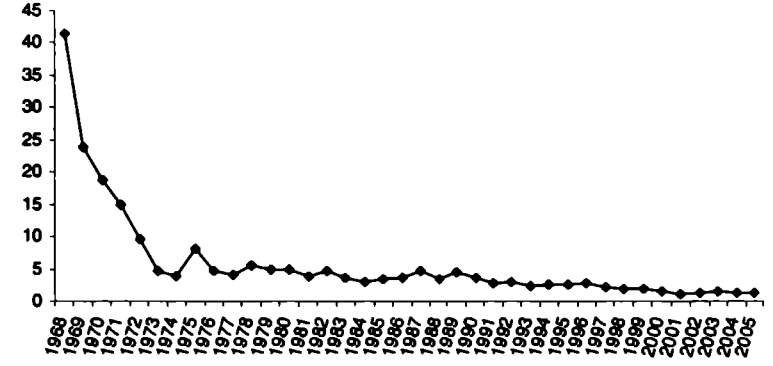
IR denotes incidence rate (number of DHF cases among 100.000 civilians) throughout the 29 Indonesian provinces in 2005. (source: Ministry of Health, Indonesia, 2006).

Figure 3. Mortality from DHF in Indonesia: 1968-2005.



The Y axis represents the total number of deaths from DHF per year during the years 1968 till 2005. (source: WHO, Sumarno 1987)

Figure 4. Case fatality rate among DHF patients in Indonesia: 1968-2006



The case fatality rates (%) are denoted on the Y axis in percentages during the years 1968-2005. (source: WHO, Sumarno 1987)

Figure 5. Case fatality rates (CFR) of DHF

CFR denotes case fatality rates (percentage of deaths DHF among reported cases) throughout the 29 Indonesian provinces in 2005 (source: Ministry of Health, Indonesia, 2006)

CONCLUSION

Epidemics of DF and DHF have become an important public health problem throughout the Indonesian archipelago since it was first recognized in 1968. The epidemiology of DHF in Indonesia is changing alarmingly given the increasing number of infections and the high percentage of adolescents and adults developing DHF [6]. The median age of DHF patients from Jakarta was 4 years and 11 months in de period from 1979-1984 [7]. Recent data from the Indonesian ministry of health, show an increasing number of DHF in children of the age of 15 and older (Unpublished data, Indonesian Ministry of Health). Also other reports show this trend [6;25].

Outbreak trends of DF/DHF are generally characterized as cyclic, however in Indonesia this pattern has become somewhat irregular. Multiple factors influence the occurrence of dengue epidemics, of these environmental, biological and demographic issues play a central role.

Dengue incidence is associated with warmer, more humid weather [5]. Higher temperatures have been shown to enhance vector efficiency [32], and mosquito biting behaviour [33]. Indeed, a marked increase in rainfall and sustained higher temperatures compared to earlier years were key factors during the Palembang outbreak [25]. Furthermore, the epidemiological pattern throughout the year shows a peak incidence of DF/DHF during the months of October trough April, usually coinciding with the rainy season [8;11;22;34].

Endemic transmission requires besides the mosquito vector, an immunological susceptible population and the circulation of dengue virus. The presence of multiple circulating strains (hyperendemicity) and the introduction of new and more virulent viruses, increase the incidence of DHF epidemics [1]. Some Indonesian studies clearly showed the relevance of secondary infections [6;23]. Of al serotypes identified in Indonesia, serotype 3 was most frequently linked to severe disease [6;25;35]. Important demographic factors contributing to the transmission of dengue viruses also concern unprecedented population growth accompanied by unplanned and uncontrolled urbanisation, a framework in which Indonesia perfectly fits.

Dengue is on the rise in Indonesia. Hence epidemiological surveillance must be established hand in hand with education campaigns and sustainable vector control programmes to control its transmission. An increasing number of severe and potentially fatal number of cases in the absence of an effective vaccine, stress the need to continue all efforts to try get a further understanding the pathophysiology of dengue disease.

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II. Clinical aspects and management

Chapter 3

DENGUE DISEASE SEVERITY IN INDONESIAN CHILDREN: AN EVALUATION OF THE WORLD HEALTH ORGANIZATION CLASSIFICATION SYSTEM.

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Submitted

ABSTRACT
Dengue disease severity is usually classified using a system set up by the World Health Organization (WHO). However, the use of this strict classification system in everyday clinical practice has not proven easy or practical. We aimed to assess the diagnostic accuracy of the WHO classification system and modifications to this system, and evaluated their potential practical usefulness. We analysed clinical data of patients, who were admitted consecutively to the hospital with a severe Dengue virus infection. Patients were classified using the WHO classification system and six modifications to this system. In addition, treating physicians were asked to classify patients immediately after discharge. We calculated the sensitivity of the various classification systems for the detection of shock and the agreement between the various classification systems and the treating physician's classification. Of 152 patients with confirmed Dengue, sixty-six (43%) had evidence of circulatory failure. The WHO classification system had a sensitivity of 86% (95%CI 76-94) for the detection of patients with shock. All modifications to the WHO classification system had a higher sensitivity than the WHO classification system (sensitivity ranging from 88% to 99%). The WHO classification system was in only modest agreement with the intuitive classification by treating physicians whereas several modified classification systems were in good agreement. The use of the strict WHO classification system to classify Dengue disease severity is to be questioned, because it is not accurate in correctly classifying Dengue disease severity and it lacks sufficient agreement with clinical practice.

INTRODUCTION

Dengue virus infections are recognized as major public health problems in tropical and subtropical regions. Each year an estimated 100 million infections occur and between 250.000 and 500.000 severe cases are reported to the World Health Organization (WHO) [1]. Infection with any of the four Dengue virus serotypes may give a broad spectrum of clinical manifestations ranging from no or minimal symptoms that cannot be easily distinguished clinically from other viral infections to a more severe form of disease that is characterized by a haemorrhagic tendency and increased vascular permeability. The WHO set up a classification system to differentiate between the self-limiting though debilitating Dengue fever (DF), and the potentially lethal Dengue haemorrhagic fever (DHF) [2]. According to these criteria, DHF is defined by the presence of fever, a haemorrhagic tendency, thrombocytopenia and some evidence of plasma leakage due to increased vascular permeability. DHF is further subdivided, with most severe cases categorized as Dengue shock syndrome (DSS) when circulatory failure is present [2].

A standardized classification system for the severity of Dengue virus infections is crucial for optimal communication of scientific data to improve our understanding of the pathogenesis and treatment of the disease. Incorrect disease severity classification may lead to faulty decision making in choosing the most appropriate treatment for the individual patient. Although the WHO classification system has been widely applied in research settings and publications, its use in everyday clinical practice has not proven easy or practical. In recent years, several studies reported difficulties with classification, inconsistencies in the WHO classification system and some found it necessary to define new categories to identify severe cases that do not meet the criteria for DHF or DSS [3-9].

These findings raise the question if the current WHO classification system is appropriate for the classification of Dengue disease severity. To answer this, we assessed the diagnostic accuracy of the WHO classification system and modifications to this system. The presence of shock was used as marker of disease severity. By comparing the various classification systems with an intuitive classification done by treating physicians, we additionally evaluated the practical usefulness of the WHO classification system and the various modified classification systems.

PATIENTS AND METHODS

Patients and clinical procedures

The study was conducted from February 2001 to April 2003 on the paediatric intensive care unit and paediatric ward of the Dr. Kariadi Hospital in Semarang, Central Java, a region in Indonesia where Dengue is endemic. All patients, aged 2 to 14 years, consecutively admitted to the hospital with suspected severe Dengue virus infection were eligible. Patients were monitored on an hourly basis by medical personnel. In case of unstable vital signs, routine laboratory test results, including platelet count and haematocrit, were performed every 3-4 hours until patients were stable. Based on periodic haematocrit determination, platelet count measurement and vital signs, treatment was reviewed and revised according to current guidelines [2]. Members of the study team recorded demographic data, medical history, physical

examination findings, clinical course and routine laboratory test results for each patient on a standard data form. Blood samples for diagnostic procedures were obtained on day of admission and on day 7 after enrolment, provided that a parent or legal guardian gave informed consent. The ethics committee of the Dr. Kariadi Hospital approved all clinical and laboratory aspects of this study.

Classification of disease severity

The study was initially designed to study pathophysiological mechanisms of Dengue virus infections. During the study, treating physicians were asked to classify patients as having DF, DHF or DSS immediately after patients were discharged. This intuitive classification was based on knowledge of medical history, physical examination findings, clinical course and all available results of routine tests (such as blood tests and chest X-ray). No structured algorithm was used to classify patients. The respective treating physicians were many different residents who worked under supervision of staff physicians at the paediatric intensive care unit and the paediatric ward of the Dr. Kariadi Hospital. Physicians were unaware of the fact that a structured classification was performed after completion of the study.

After completion of the study, two investigators (PK and ATAM) determined the presence of the following four clinical and laboratory manifestations on admission and during follow up in every patient using the standard data form: 1) fever or a history of acute fever, 2) haemorrhagic manifestations (at least a positive tourniquet test), 3) thrombocytopenia (platelet count $\leq$100.000 cells/mm^3), and 4) signs of plasma leakage (haematocrit $\geq$ 20% above average for age, haematocrit $\geq$ 20% compared to the haematocrit at hospital discharge, pleural effusion, hypoproteinemia or hypoalbuminemia). This was performed independently of each other and blind to the treating physician's classification.

Patients were subsequently classified as having DHF using these manifestations as proposed by the WHO [2]. If patients did not have one or more of these manifestations, they were considered to suffer from DF. In addition, several modifications to the WHO classification system were made as previously described [8]. Since all patients had fever or had a history of acute fever, the remaining three manifestations (haemorrhagic manifestations, thrombocytopenia and signs of leakage) were used to make the modifications. This resulted in six modified classification systems (Table 1). DSS was diagnosed when all DHF criteria were met plus evidence of circulatory failure defined as hypotension for age (systolic pressure <90 mmHg for those $\geq$ 5 years of age and <80 mmHg for those <5 years of age) or narrow pulse pressure (<20 mmHg) [2].

Table 1. Modified systems for the classification of Dengue haemorrhagic fever.

Bleeding and thrombocytopenia
Bleeding and haemoconcentration
Haemoconcentration and thrombocytopenia
Bleeding and thrombocytopenia or haemoconcentration
Thrombocytopenia and haemoconcentration or bleeding
Haemoconcentration and bleeding or thrombocytopenia

DIAGNOSTIC PROCEDURES

Paired blood samples were tested for serologic evidence of acute Dengue infection. Commercially available enzyme-linked immunosorbent assays (Focus Technologies, Cypress, Calif., USA) were used for the detection of Dengue virus specific IgG and IgM antibodies, according to the procedures described by the manufacturer. The sensitivity and specificity of these tests have been evaluated previously [10]. Cases were considered serologically-confirmed if the IgM ELISA was positive during the acute phase of disease (optical density of the sample higher than the optical density of the cut off serum provided by the manufacturer) and/or if a four-fold increase in IgG titre was demonstrated in paired acute and convalescent sera. For some patients, a definitive serodiagnosis was not possible because no convalescent sample was obtained. Detection of Dengue virus antigen and/or viral RNA was attempted in these cases using a dot blot immunoassay and a Dengue serotype specific reverse transcription PCR respectively [11]. Patients with definitive serodiagnosis and/or positive dot blot and/or positive PCR were considered to have confirmed Dengue virus infection. The absence of Dengue virus antigen, viral RNA and negative serology, was indicative of a non-Dengue virus infection. When an alternative clinical diagnosis was absent and serology was inconclusive, patients were characterized as indeterminate.

Statistical analysis

The evaluation of the diagnostic accuracy of the various classification systems was based on the presence of circulatory failure on admission and during follow up as the "reference standard". We calculated the proportion of patients with circulatory failure who were correctly classified as DHF (sensitivity). In addition, we calculated the proportion of patients classified as DF by the WHO classification system and without circulatory failure who were reclassified as having DHF when the modified classification systems were applied. The corresponding exact 95% confidence intervals (95% CI) were calculated from the binomial distribution.

As a measure of agreement between various classifications systems and the intuitive classification by the treating physician, we calculated the weighted kappa (k_w) statistic with a 95% confidence interval. The k_w values were interpreted as: poor agreement, $\leq$0.20; fair agreement, 0.21 to 0.40; moderate agreement, 0.41 to 0.60; good agreement, 0.61 to 0.80; or very good agreement, $\geq$0.81 [12]. A P-value $\leq$0.05 was considered to indicate statistical significance. Analyses were performed using SPSS 12.0.0. Agreement between the various classification systems and classification by treating physician was performed using MedCalc version 8.0.0.0.

RESULTS

A total of 198 patients were initially enrolled in this study. Parents from thirteen patients withdrew informed consent during follow up. Two patients appeared not to suffer from Dengue during follow up (1 patient was diagnosed with measles and 1 patient was diagnosed with malaria). Of the remaining 183 patients, 28 patients (15%) had inconclusive serology and were therefore categorised as indeterminate. Three patients (2%) had definitive negative serology and were categorized as not

Dengue. The presence of Dengue was objectively confirmed in 152 patients (83%). Of the patients with confirmed Dengue, 66 had evidence of circulatory failure: 52 (34%) had shock on admission and 14 (9%) went on to develop shock during the hospital admission. Six patients (4%) died. Patient characteristics on admission are summarised in Table 2.

Table 2. Baseline characteristics at admission of 152 patients with confirmed acute Dengue.*

Variable	
Age, median (IQR)	7 years (5-10)
Male sex, n (%)	74 (49)
Duration of illness, median (IQR)	4 days (3-4)
Haemorrhagic tendency, n (%)	133 (88)
Positive tourniquet test, n (%)	103 (68)
Spontaneous haemorrhage, n (%)	93 (61)
Hepatomegaly, n (%)	105 (69)
Systolic blood pressure, median (IQR)	90 mmHg (80-100)
Hypotension for age, n (%) †	50 (33)
Pulse pressure <20 mmHg, n (%)	12 (8)
Pulse rate, median (IQR)	120 beats/min (104-128)
Presence of pleural effusion, n (%) ‡	102 (67)
Haematocrit, median (IQR)	41% (36-45)
Platelet count, median (IQR)	58.000 cells/mm^3 (37.000-85.000)
Platelet count <100.000 cells/mm^3, n (%)	131 (86)

* n, denotes number; IQR, denotes interquartile range
† hypotension is defined to be a systolic pressure of <80 mmHg for those <5 years of age, or <90 mmHg for those >5 years [2]
‡ the presence of pleural effusion was assessed through a chest X-ray

Diagnostic accuracy of the various classification systems
According to the WHO classification system, 20 (13%) patients were classified as having DF and 132 (87%) as having DHF. Of the DHF group, 57 (43%) could be classified as having DSS. Of the 66 patients with confirmed Dengue and circulatory failure, 9 (14%) failed to meet all four criteria necessary for a diagnosis of DHF, and were thus classified as having DF. Six patients had a negative tourniquet test result and no bleeding manifestations during hospital admission, 1 patient never had a platelet count less than 100.000 cells/mm^3, and the remaining 2 had no evidence of haemoconcentration or other signs of plasma leakage. The WHO classification system had a sensitivity of 86% (95%CI 76-94). The number of patients with circulatory failure classified as DHF changed when the WHO classification system was modified. Interestingly, all modifications had a higher sensitivity than the WHO classification system. The sensitivities of the various classification systems are shown in Table 3. Eleven patients without circulatory failure were classified as having DF according to the WHO classification system. Modification by the various systems came at the expense of some DF patients being reclassified as DHF as shown in Table 4.

Table 3. Sensitivity of the various disease classification systems in 152 patients with confirmed acute Dengue virus infection.*

Criteria used for the classification of DHF patients	Number of patients with circulatory failure (n= 66) classified as DHF	Sensitivity (95% CI)
WHO classification system	57	86 (76-94)
Bleeding and thrombocytopenia	59	89 (79-96)
Bleeding and haemoconcentration	58	88 (78-95)
Haemoconcentration and Thrombocytopenia	63	96 (87-99)
Bleeding and thrombocytopenia or haemoconcentration	60	91 (81-97)
Thrombocytopenia andhaemoconcentration or bleeding	65	99 (92-100)
Haemoconcentration and bleeding or thrombocytopenia	64	97 (89-100)

* n, denotes number; CI, denotes confidence interval; DF, denotes Dengue fever; DHF, denotes Dengue haemorrhagic fever; WHO, denotes World Health Organization.

Table 4. Proportion of patients reclassified from DF to DHF when the various modified classification systems are applied.*

Criteria used for the classification of DHF patients	Number of DF patients without circulatory failure reclassified to DHF	Proportion (95% CI)
Bleeding and thrombocytopenia	5	45 (17-77)
Bleeding and haemoconcentration	1	9 (0-41)
Haemoconcentration andThrombocytopenia	2	18 (2-52)
Bleeding and thrombocytopenia or haemoconcentration	6	55 (23-83)
Thrombocytopenia andhaemoconcentration or bleeding	7	64 (31-89)
Haemoconcentration and Bleeding or thrombocytopenia	3	27 (6-61)

* DF, denotes Dengue fever; DHF, denotes Dengue haemorrhagic fever; WHO, denotes World Health Organization.

Disease classification by treating physicians

Treating physicians classified 8 patients (5%) as having DF and the remaining 144 patients (95%) as having DHF. Of the 144 patients diagnosed as having DHF, 91 (63%) were considered to have circulatory failure at admission or at some point in time during admission in the hospital. In addition to the 66 patients with hypotension or narrow pulse pressure, treating physicians considered 25 patients with tachycardia, restlessness and cold and clammy skin as having compensated shock

and subsequently diagnosed them as having circulatory failure. Agreements between the various classification systems and disease classification by treating physicians are shown in Table 5. The WHO classification system and three modified classification systems showed moderate agreement with classification by the treating physician: bleeding + thrombocytopenia, k_w value 0.53 (95% CI 0.41-0.64); bleeding + thrombocytopenia or haemoconcentration, k_w value 0.55 (95% CI 0.43-0.66); WHO classification system, k_w value 0.57 (95% CI 0.45-0.68) ; and bleeding + haemoconcentration, k_w value 0.59 (95% CI 0.47-0.71). The remaining three modified classification systems showed good agreement with classification by the treating physician: thrombocytopenia + bleeding or haemoconcentration, k_w value 0.64 (95% CI 0.53-0.74); thrombocytopenia + haemoconcentration, k_w value 0.67 (95% CI 0.56-0.78); and haemoconcentration + thrombocytopenia or bleeding, k_w value 0.70 (95% CI 0.59-0.81).

Table 5. Agreement between the various classification systems and classification by the treating physician.*

		Classification by treating physician		
		DSS	DHF	DF
WHO classification system	DSS	57	0	0
	DHF	23	52	0
	DF	11	1	8
Bleeding and thrombocytopenia	DSS	59	0	0
	DHF	23	52	5
	DF	9	1	3
Bleeding and haemoconcentration	DSS	58	0	0
	DHF	23	53	0
	DF	10	0	8
Haemoconcentration and	DSS	63	0	0
Thrombocytopenia	DHF	25	52	0
	DF	3	1	8
Bleeding and	DSS	60	0	0
thrombocytopenia or haemoconcentration	DHF	23	53	5
	DF	8	0	3
Thrombocytopenia and	DSS	65	0	0
Haemoconcentration or bleeding	DHF	25	52	5
	DF	1	1	3
Haemoconcentration and	DSS	64	0	0
bleeding or thrombocytopenia	DHF	25	53	0
	DF	2	0	8

* DF, denotes Dengue fever; DHF, denotes Dengue haemorrhagic fever; DSS, denotes Dengue shock syndrome; WHO, denotes World Health Organization.

DISCUSSION

Our results show that a considerable number of Dengue virus infected patients with circulatory failure is not identified correctly when the WHO classification system is applied. By modifying the combination of criteria that are included in the WHO classification system, we were able to identify more patients with circulatory failure. Overall, 86% of patients with circulatory failure were identified correctly by the strict WHO classification system. By contrast, four modified classification systems recognized more than 90% of patients with circulatory failure as DHF.

These findings are largely in line with observations made by Phuong and colleagues who studied a considerable larger group of Vietnamese patients [8]. They also demonstrated that all modified classification systems outperformed the WHO classification system in the identification of 631 patients with confirmed Dengue and circulatory failure. A high sensitivity (> 90%) was found with the following modified classification systems: a haemorrhagic tendency with either thrombocytopenia or haemoconcentration (93%), haemoconcentration with either thrombocytopenia or a haemorrhagic tendency (94%) and thrombocytopenia with either haemoconcentration or a haemorrhagic tendency (95%). If the purpose of classifying Dengue disease severity is to identify the maximum number of patients with circulatory failure, then it would seem, that on the basis of Phuong's and our results, one of these modified classification systems is to be preferred.

Likewise, we found that the WHO classification system was in only modest agreement with the intuitive classification by treating physicians. Treating physicians were inclined to classify patients with evidence of plasma leakage as having DHF even in the absence of both thrombocytopenia and a haemorrhagic tendency. As a result, the modified classification systems haemoconcentration and thrombocytopenia, and haemoconcentration with either thrombocytopenia or a haemorrhagic tendency demonstrated good agreement with the intuitive classification by treating physicians. Phuong and colleagues noted that in clinical practice many physicians use the modified system of a haemorrhagic tendency usually together with thrombocytopenia rather than haemoconcentration. In our study we showed that this is not the case, all the more because the intuitive classification by treating physicians was also the same as the final clinical diagnosis given on discharge.

Although several modified classification systems were in good agreement with the intuitive classification by treating physician, they still did not reach the magnitude of agreement we had expected (maximum k_w value 0.70). Two separate views of treating physicians played a prominent part in this. First, a considerable number of patients were classified as shock, although they did not meet the WHO criteria for shock. Treating physicians classified patients as having shock when symptoms and signs, such as cool and clammy skin, rapid pulse, decreased urinary output and confusion, indicating a stage I or compensated shock were present. This was done even in the absence of hypotension for age or a narrow pulse pressure. Second, patients who had no evidence of plasma leakage but did have a haemorrhagic tendency and thrombocytopenia were classified as DF. However, when circulatory failure was present a classification of DSS was given. To make a distinction between patients with and without evidence of plasma leakage an additional classification could be useful [4;5].

Several potential limitations of this study should be noted. First, grading of Dengue disease severity was performed at the time of discharge or after completion of the study. By determining disease severity retrospectively, the classification systems may only serve a limited number of purposes. For example, they may be used to collect public health data on incidence of severe disease in different locations or over time. The classification systems are not suited for the identification of those at risk for developing severe disease, but they can be used as outcomes of interest in clinical trials and observational studies focusing on for example risk factors that may predict poor outcome. Second, patients' clinical course was assessed daily using standard data forms, regular haematocrit and thrombocyte measurements and additional diagnostic testing (i.e. chest radiography). This enabled us to collect all the data necessary for classification of disease severity. If regular laboratory testing or additional diagnostic tests are not performed because of for example limited resources, it may be difficult to demonstrate the presence of haemoconcentration with the possibility of misclassification. Finally, our analysis was confined to Javanese patients. As far as we know, only one other study performed in a Vietnamese population assessed the same modifications [8]. We can therefore only speculate on the possibility of extrapolating our findings to populations originating from other Dengue affected areas like for example the American region.

An increasing number of Dengue infections have been related to other unusual manifestations. These include fulminant liver failure, cardiomyopathy, ocular manifestations and neurological phenomena such as altered consciousness, convulsions, and coma resulting from encephalitis and encephalopathy [1;13-16]. Neurological manifestations were initially ascribed to complications secondary to DHF and DSS, although recently the invasion of the central nervous system by Dengue viruses has been suggested [16]. Patients with symptoms or signs of a suspected infection of the central nervous system were not considered for inclusion in our study nor was additional diagnostic testing (i.e. lumbar puncture) done in included patients with minor neurological symptoms. We are therefore unable to pronounce upon the possibility of an increase in patients with encephalitis caused by Dengue viruses. If additional studies show that such is the case than this would highlight the fact that the WHO classification needs revision.

In conclusion, our results show that the WHO classification system is less accurate in correctly classifying Dengue disease severity than all other modified classification systems. The implication of these findings is to question the use of the strict WHO classification system to classify Dengue disease severity, all the more because it lacks sufficient agreement with how patients are classified in clinical practice. Additional prospective clinical reports from as many centres as possible are needed to learn the extent of the variability, ultimately leading to an improvement of the clinical usefulness of the system.

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Chapter 4

REDUCED MORTALITY AFTER USE OF A COLLOID FLUID REGIMEN FOR PRIMARY RESUSCITATION OF CHILDREN WITH SEVERE DENGUE SHOCK SYNDROME

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ABSTRACT
Dengue shock syndrome (DSS) causes significant morbidity and mortality among Asian children. The cornerstone of treatment is adequate fluid replacement therapy, but only few studies compared different fluid replacement strategies in properly conducted clinical trials, showing variable results. The aim of this study was to evaluate the effects of a colloid fluid regimen containing 6% hydroxyethyl starch (HES) of 200 kD molecular weight in comparison to currently by the WHO recommended treatment with Ringer's Lactate (RL) in an open-labeled, randomized clinical trial. Sixty Indonesian children admitted to the hospital with severe DSS were included. Children were assigned initial fluid resuscitation for 10-30 minutes with HES (n=30) or RL (n=30). Several clinical and laboratory data were collected. Treatment with HES significantly reduced mortality from 27% to 7%, which is a reduction of 74%. In addition, organ complication rates, shock recovery times and duration of vascular leakage were shortened in the intervention group, as well as duration of mechanical ventilation and length of stay at the PICU. No adverse reactions were noted. We conclude that a short, initial infusion of colloid fluids like HES in children with severe DSS is safe and can improve mortality in a cost-effective way.

INTRODUCTION

Dengue virus infections give a broad spectrum of clinical manifestations ranging from no or minimal symptoms that cannot be easily distinguished clinically from other viral infections to the relatively new but potentially fatal Dengue hemorrhagic fever (DHF) and Dengue shock syndrome (DSS) [1]. DHF and DSS are characterized by a hemorrhagic tendency and increased systemic vascular permeability and may develop after infection with any of four Dengue viral serotypes [2]. An estimated 50-100 million cases of Dengue virus infection and 500.000 cases of DHF resulting in around 24.000 deaths are reported to the World Health Organization (WHO) annually [1,2].

The pathophysiological mechanisms underlying the increased vascular permeability are poorly understood. Activation of inflammatory pathways and the coagulation system seem to be involved in the process, resulting in disruption of the normal endothelial barrier [3,4]. Currently, no specific treatment is available to combat any of these phenomena [5,6]. Treatment is limited to supportive measures and predominantly relies on adequate fluid resuscitation in cases of circulatory failure. WHO management guidelines recommend replacement of plasma losses with crystalloid solutions like Ringer's lactate initially, followed by boluses of colloid fluids like dextran for patients who have recurrent or refractory shock [1].

There has been considerable debate in the medical literature on the use of crystalloids or colloids for volume replacement strategies in critically ill patients [7,8]. Crystalloid solutions stay within the intravascular space only for a short time since they are rapidly distributed to the interstitium [8]. Because colloid fluids typically are solutions of large molecules with high osmotic values they do not tend to pass through membranes like the endothelial barrier. Therefore, colloid fluids remain in the intravascular space much longer, and it takes less time and requires less volumes of fluid to correct for hypovolemic disturbances. As a group, colloids are very effective volume expanders, but no single colloid is ideal. Hydroxyethylstarch (HES), albumin, dextran, and other less commonly used colloids each have specific, significant toxicities that must be considered when using them. Because of financial aspects, potential toxicity and the lack of clinical trials supporting there use, current WHO guidelines still recommend crystalloid fluids for the initial fluid resuscitation of DSS. The few clinical trials performed thus far, suggest a possible beneficial effect of several colloids on haemodynamic parameters in severe DSS, but the results were variable [9,10,11].

HES is a frequently studied, cost-effective colloid and it only evokes allergic reactions in very rare cases. The colloid is used in different forms and concentrations. HES with a molecular weight (MW) of 200 kD has a positive effect on cardiac output, microcirculation, oxygen delivery and perfusion pressure [12]. Potent anti-inflammatory properties have been described to HES in patients with sepsis. Levels of the adhesion proteins ICAM-1 and VCAM-1 did not increase during sepsis, whereas levels of these proteins were increased in patients treated by any other fluid regimen [13]. One of the drawbacks of HES, however, is that the colloid has also been associated with an increased bleeding tendency. High molecular weight HES 480 kD MW was associated with a significantly longer partial thromboplastin time and

subsequently with increased rates of serious post-operative bleeding complications after neurosurgery and cardiac surgery [14,15]. Medium molecular weight HES 200 kD and low molecular weight HES 70 kD have also been associated with coagulation disturbances like an increased partial thromboplastin time and decreased levels of Factor VIII/von Willebrand factor complexes, but all to a lesser extent and resulting in less serious bleeding complications [16].

To address these issues, we conducted an open-labeled, prospective, randomized clinical trial evaluating the use of HES 200 kD MW in comparison to the currently recommended use of Ringer's Lactate as initial fluid regimen for the primary resuscitation of children with DSS in an endemic region in Indonesia. We evaluated the effects of both solutions on mortality, organ dysfunction, shock recovery time and markers of vascular leakage.

PATIENTS AND METHODS

Study Design

The study was a single-center, open-labeled, randomized comparison of two fluid regimens for the initial resuscitation of children with a severe Dengue virus infection: a colloid solution [6% hydroxyethyl starch 200 kD MW (HAES-Steril 6%)] and an isotonic crystalloid solution (Ringer's Lactate). The study was conducted at the pediatric intensive care unit (PICU) and high nursing dependency unit (HND) of the Dr. Kariadi Hospital in Semarang, Indonesia. The ethics committee of the Dr. Kariadi Hospital approved the protocol.

Study population

All children, 3 to 14 years of age, consecutively admitted to the hospital with clinical Dengue shock syndrome were considered eligible for inclusion provided that a parent or legal guardian gave informed consent. The following criteria were used for a clinical diagnosis of Dengue shock syndrome: 1) fever or a history of acute fever, 2) haemorrhagic manifestations (at least a positive tourniquet test), 3) thrombocytopenia (platelet count $\leq$100.000 cells/mm^3), 4) signs of plasma leakage (haematocrit $\geq$20% above average for age, haematocrit ^{3} 20% compared to the haematocrit at hospital discharge, pleural effusion, hypoproteinemia or hypoalbuminemia), and 5) evidence of circulatory failure defined as hypotension for age (systolic pressure <90 mmHg for those $\geq$5 years of age and <80 mmHg for those <5 years of age) or narrow pulse pressure (<20 mmHg) [WHO]. Patients with multiple organ dysfunction syndrome (MODS), i.e. more than 2 organ dysfunctions, or patients not expected to survive at least 24 hours at the time of entry in the study (Pediatric Index of Mortality [17,18]> 70%), were excluded from this study.

Clinical procedures

Children meeting the inclusion criteria were allocated to any of the two treatment strategies using a computer-generated list of random numbers. The randomization process was carried out by independent research staff not involved in clinical care. Patients were randomly assigned to receive either Ringer's Lactate (control group) or hydroxyethyl starch (intervention group). Patients in the control group were given 20-60 ml per kilogram of body weight of fluid or more within 10-30 minutes, to keep central venous pressure at a level of 12-15 cm H_2O. Patients in the intervention group

were given 10-30 ml per kilogram of body weight of fluid within 10-30 minutes, to keep central venous pressure at a level of 15-18 cm H_2O. Fresh frozen plasma was given to both groups when clinically significant bleeding occurred and when coagulation screening tests were significantly prolonged. This was left to the discretion of the treating physician.

After infusion of the study fluid, patients received standard schedules of Ringer's Lactate according to current WHO guidelines, and were monitored on an hourly basis by medical personnel. In case of unstable vital signs, routine laboratory test results, including platelet count and haematocrit, were performed every 3-4 hours and, if necessary, treatment was given according to WHO guidelines. Fluid resuscitation was stopped when patients were stable. A stable situation was defined by 1) good tissue perfusion with signs of good mental status, normal systolic blood pressure (SBP) and mean arterial pressure (MAP) for age, diuresis more than 1ml per kilogram of body weight per hour, SaO2 > 92%, capillary refill time < 2 seconds, and serum lactate acid level < 2.2 mmol/l, and by 2) a good plugging effect with normalization of serum protein and albumin level, no signs of respiratory distress and minimal signs of pleural effusion and/or pulmonary edema.

Intravascular volume status was monitored on admission and every 2 hours until 24 hrs after admission by measuring central venous pressure and mean arterial pressure. Chest roentgenogram and blood gas analysis were performed on admission and before discharge from PICU/HND. The degree of vascular leakage was monitored by serum protein and albumin levels and the severity of pleural effusion at chest roentgenogram. Serum protein and albumin levels were measured on admission, upon recovery from shock, at 48 hrs after admission, and before discharge from PICU. Severity of pleural effusion and organ complications (pulmonary and hematologic dysfunction) were determined at admission and at 48 hrs after admission. Severity of pleural effusion was graded as severe (either 2/3 of the right hemithorax or bilateral pleural effusion), moderate (more than 1/3 of the right hemithorax) or mild (less than 1/3 of the right hemithorax).

Members of the study team recorded demographic data, medical history, physical examination findings, clinical course and routine laboratory test results for each patient on a standard data form.

Diagnostic procedures

A clinical diagnosis of dengue infection was confirmed using a rapid dengue blot IgG and IgM (Pan-Bio) and a hemagglutination inhibition test (HI) [19,20]. The rapid dengue blot test was performed 5 days post onset of fever and the HI test was done on admission and before discharge from the hospital. A fourfold or greater increase in HI titer between paired specimens was indicative of a recent infection.

Outcome measures

The primary outcome measures were mortality rate, organ complications (pulmonary and hematological dysfunction), and levels of parameters of vascular leakage. Lung dysfunction was dichotomized in mild dysfunction (Acute Lung Injury/ALI: PO_2/FiO_2 200-300 mmHg) and severe dysfunction (Acute Respiratory Distress Syndrome/

ARDS: PO_2/FiO_2 < 200 mmHg). Hematological dysfunction was also dichotomized in mild dysfunction (platetelet count < 100.000/mm^3 with or without prolonged PTT or PT > 1.5x normal value, fibrinogen level < 1.3 g/dl), and severe dysfunction (platelet count < 30.000/mm3 with or without prolonged PTT or PT > 2x normal value, fibrinogen level < 1.0g/dl). Secondary outcome measures were duration of shock, number of days in PICU, ventilator days and blood transfusion needed.

Statistical Analysis

Data are presented as means with standard deviation or as numbers with percentage. Continuous variables were compared by the Student T-test. For categorical variables, the chi-square or Fisher's exact tests were applied. Two-sided P-values less than 0.05 were considered to indicate statistical significance. Analyses were performed with use of SPSS software, version 10.

RESULTS

Patient characteristics

Before randomization, 73 patients with suspected DSS were recruited into the study. Five patients were excluded because their pediatric index of mortality score exceeded 70% and 3 patients with bacterial sepsis were not included, as well as 2 patients with severe liver dysfunction and 3 patients with negative serological test results. Finally, a group of 60 patients fulfilling the selection criteria was enrolled. The patients were randomly assigned to receive either protocol treatment (n=30) or control treatment (n=30). None of the patients withdrew from the study after randomization. Demographic information and clinical characteristics at admission are shown in table 1. Age, gender and nourishing status of the patients were similarly distributed among the groups and the groups were comparable for occurrence rates of various hemorrhagic manifestations and pleural effusion.

Table 1. Patient characteristics at inclusion.*

	Control (n= 30)	Intervention (n=30)	P value
Male sex, n (%)	16 (53)	13 (43)	NS
Age, mean (SD)	6.6 years (2.0)	6.9 years (2.0)	NS
Undernourished, n (%)	5 (17)	7 (23)	NS
Positive Tourniquet test, n (%)	10 (33)	12 (40)	NS
Petechie, n (%)	3 (10)	3 (10)	NS
Epistaxis, n (%)	3 (10)	4 (13)	NS
Gum bleeding, n (%)	3 (10)	5 (17)	NS
Hematemesis, n (%)	8 (27)	11 (37)	NS
Melena, n (%)	8 (27)	10 (33)	NS
Patients with pleural effusion, n (%)	25 (83)	28 (93)	NS
Severe, n (%)	13 (43)	14 (46)	NS
Moderate, n (%)	8 (27)	12 (40)	NS
Mild, n (%)	4 (13)	2 (7)	NS

* n, denotes number; SD, denotes standard deviation

Clinical outcomes

Clinical outcomes of the study are presented in table 2. The overall mortality during hospital stay was 17%. The mortality rates differed significantly between the control group and the intervention group (8 patients (27%) vs. 2 patients (7%), respectively; $p<0.001$). All non-survivors met criteria for severe DIC. Pulmonary dysfunction occurred more frequently in the control group. In addition, the duration of shock was reduced in the intervention group, as well as length of stay at the paediatric intensive care unit and number of days that patients required mechanical ventilation. Furthermore, severe pleural effusions during hospital stay were found less frequently in the intervention group, and blood transfusions were given to all patients in the control group but to only 7 patients (23%) of the intervention group.

Table 2. Clinical outcomes.*

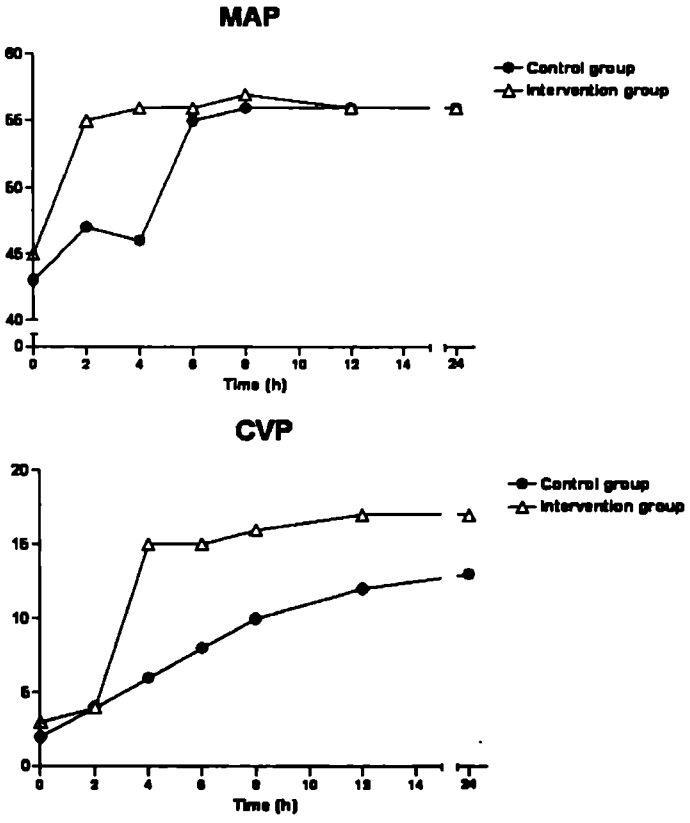
	Control (n=30)	Intervention (n=30)	P value
Mortality rate, n (%)	8 (27)	2 (7)	p< 0.05
Pulmonary dysfunction, n (%)	10 (33)	3 (10)	p< 0.05
Acute lung injury, n (%)	4 (13)	1(3)	NS
Acute Respiratory Distress Syndrome, n (%)	6 (20)	2 (7)	NS
Diffuse Intravascular Coagulation, n (%)	8 (27)	2 (7)	p< 0.05
Duration of shock, mean (SD)	4.9 hours (2.8)	2.3 hours (1.3)	p< 0.01
PICU length of stay, mean (SD)	14.0 days (2.1)	7.2 days (1.3)	p< 0.01
Duration of mechanical ventilation, mean (SD)	8 days (1.1)	4 days (0.7)	p< 0.05
Severe pleural effusion, n (%)	21 (70)	2 (6)	p< 0.01
Blood transfusion, n (%)	30 (100)	7 (23)	p< 0.01

* n, denotes number; SD, denotes standard deviation

Shock recovery

Shock recovery was monitored by hourly measurements of the mean arterial blood pressure (MAP) and central venous pressure (CVP) (figure 1). Initially, MAP was decreased in both groups [mean values (SD): 43 (2) and 45 (2) mm Hg in the control group and the intervention group, respectively], but recovered more quickly in the intervention group, reaching baseline plateau values sooner (control group vs. intervention group: recovery at t=4h vs. t=2h after admission, respectively). Similarly, CVP was decreased in the beginning [2 (1.1) and 3 (1.2) mm Hg in the control group and the intervention group, respectively] and recovered more quickly in the protocol group (control group vs. intervention group: recovery at t>24h vs. t=4h after admission, respectively).

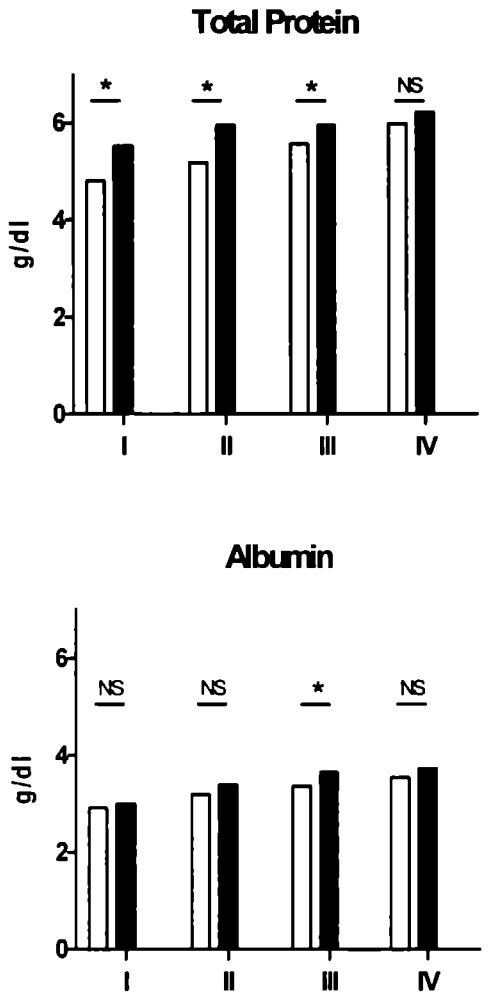
Figure 1. Mean arterial blood pressure (MAP) and central venous pressure (CVP).



Vascular leakage

Serum protein and albumin levels were measured as markers of vascular leakage (figure 2). Protein and albumin levels were low in both groups at admission, especially in the control group, but in the intervention group serum protein levels returned to baseline levels at the moment of shock recovery, whereas levels in the control group only returned to baseline at time of discharge from PICU. Serum albumin level in the protocol group slightly increased during observation days and there was a significant increase on the 48 hours of admission compared to the control group.

Figure 2. Markers of vascular leakage.



Mean plasma levels of total protein and albumin from patients (n=60) with dengue shock syndrome. Patients were compared at sequential time-points. Patients were treated by a study protocol based upon treatment with HES (black bars) or with Ringer's lactate (white bars). A P value <0.05 was considered statistically significant (*). NS= Not Significant. I= admission. II= moment of shock recovery. III= 48 hours after admission. IV= Discharge from PICU.

DISCUSSION

This open-labeled, randomized clinical trial showed a beneficial effect of initial treatment of children with severe DSS in the first 10-30 minutes after hospital admission with a colloid solution containing HES 200 kD MW in comparison to treatment with an isotonic crystalloid solution (Ringer's Lactate). The study showed a significant, impressive decrease in mortality from 27% in the control group to 7% in the intervention group, which is a reduction by 74%. In addition, organ complication rates were lower and shock recovery time and duration of vascular leakage were shortened in the intervention group. The improved parameters were also reflected in a decreased duration of mechanical ventilation and length of stay at the PICU. Together, these results suggest that substitution of currently recommended isotonic solutions by colloid solutions for the initial fluid resuscitation in DSS results in a better sealing effect with less complications and an improved clinical outcome.

The discussion about fluid replacement strategies has a long history [7,8]. In severe dengue virus infections however, the debate is relatively new [21]. An initial pilot study in 50 Vietnamese children with DSS comparing the colloids dextran and gelatin with crystalloids showed more rapid restoration of cardiac index, blood pressure and hematocrit levels after use of colloids [9]. A second study was subsequently performed in 230 children with DSS which showed no clear advantages of colloids, but the study was hampered by disease severity differences between the study groups at inclusion despite randomization [10]. The analysis showed some beneficial effects of dextran and gelatin on hematocrit reduction, pulse pressure recovery time and rescue colloid requirement, but also negative effects, because colloid use was associated with allergic reactions. A post-hoc logistic regression analysis correcting for the baseline differences only showed a beneficial effect of gelatin in comparison to Ringer's lactate in a subgroup of severely ill children with a pulse pressure d+10 mmHg. Finally, a third, major, randomized double blinded clinical trial in 512 children with DSS was conducted by the same research group [11]. Here, no differences were found between the colloids dextran or HES and the crystalloid Ringer's lactate in children with moderately severe shock. Furthermore, no differences were found between the two colloids, without comparison to crystalloids, in children with severe shock, as defined by a pulse pressure <10 mmHg. Therefore, the authors concluded that, because of lack of evidence of beneficial properties of colloids in children with moderately severe shock, Ringer's lactate should be recommended in this patient group. Furthermore, based upon results from their second study, they recommended use of colloids, preferably HES, for the treatment of children with severe DSS with pulse pressures <10 mmHg.

The results from the current study support the thesis that HES is more beneficial than Ringer's lactate in children with severe DSS. Patients in the current study were not stratified according to pulse pressure, because at the time of initiation of this study, a pulse pressure <10 mmHg was not yet identified as a strong prognostic marker. Still, mortality in the study was high, about 17%, whereas mortality in the Vietnamese studies was below 1% [9-11]. Because treatment strategies were comparable, these data suggest that the patients in this study were indeed suffering from severe DSS and perhaps an even more serious form of DSS.

The current study showed no evidence of adverse effects. The use of colloids can be complicated by allergic reactions, although this complication has rarely been reported in patients receiving HES [8]. In this study, no allergic reactions were noted. Furthermore, the use of various HES solutions, in particular high molecular weight variants, has been associated with bleeding complications in surgery patients in the past [15]. These bleeding complications depended strongly on the specific composition of the molecule which influences its degradation time [16]. The current study showed no evidence of increased rates of complications. On the contrary, all hemodynamic parameters, including occurrence of DIC and blood transfusion requirement, were improved after HES infusion.

The impressive beneficial effects of HES after only a short time of 10-30 minutes of initial fluid resuscitation suggest that HES may have more beneficial effects in DSS than those related to improved hemodynamics alone. Indeed, anti-inflammatory properties of HES have been demonstrated in septic trauma patients and patients undergoing abdominal surgery [13,22]. Expression of the adhesion molecules VCAM-1 and ICAM-1 on endothelial cells was reduced in these patients. The function of the endothelial lineage of the vessel wall is not limited to a sole barrier function because it also plays an important role in the activation of inflammation and coagulation [23]. Because both processes are involved in the pathophysiology of DSS [3,24-27], suppression of endothelial cell activation by HES as indicated by reduced expression of VCAM-1 and ICAM-1 may well add to the beneficial properties of HES.

The study was an open-labeled, randomized study. Blinding of investigators or patients was not performed because of practical reasons and this may theoretically have influenced the study results. On the other hand, randomization was carried out carefully, resulting in comparable groups at inclusion. Moreover, most study endpoints were objective measurements like mortality rates. If lack of blinding did affect the study outcomes, it did probably only affect minor outcomes based upon subjective treatment decisions like PICU length of stay and mechanical ventilation days or subjective interpretations of diagnostic imaging. Regarding this limitation of the study, most of these decisions were defined prior to the study, but subjective influences cannot be excluded for these minor endpoints.

In summary, this study showed impressive beneficial effects of a colloid fluid regimen with HES 200 kD MW for initial resuscitation of 60 children with severe forms of DSS. Mortality was significantly reduced by 74%. Other endpoints including organ dysfunction, shock recovery time and levels of markers of vascular leakage were all reduced after HES infusion. No adverse reactions were noted. Because of lack of stratification to pulse pressure, it is not sure which patient group profited the most of HES infusion. The effect of HES may have been mediated by both hemodynamic and anti-inflammatory properties. Together, these results suggest that short, initial infusion of colloids in children with severe DSS is safe and can improve mortality in a cost-effective way. Further studies should define the optimal fluid replacement strategies and specific patient groups that gain most benefit by colloid fluid resuscitation in order to make more specific recommendations to adjust current WHO guidelines for the treatment of DSS.

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III. Coagulation and endothelium

Chapter 5

IS CLINICAL OUTCOME OF DENGUE VIRUS INFECTIONS INFLUENCED BY COAGULATION AND FIBRINOLYSIS? A CRITICAL REVIEW OF THE AVAILABLE EVIDENCE.

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ABSTRACT
Despite increasing effort to unravel the pathogenesis of Dengue, the deterioration of this infection into severe disease remains poorly understood. Recent in vitro data suggest coagulopathy and disturbances in fibrinolysis to play a more pivotal role in the pathophysiology of Dengue than currently perceived. If disturbances in coagulation and fibrinolysis are predictors of clinical outcome of Dengue virus infections this could have important consequences for both diagnosis and treatment. We therefore critically reviewed the literature to assess an association between coagulation and fibrinolysis activation, and clinical outcome of Dengue virus infections. In general, the selected studies demonstrated activation of both the coagulation and the fibrinolytic system in Dengue virus infection. This activation of coagulation and fibrinolysis was more pronounced in severe infections and in cases with ultimately a poor clinical outcome. However, the findings were not consistent and due to a lack of detailed information on patient, disease and study design characteristics, we were unable to ascertain whether inconsistencies were caused by differences in these characteristics, selection bias or confounding factors. We therefore conclude that an association between activation of coagulation and fibrinolysis, and clinical outcome of Dengue virus infections is conceivable but inadequately assessed and that a true association will need to be evaluated by methodological sound studies, complemented with complete and reliable reporting.

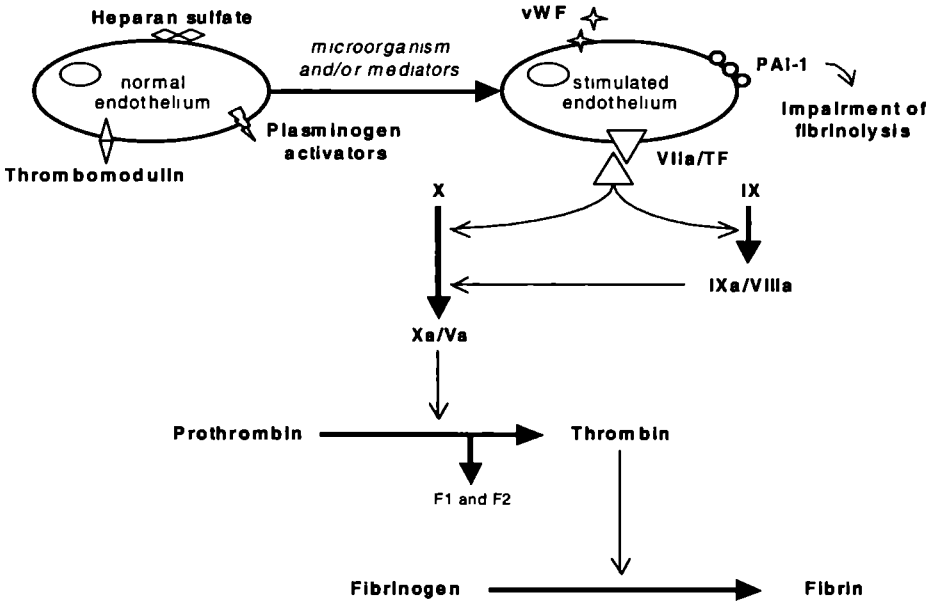
INTRODUCTION

Since its first recognition as a potentially life threatening infectious disease in the 1950's, numerous studies have been conducted to unravel the mechanism underlying the deterioration of Dengue virus infections into Dengue haemorrhagic fever (DHF) and Dengue shock syndrome (DSS). The most prominent pathophysiological change in these syndromes, which distinguishes them from Dengue fever (DF), is increased vascular permeability resulting in loss of plasma from the vascular component with impending shock when plasma loss becomes critical. An elevated haematocrit, hypoproteinemia or evidence of serous effusion are distinctive indicators of plasma leakage [1-3].

The characteristic haemorrhagic manifestations, such as epistaxis, gingival bleeding, gastrointestinal bleeding, haematuria and menorrhagia, are primarily thought to be due to vasculopathy, thrombocytopenia and thrombocytopathy. Defects in coagulation and fibrinolysis are believed to be related to disseminated intravascular coagulation, a feature which is only seen in the severest form of Dengue infection and which is generally felt not to be central to the pathogenesis of DHF and DSS [4]. However, the defective vascular function resulting in increased permeability seen in all grades of DHF, may also lead to alterations in other properties of the endothelium. The normal endothelium produces inhibitors of blood coagulation and modulators of fibrinolysis, like for example thrombomodulin, heparan sulphate and plasminogen activators, all inhibiting thrombus formation and therefore contributing to the maintenance of vascular patency. Upon stimulation by cytokines and micro-organisms, the endothelium could lose its nonthrombogenic protective property by expressing Tissue Factor, plasminogen activator inhibitor-1 (PAI-1) and von Willebrand factor (vWF) (Figure 1). These responses act in concert with platelets, plasma coagulation, fibrinolytic and inhibitory factors in a highly integrated and carefully orchestrated way to maintain normal haemostasis. However, if responses are excessive, intravascular thrombosis, bleeding or both may follow. Recent in vitro data also suggest that the Dengue virus as well as antibodies obtained from Dengue infected patients may directly influence the haemostatic system [5-9].

If disturbances in coagulation and fibrinolysis do contribute to the severity of Dengue virus infections and subsequent risk of mortality, this could have consequences for both diagnosis and therapy. The presence of a haemostatic dysfunction in an early stage of disease could forewarn the practitioner of the development of severe disease or overt DIC and allow for measures to provide adequate surveillance and symptomatic support. Additionally, new supportive interventions could be sought to supplement the currently recommended fluid replacement therapy [3]. In order to determine whether haemostatic disturbances are associated with an increased risk for morbidity and mortality in Dengue virus infections, we reviewed the literature using current recommendations regarding the selection and appraisal of relevant observational studies [10-12].

Figure 1. Endothelial cells in infection.



The normal endothelium produces inhibitors of blood coagulation such as thrombomodulin, heparan sulfate and plasminogen activators. The nonthrombogenic properties are lost by expressing tissue factor when endothelial cells are stimulated by micro organisms (dengue virus?) and/or mediators (i.e. cytokines). Plasminogen activator inhibitor-1, von Willebrand Factor and Tissue factor, the critical inducer of *in vivo* blood coagulation, are expressed. VIIa/TF, activated factor VII/tissue factor complex; X, factor X; Xa/Va. activated factor X/activated factor V complex, IX, factor IX; Ixa/VIIIa, activated factor IX/activated VIII complex; PAI-1, plasminogen activator inhibitor-1; vWF, von Willebrand Factor.

METHODS

Literature Search and Data Sources

A systematic review was performed to identify eligible articles. The MEDLINE database from 1966 through October 2002 was searched by one of the reviewers (ATAM). This was performed, by combining the Medical Subject Headings (MeSH terms) and text words *Dengue, Dengue Virus, Blood Coagulation, Blood Coagulation Disorders, Blood Coagulation Factor Inhibitors, Blood Coagulation Factors, Blood Coagulation Tests, DIC, disseminated intravascular coagulation* and *fibrinolysis.* In addition, cross-references cited in selected articles and reviews were hand-searched for relevant articles.

Table 1. Laboratory tests for the evaluation of the coagulation system.

	Coagulation	Fibrinolysis
Screening tests	Activated partial thromboplastin time (aPTT)	Euglobulin clot lysis time (ECLT)
	Prothrombin time (PTT)	Dilute whole blood clot lysis time (DWBCLT)
	Thrombin time (TT)	
	Fibrinogen	
Activation markers	Factor XIIa-C1-inhibitor complex	Plasmin-α2-Antiplasmin complex (PAP)
	Kallikrein-C1-inhibitor complex	D-dimer
	Thrombin-Antithrombin complex (TAT)	Fibrinogen (fibrin) degradation products (FDP)
	Prothrombin activation fragment F1+2	

Study Selection and Data Extraction

All identified epidemiological studies and case series, evaluating coagulation and fibrinolytic activity in patients with laboratory confirmed Dengue virus infections, were selected. Standard definitions were used to define epidemiological studies [13]. Studies were considered for inclusion if laboratory tests were used to evaluate coagulation and fibrinolytic activity. These tests can be conveniently divided in global screening tests, tests for the detection of activation markers and tests for the detection of specific components of the coagulation system [14-18]. A list of some examples of screening tests and assays to detect activation markers of coagulation and fibrinolysis is shown in Table 1.

Inclusion was restricted to studies in which primary data (e.g. median or mean, and if stated measures of variability of data) on results of one of the above-mentioned assays in association with morbidity or mortality could be extracted. The quality of selected studies was appraised by reviewing them against a set of methodological criteria [11;19]. Key study features were assessed in detail using a checklist as outlined in Table 2 [11] and primarily involved the reporting of study subject characteristics, selection of cases and controls, confounding factors and the adjustments made for confounding.

Selection of studies, quality appraisal and data extraction were independently performed by two of the authors (ATAM and MRM). Disagreement was resolved by discussion and if necessary by adjudication of a third reviewer (ECMG). Interanalyst agreement in selection of studies and data extraction was not quantified.

Statistical analysis

A formal meta-analysis was not considered appropriate because the selected studies were highly heterogeneous with respect to selection of cases and controls, number of cases and controls, criteria for classification of cases, assays performed and description of data. If possible, we used the one-way factorial ANOVA test to analyse statistical significant differences in means between 2 or more samples. For this analysis the study had to state total number of observations, the mean and either the standard deviation (SD) or the standard error of the mean (SEM) for the observations in each group. For the statistical comparison of proportions derived from two groups or more, we used the Chi squared test. Alternatively we used Fisher's exact test for tables with very small expected frequencies. A two-sided p-value < 0.05 was considered to indicate statistical significance.

RESULTS

Literature search and Data Sources

The computer search yielded 104 references. Sixty-one articles were excluded, after reading title and abstract; one editorial, ten case reports, eleven reviews, six in vitro studies and thirty-three that did not address the topic under investigation. A list of these excluded articles is available from the authors. The full article of the remaining forty-three potentially relevant references was read to assess selection for this review.

Several articles reported on the same study and study results [20-26]. In order to avoid inclusion of duplicate reports, the most detailed article was selected [20;23;26]. Two articles reported on the same study although different study results were presented [27;28]. These articles were therefore included. Twelve articles did not describe epidemiological studies or case series, but were either case reports [29-32], reviews [33-38], a treatment study [39] or a report of a seminar [40]. Five articles [41-45] described studies which did not assess coagulation or fibrinolysis activity and one article studied patients with unconfirmed Dengue virus infections [46]. These articles were therefore excluded. Data on severity of disease or mortality as outcome was not reported in three articles [47-49] and five articles [50-54] provided insufficient data. Hand searching of the bibliographies of reviews and selected studies resulted in 2 additional articles [55;56]. Thus, a total of thirteen articles [20;24;26-28;55-62] was selected for this review.

Table 2. Checklist to appraise the quality of selected articles

Study identification (author, title, year of publication)
What is the study type?
What coagulation and/or fibrinolysis factors are considered?
What outcomes are considered? Dengue severity or mortality?
What other factors could affect the outcome(s)?
What are the characteristics of the population and study setting?
Are the study participants (cases and controls) well defined in terms of time, place and person?
What percentage of individuals refused to participate?
If severity of disease was considered as outcome, how was it measured?
Are risk factors and outcomes measured independently (blind) of each other?
Are all important risk factors included in the analysis?
What percentage of individuals recruited into the study are not included in the analysis (loss to follow up)?
How well was the study done to minimise bias? What is the likely direction in which bias might affect the study results?

Study appraisal

Table 3 summarises the designs of the selected studies and the characteristics of the study subjects. Three studies were cohort studies, seven had a case control design and two were case series. The number of cases enrolled in these studies ranged from 11 to 187, and the corresponding number of controls ranged from 5 to 50. Four studies [20;27;28;59;62] reported criteria used to select cases. Although the remaining studies did provide some details on selection of cases, a clear strategy on inclusion and details on number of excluded cases and reasons for exclusions was missing. The majority of cases concerned hospitalised patients with DHF or DSS. In addition to these severe cases, Krishnamurti et al [59] and Huang et al [58] studied cases with a diagnosis of DF. Krishnamurti et al [59] also studied cases in an early stage of disease, before their illness could be classified as DF or DHF. Classification of disease severity in the selected studies was based on different case definitions in contrast to the study done by Weiss et al [56] who only reported raw data of those under study. The case definitions used were in particular those proposed by Nimmannitya [63] and WHO [3;64], although some articles classified cases according to other case definitions [55;65]. Differences between these case definitions are based on the inclusion of laboratory abnormalities, i.e. number of thrombocytes or elevated haematocrit, to differentiate DF from DHF. Main outcome measures were disease severity based on these classifications in the majority of studies and mortality in only one study [27;28]. Other clinical endpoints, like bleeding scores, time to recover from shock and pleural effusion index were reported although primary data on laboratory test results were missing [59;62]. These data could therefore not be reviewed.

Table 3. Characteristics of selected studies*

Test	Reference	DF	DHF I	DHF II	DHF III	DHF IV	Controls
aPTT	Wills et al	-	-	-	median 37.7, range 18.8-58.1		-
	van Gorp et al	-	-	-	median 52.0, range 43.4-64.1		-
	Krishnamurti et al	46.1, SEM 3.0	48.0, SEM 1.7	45.8, SEM 2.2	46.5, SEM 0.9	-	-
	Huang et al	31.5, SEM 2.0	60.2, SEM 10.9				23-25
	Imrangkura et al	-	65, SD 4	68, SD 5	81, SD 8		36, SD 65
	Furushara et al	-	-	47.4, SD 12.2	-	-	29.2, SD 3.1
	Srichaikul et al		-	56, SD 10	62, SD 17		normal 44, SD 8; febrile 46, SD 8
PT	Wills et al	-	-	-	median 13.4, range 11.3-15.9		-
	van Gorp et al	-	-	-	median 13.2, range 11.9-15.4		-
	Imrangkura et al	-	81, SD 1	72, SD 5	72, SD 5		60 – 160
	Furushara et al	-	-	52.4, SD 14.7	-	-	100
	Wenn et al	13, SD 1.1			13.9, SD 2.2		-
TT	Krishnamurti et al	23.9, SEM 0.6	22.8, SEM 0.7	24.1, SEM 1.2	25.1, SEM 2.2	-	-
	Imrangkura et al	-	8, SD 3	8, SD 1	10, SD 1		2 – 12
Fibrinogen	Wills et al	-	-	-	median 1.59, range 0.7-5.63		-
	van Gorp et al	-	-	-	median 1.6, range 1.3-1.9		-
	Krishnamurti et al	280, SEM 16	224, SEM 19	256, SEM 17	221, SEM 35	-	-
	Imrangkura et al	-	202, SD 11	197, SD 19	144, SD 12		330, SD 67
	Furushara et al	-	-	97.4, SD 32.3	-	-	362, SD 96
	Srichaikul et al	-	-	218, SD 88	194, SD 67		normal 205, SD 60; febrile 251, SD 40
	WHO	-	1.51	1.2	0.98	0.86	1.72
	Wenn et al	192, SD 47.3			165, SD 25.9		-
TAT	van Gorp et al	-	-	-	median 27.2, range 13.3-65.7		-
F1+2	Krishnamurti et al	2.93, SEM 0.46	4.30, SEM 0.84	3.60, SEM 0.37	3.82, SEM 0.85	-	-
	van Gorp et al	-	-	-	median 3.2, range 2.0-5.0		-
Xla-Clarh	van Gorp et al	-	-	-	median 13.5, range 0-31.8		-
Kall-Clarh	van Gorp et al	-	-	-	median 13.2, range 5.9-47.8		-
	Edelman et al	-	0.92, SD 0.17		0.85, SD 0.37		afebrile 0.99, SD 0.22; febrile 1.01, SD 0.25
TAFI-antigen	van Gorp et al	-	-	-	46.0, range 30.4-61.8		-
TAFI-activity	van Gorp et al	-	-	-	31.8, range 21.0-39.9		-
Tissue Factor	Wills et al	-	-	-	median 81.0, range 7.7-302.4		-
Factor II	Furushara et al	-	-	82.3, SD 9.3	-	-	100
Factor V	Mitrakul et al	-	-	95.1, SD 35.8	57.9, SD 42.5	136.8, SD 24.7	-
Factor VII	Furushara et al	-	-	100.5, SD 25.7	-	-	100
	Mitrakul et al	-	-	71.9, SD 24.6	60.5, SD 6.5	68.3, SD 19.6	-
Factor VIII	Furushara et al	-	-	9.6, SD 3.3	-	-	100
	Mitrakul et al	-	-	116, SD 53.8	74.7, SD 43.9	89.5, SD 28.1	-
Factor IX	Mitrakul et al	-	-	102.5, SD 3.5	88.3, SD 50.5	122	-
Factor X	Mitrakul et al	-	-	100	77, SD 32.5	100	-
Total TFPI	Wills et al	-	-	-	median 55.4, range 25.9-128.3		-
Free TFPI	Wills et al	-	-	-	median 4.6, range 0-25.4		-
Prekallikrein	Edelman et al	-	56, SD 12		41, SD 18		afebrile 91, SD 16; febrile 74, SD 15
Factor XII	Edelman et al	-	84, SD 38		59, SD 43		afebrile 84, SD 36; febrile 117, SD 43
AT III	Wills et al	-	-	-	median 0.62, range 0.43-1.25		-
	Furushara et al	-	-	59.6, SD 8.2	-	-	100
Protein C	Wills et al	-	-	-	median 0.36, range 0.18-0.90		-
	van Gorp et al	-	-	-	median 52.5, range 36.8-66.3		-
Protein S act.	van Gorp et al	-	-	-	median 51.0, range 39.0-64.3		-
Protein S free	Wills et al	-	-	-	median 0.40, range 0.23-0.59		-
	van Gorp et al	-	-	-	median 23.0, range 19.0-28.0		-

* DF, Dengue fever; DHF, Dengue haemorrhagic fever; DSS, Dengue shock syndrome; WHO, world health organization; m, month; y, year; S.D., standard deviation

The population, from which the control groups were selected, generally concerned hospitalised patients, patients with other infectious diseases than Dengue and healthy individuals. The majority of selected articles did not report patient characteristics, like age, sex or ethnicity. Information on characteristics of disease (e.g. duration of illness prior to admission, disease characteristics at admission) was only briefly discussed or not stated at all. Whether these variables could have influenced the results of the assays or outcome of disease is unclear, since no attempt was made to look for potential confounders and to account for them by means of statistical adjustment.

Coagulation

The tests which were performed in the acute phase to assess coagulation activity in Dengue virus infected cases and controls are listed in Table 4 and consisted of assays measuring markers of coagulation activation, global screening tests and assays measuring individual factor activity. The results show an activated coagulation system in Dengue virus infections, which is most pronounced in severe disease. Several studies underlined this by measuring fibrinogen levels with the lowest mean values observed in the severest forms of Dengue virus infections as demonstrated by Isarangkura et al [26] ($P<0.01$, ANOVA), Srichaikul et al [61] ($P=0.05$, ANOVA) and the WHO [20]. The questions whether decreased fibrinogen levels are evident at admission and subsequently may be used as a marker of severe disease or poor outcome are left without reply. Krishnamurti et al [59], Srichaikul et al [61] and Funahara et al [23] are conflicting in their reports, since the first two observed minor differences between the several groups (respectively $P=0.19$ and $P=0.52$, ANOVA) whereas the latter revealed a large difference between controls and Dengue virus infected cases ($P<0.01$, ANOVA). Two additional studies measured moderately low fibrinogen levels in severe Dengue virus infections [28;62]. An analysis of the actual proportion of values outside the normal range showed differences between the several groups, which did not reach statistical significance. Weiss et al [56] reported decreased fibrinogen levels in 83% of hypotensive cases compared with 50% of normotensive cases ($P=0.2$, Fisher's exact test) and Srichaikul et al [61] found abnormal fibrinogen levels in 17.6% of DHF grade III & IV cases, 16.7% of DHF grade II cases and 25% of fever controls ($P=0.31$, Fisher's exact test). Although measurement of fibrinogen levels is advocated to study coagulation activity, one should bear in mind that plasma levels may remain in the normal range, because this protein is an acute-phase reactant. A finding of hypofibrinogenemia has diagnostic value in very severe cases of for example disseminated intravascular coagulation [66].

An activated coagulation system in Dengue virus infections is further substantiated by increased levels of thrombin-antithrombin complexes (TAT) and prothrombin activation fragments F_{1+2}. Van Gorp et al [27] found these coagulation activation markers to be elevated in severe Dengue virus infections on the day of admission and in particular in non-survivors when compared with survivors (median TAT levels respectively 50.9 and 21.0, median F_{1+2} levels respectively 4.9 and 2.8). The observation of a more pronounced decrease of both antigen and activity of thrombin-activatable

fibrinolysis inhibitor (TAFI) in non-survivors (median TAFI antigen/activity: non-survivors 33.5/20.1 and survivors 48.7/34.0) also suggests excessive thrombin formation with resulting consumption of TAFI. Results from the study done by Krishnamurti et al [59] revealed increased F_{1+2} levels which did not statistically differ between DF and DHF in the acute phase (P=0.39, ANOVA). The differences between the above-mentioned studies are a result of the outcomes used and also probably due to differences in stage of disease of included study subjects. Van Gorp et al [27;28] selected cases with a clinical diagnosis of DSS and reported mortality as outcome, whereas Krishnamurti et al [59] stated that most of their cases entered the study at an earlier stage of disease before their illness could be classified as DF or DHF and used this classification as outcome.

The results of the retrieved studies suggest that the intrinsic pathway is primarily the most affected part of the coagulation system and that this occurs in advanced stages of disease. The screening test that evaluates the intrinsic pathway of coagulation, the aPTT, tends to be abnormal in severe Dengue infected cases. Prolonged mean aPTT levels in Dengue infected cases were reported by Krishnamurti et al [59] (P=0.96, ANOVA), van Gorp et al [28], Huang et al [58] (P<0.01, ANOVA), Isarangkura et al [26] (P<0.01, ANOVA), Funahara et al [23] (P=0.01, ANOVA) and Srichaikul et al [61] (P=0.04, ANOVA). Srichaikul et al [61] also demonstrated that the proportion of cases with a prolonged aPTT during the course of illness was higher in severe Dengue virus infections compared to milder forms and fever controls (P<0.01, Fisher's exact test). However, not all studies were conclusive since Wills et al [62] did not find very prolonged aPTT test results in their patients suffering from severe Dengue virus infection. Their results show marginally prolonged aPTT when compared to 1-month follow up.

Table 4. Results of coagulation assays in different grades of Dengue virus infections*

Test	First Author	DF	DHF I	DHF II	DHF III	DHF IV	Controls
FDP	Isarangkura et al	-	11 8, SD 9	26, SD 12	50.4, SD 18		normal 0 6, SD 1 4, children with URTI 8.5, SD 10
	Srichaikul et al	-	-	18, SD 27	24, SD 16		normal 6, SD 10; febrile 22, SD 4
	WHO	-	1 7	3.2	2 9	5 1	-
	Suvatte et al	-	11.2, SD 7 06	15.2, SD 11.29	16 49, SD 14.25	23 4, SD 23.08	3.7, SD 1.7
D-dimer	van Gorp et al	-	-	-	median 241.0, range 197.3-761 0		-
PAP	van Gorp et al	-	-	-	median 8 6, range 6.3-12.0		-
Plasminogen	Krishnamurti et al	84, SEM 3	73, SEM 5	74, SEM 5	62, SEM 8	-	-
tPA	van Gorp et al	-	-	-	median 52.5, range 41.0-61.0		-
	Huang et al	21 1, SD 5 1	50 4, SD 6.0				10.4, SD 1.2
PAI-1	Wills et al	-	-	-	median 114 6, range 19.8- 674.4		-
	van Gorp et al	-	-	-	median 183.0, range 86.8-392.5		-
	Huang et al	63.2, SD 8.3	38 6, SD 6.3				38.6, SD 3 9
Alpha2-antiplasmin	Krishnamurti et al	111, SEM 6	97, SEM 10	89, SEM 5	95, SEM 15	-	-
	Funahara et al	-	-	32, SD 22	-	-	100

* Data are presented as mean with corresponding standard deviation (SD) or standard error of the mean (SEM), unless stated otherwise. DF, Dengue fever; DHF, Dengue haemorrhagic fever; TAT, thrombin-antithrombin complexes; TAFI, thrombin-activatable fibrinolysis inhibitor; aPTT, activated partial thromboplastin time; PT, prothrombin time; TT, thrombin time; XIa-C1inh, factor XIa-C1-inhibitor complex; Kall-C1inh, kallikrein-C1-inhibitor complex; AT III, antithrombin III.

A prolonged aPTT is likely the result of acquired deficiencies or abnormalities of almost all coagulation factors with the exception of factors VII and XIII. Activities of about 20% or less of factors VIII and IX may be sensitively detected by most aPTT tests [67]. Funahara et al [23] demonstrated mean activity levels of factor VIII as low as 9.6%. Other investigators could not confirm these findings. Mitrakul et al [60] measured the activity of several coagulation factors and found them, factors VIII and IX in particular, to be variably decreased, but not consistently below 20%. Mean activity levels of both factor VIII and IX in the several groups slightly differed (respectively P=0.5 and P=0.79, ANOVA). If the observed abnormal levels of factor XII and prekallikrein in the study performed by Edelman et al [57] contribute to intrinsic coagulation activation seems unlikely, since contact factors do not play a role in the current concept of physiological blood coagulation [68]. This is emphasised by Edelman et al [57] and van Gorp et al [27], who reported hardly any differences in levels of Kallikrein-C1-inhibitor complexes (Kall-C1inh) between the groups suggesting no involvement of the contact system. Edelman et al [57] found no differences between their DHF cases and controls (P=0.53, ANOVA) and van Gorp et al [27] found no significant differences between survivors and non-survivors (median Kall-C1inh levels of respectively 9.5 and 24.4 with a reported P-value of 0.3).

There is limited and conflicting evidence for activation of the Tissue factor pathway of coagulation in Dengue virus infections. The findings by Weiss et al [56] and Isarangkura et al [26] of normal PT values (respectively P=0.2 and P=0.71, ANOVA), do not support a derangement of this part of the coagulation system. This is in contrast to the findings by Funahara et al [23] and van Gorp et al [28] who found a prolonged PT in their group of Dengue infected cases where cases with a poor clinical outcome were more likely to have evidence of a deranged Tissue Factor pathway (median PT levels non-surv. 16.3 vs. surv. 12.6). Wills et al [62] also found marginally prolonged PT values and increased Tissue Factor concentrations on day of admission. These results and the observed activity of factor VII in Dengue infected cases by Funahara et al [23] and Mitrakul et al [60] (P=0.68, ANOVA), suggest a continuous activated Tissue Factor pathway in advanced stages of Dengue virus infections.

Fibrinolysis

The results of assays evaluating fibrinolysis are depicted in Table 5 and, in analogy with the coagulation data, demonstrate a modestly accelerated fibrinolytic state in Dengue virus infected cases. Increased levels of fibrinogen and fibrin degradation products (FDP) were observed in four studies of which only Isarangkura et al [26] and Suvatte et al [55] found the observed differences between the groups under study to be statistically significant (both P<0.01, ANOVA). Suvatte et al [55] demonstrated that FDP levels were not as high as in disseminated intravascular coagulation associated with bacterial sepsis. Positivity for D-dimers did differ between the study groups of Krishnamurti et al [59] although this was not statistically significant (25% in DF cases, 25% in DHF I cases, 67% in DHF II cases and 100% in DHF III cases; P=0.33, Fisher's exact test). D-dimer levels measured by van Gorp et al [28] were shown to be elevated in all cases, but no association could be detected between clinical outcome.

Evidence of fibrinolysis activation is further substantiated by the presence of elevated concentrations of plasmin-antiplasmin complexes (PAP) by van Gorp et al [27], increased t-PA levels by Huang et al [58] ($P<0.01$, ANOVA) and decreased plasminogen levels by Krishnamurti et al [59] ($P=0.07$, ANOVA). The results on the activity of the principal inhibitor of fibrinolysis, α_2-antiplasmin, are however inconsistent, since Funahara et al [24] found decreased activity in Dengue virus infected cases where Krishnamurti et al [59] did not find any statistical significant difference ($P=0.1$, ANOVA). Activities remained within normal values in the latter study. Interestingly, another important inhibitor of fibrinolysis, plasminogen activator inhibitor-1 (PAI-1), was elevated in severe Dengue virus infected cases. Both Wills et al [62] and van Gorp et al [28] found this endothelial derived protein to be circulating in elevated levels in particular in cases with a poor clinical outcome.

Table 5. Results of fibrinolysis assays in different grades of Dengue virus infections*

Reference	Year and location of study	Study design	Patient selection	Characteristics of study subjects	Clinical outcome	Controls
Wills BA et al	1998-1999, Vietnam	Cohort	All children who directly presented to the hospital with a clinical diagnosis of DHF grade III & IV according to WHO criteria	187 cases, of which 48 (21 male) were chosen for detailed study, age range whole group 1-14 years	Serial measurements of coagulation tests on day 1, day 2 and 1 month	-
Krishnamurti C et al	1997-1998, Thailand	Cohort	Children hospitalized with suspected Dengue, fever within 8 hours before enrolment and no obvious non-dengue source of infection	36 male, 32 female, mean age DF cases 8.3 (SD 0.6), DHF cases 8.5 (SD 0.5)	Grade of disease: 21 DF, 8 DHF grade I, 30 grade II & 9 grade III	-
van Gorp EC et al	1998, Indonesia	Cohort	Consecutive patients with a clinical diagnosis of DHF grade III & IV according to WHO criteria	24 male, 26 female, mean age non-survivors 6.0 (SD 2.8), survivors 6.8 (SD 2.8)	Mortality: 13 non-survivors & 37 survivors	-
Huang YH et al	1998, Taiwan	Case control	Laboratory confirmed Dengue virus infected patients	19 male, 6 female; age range 4 to 75 y	Grade of disease: 17 DF & 8 DHF grade I - IV	17 normal individuals
Isarangkura PB et al	Indonesia	Case control	Laboratory confirmed Dengue virus infected patients	20 male, 20 female, age range 6 m to 14 y	Grade of disease: 4 DHF grade I, 20 grade II & 16 grade III	30 normal children of similar age group
Funahara Y et al	Thailand	Case control	Laboratory confirmed Dengue virus infected patients	11 cases, age range 2 to 9 y	Grade of disease: 8 DHF grade II, 2 grade II & 1 grade IV	5 hospitalized non-DHF patients, with no hemorrhagic tendency
Srichaikul T et al	Thailand	Case control	Laboratory confirmed Dengue virus infected patients	22 male, 7 female; age range 4 to 13 y	Grade of disease: 12 DHF grade II, 13 grade III & 4 grade IV	5 normal children and 4 patients with acute infectious diseases other than dengue
Halstead R et al	1972, Thailand	Case control	Patients with a clinical diagnosis of DHF according to Nimmannitya s criteria	18 cases	Grade of disease: 7 DHF grade I/II & 11 grade III/IV	8 hospitalized and febrile non-Dengue patients and 18 afebrile children (16 hospitalized and 2 healthy)
WHO	1971, Thailand	Case control	All patients with a clinical diagnosis of DHF according to Nimmannitya s criteria	52 cases	Grade of disease: 4 DHF grade I, 14 grade II, 22 grade III & 12 grade IV	5 children out of a group of 43 controls (30 normal and 13 non-Dengue)
Suvatte et al	1971, Thailand	Case control	Laboratory confirmed Dengue virus infected patients	128 cases	Grade of disease: 18 DHF grade I, 77 grade II, 29 grade III & 4 grade IV	50
Mitrakul C et al	Thailand	Case series	Laboratory confirmed Dengue virus infected patients	61 cases	Grade of disease: 28 DHF grade II, 17 grade III & 16 grade IV	-
Wenz et al	1962, Thailand	Case series	Laboratory confirmed Dengue virus infected patients	27 cases, age range 4 m to 12 y	Not classified, raw data was reported	-

* Data are presented as mean with corresponding standard deviation (SD) or standard error of the mean (SEM), unless stated otherwise. DF, Dengue fever; DHF, Dengue haemorrhagic fever; FDP, fibrin degradation products; PAP, plasmin-antiplasmin complexes; tPA, tissue-type plasminogen activator; PAI-1, plasminogen activator inhibitor-1; URTI, upper respiratory tract infection; DIC, disseminated intravascular coagulation.

DISCUSSION

We critically reviewed the evidence for an association between coagulation and fibrinolysis disturbances and clinical outcome of Dengue virus infections. This resulted in a selection of studies, of which the majority was published in 1970-1980, demonstrating a variable degree of coagulation activation in Dengue virus infections, which tended to be more pronounced in severe disease through a deranged intrinsic pathway and in particular in cases with a poor clinical outcome. The fibrinolytic system was also found to be more disturbed in the severest forms of disease. Coagulant and fibrinolytic derangements like these are highly suggestive of a stressed haemostatic system that may ultimately result in overt disseminated intravascular coagulation. The observations in the selected studies and previous characteristic findings of widespread intravascular formation of fibrin and thrombotic occlusion of small and midsize vessels [69], highlight the fact that diffuse coagulation is triggered in Dengue virus infections. If haemostatic disturbances are present in patients in the same stage of disease and are shown to coincide with clinical outcome, they can subsequently be used as a valid marker of risk and form a useful part of a risk prediction model, whether causally related or not [11]. Separation of individuals with the same disease into those at high and low risk may be extremely valuable in appropriately targeting therapy.

The contribution of non-overt and overt disseminated intravascular coagulation to morbidity and the risk of mortality however, could not be specified in detail, since none of the selected studies combined test results to diagnose disseminated intravascular coagulation. In clinical disorders known to be associated with disseminated intravascular coagulation, the combination of platelet count, global clotting screening tests, a test for fibrin degradation products and measurement of coagulation inhibitors is advocated to establish or rule out this syndrome with reasonable certainty [66]. In addition, study results were inconsistent and sometimes contradicted by other findings, which was likely a result of differences in time-point of inclusion of study subjects or variations in disease or patient characteristics. In our appraisal of the selected studies special care was taken to assess the design of the studies on which an association could be based and to search for explanatory factors resulting in these inconsistencies. Overall the study features considered most important were those that involved selection bias, sources for controls and confounding [11;19]. Our appraisal of the relevant studies resulted in several inferences.

First, the cohort design was found to be the strongest available study design by which an association between coagulation and fibrinolysis disturbances and clinical outcome of Dengue virus infections could be evaluated. The three cohort studies only comprised small groups of cases (68 cases by Krishnamurti et al [59], 50 cases by van Gorp et al [27;28] and 48 cases out of a group of 187 cases by Wills et al [62]), whereas the number of included cases in the case control studies differed between 11 and 128. Although these study designs may be subject to several forms of biases related to the assembly of cases [70-72], we were unable to ascertain whether this could have distorted the study results due to a lack of detailed information on selection of both cases and controls. Were only those cases selected of which blood samples on

different time points was available? A valid assessment could be impaired if this was the case, since a true association would be underestimated if non-survivors were excluded from analysis due to missing data on all time points.

Second, in addition to the above-mentioned inference and related to the selection of cases and controls are confounding factors resulting from differences between the study subjects in personal and disease characteristics. From the majority of studies it remains unclear whether cases entered the study at a similar stage of disease and if supportive measures were already applied on inclusion or not. Since it is known that Dengue virus infections may rapidly develop in a serious life threatening disease and early treatment may result in rapid recovery [1], it is not unimaginable that slight unrecognised and unmentioned differences in one of these aspects confounded study results. Delayed hospitalisation for example could play an important factor on clinical outcome and could therefore lead to different conclusions when not taken into account. Poorly defined personal characteristics could also not warrant differences in clinical outcome to be independent of age, sex or race. Epidemiological studies even suggest severe Dengue virus infections to be more frequent in certain races and certain specific age groups [1;3;73].

Third, in assessing an association between coagulation and fibrinolysis activation and clinical outcome of Dengue virus infections one could speculate on a direct or indirect effect of the Dengue virus. Is a variable degree of coagulation activation a direct result of Dengue virus or is it a result of other factors, i.e. cytokines, known to independently influence both clinical outcome and coagulation activation [49;74]? Although several types of controls were used, one can argue whether it is appropriate to use a healthy control group in particular when studying the supposed direct effects of the virus or antibodies derived from Dengue infected cases on coagulation and fibrinolysis [5;9]. A distinction could not be made between the independent effects of the Dengue virus and non-specific features of infection on alterations in coagulation and fibrinolysis activity. In our view it is therefore only justified to include a group of healthy individuals to obtain reference values and that it is more appropriate to consider drawing controls from the same hospital or clinic diagnosed with other diseases [75].

Despite using combinations of keywords related to the disease and the exposure of interest without any restrictions or adding any methodological filter, the search only yielded fourteen articles. This indicates the lack of studies performed to date and in combination with the inconsistencies mentioned above, which is mostly due to published studies derived from 1970-1980, emphasises the fact that more studies are needed on this subject. Two of the articles were case series [56;60] which suggested coagulation and fibrinolysis activation to be associated with clinical outcome. It should however be noted that due to a lack of a comparison group the only conclusion one can draw is that haemostatic disturbances do occur in patients with Dengue virus infections while a true association cannot be established. Descriptive studies can generally provide interesting biological information if a group of subjects is adequately followed-up over time and generate hypotheses that need to be tested in methodologically rigorous studies [71;76]. We decided not to broaden or adjust our

search strategy based on the retrieval of two articles, which were identified by hand searching the reference lists of selected articles and reviews [55;56]. These articles described data obtained from patients suffering from severe Dengue virus infections that went by the name of Thai haemorrhagic fever, a name that was gradually replaced by Dengue haemorrhagic fever and Dengue shock syndrome in the early 1970s. Since it is most likely that unidentified articles, which were published before the 1970s, will not change the conclusion of our analysis, our search strategy was not extended with for example the keywords Thai haemorrhagic fever.

We conclude that increasing Dengue virus disease severity is accompanied by coagulation and fibrinolysis disturbances likely to result in disseminated intravascular coagulation in advanced stages of disease. However, an estimate of association between haemostatic disturbances and clinical outcome in Dengue virus infections based on selected studies was not possible. Additional studies should evaluate to what extent activation of coagulation and fibrinolysis contribute to morbidity and the risk of mortality in Dengue virus infections, whether this activation follows the current hypothetical concept of coagulation, and whether the observed responses are a result of a generalised inflammatory response or result from an interaction with the Dengue virus and Dengue virus-specific antibodies as demonstrated by previous in vitro test results [5-9]. The best study design for estimating the association between coagulation and fibrinolysis and clinical outcome of Dengue virus infections would be a prospective cohort study. One should keep in mind that an appropriate number of patients selected at a similar time point in disease is required. Measurement of biochemical markers of coagulation activation will provide valuable information on characteristic haemostatic responses in Dengue virus infections and by combining test results of platelet count, aPTT, PTT, tests for fibrin degradation products and coagulation inhibitors, one could determine whether disseminated intravascular coagulation is associated with clinical outcome. This information will help us to improve our understanding of the pathophysiological mechanism of haemorrhage in Dengue virus infections, to separate Dengue virus infected patients into those at high and low risk for poor clinical outcome, and to search and find alternative forms of therapy.

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Chapter 6

ARE DENGUE VIRUS ASSOCIATED COAGULATION ABNORMALITIES RELATED TO DISSEMINATED INTRAVASCULAR COAGULATION?

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Submitted

ABSTRACT
Dengue viruses may cause a severe and potentially fatal hemorrhagic disease in humans. Whether disseminated intravascular coagulation (DIC) plays an important role in disease severity is subject of discussion. To clarify the role of DIC in Dengue, we examined coagulation abnormalities in Dengue virus-infected patients throughout time. Eighty-eight patients with severe Dengue virus infection were enrolled. Blood samples were obtained on day of admission, days 1, 2 and 7 after admission and at a 1-month follow-up visit. Coagulation screening tests and measurement of anticoagulant factors were performed on serial samples of included patients. The majority of patients had coagulation abnormalities on admission and on the first two days following admission. Abnormal coagulation screening tests and alterations in levels of anticoagulant factors were associated with disease severity. A diagnosis of overt DIC was present on admission in 16% and during follow up in 28% of our patients. This was associated with an increased risk of severe disease (DIC present on admission: OR 5.2, 95%CI 1.3-21.2; DIC present during follow up: OR 4.4, 95%CI 1.6-12.1). Anticoagulant and fibrinogen patterns, however, were substantially different from bacterial associated DIC and were rather in line with observations made in patients with liver disease. Together, these data indicate that Dengue virus infections may cause gross coagulation abnormalities. Although the observed coagulation abnormalities lead to a positive overt DIC score, they cannot be explained by DIC alone. Additional mechanisms need to be taken into account.

INTRODUCTION

Dengue ranks high among emerging infectious diseases in public health significance and is the most important of arthropod-borne viral diseases [1]. At present, almost 30% of the world population is at risk for Dengue virus infection and it is expected that this number will increase substantially as its transmission spreads to unaffected geographic regions [2]. Infection with any of the four Dengue virus serotypes may go unnoticed or may induce clinical manifestations ranging from uncomplicated Dengue fever (DF) to the potentially life-threatening Dengue haemorrhagic fever (DHF) and Dengue shock syndrome (DSS) [1]. The cardinal feature that distinguishes DHF and DSS from DF is increased vascular permeability, resulting in the loss of plasma from the vascular compartment with impending shock when plasma loss is excessive [3]. The characteristic haemorrhagic manifestations are thought to be due to thrombocytopenia and thrombocytopathy. Recent data suggest that other abnormalities in blood coagulation and fibrinolysis may also play an important role in disease severity [4-6]. Whether these abnormalities are a result of a consumptive coagulopathy (disseminated intravascular coagulation (DIC)), a failure to synthesise coagulation factors by the liver, or a loss of coagulation factors from the vascular compartment due to increased vascular permeability, is subject of discussion. Identification of the precise nature of Dengue virus associated coagulation abnormalities is important since it may have consequences for surveillance, prognosis and symptomatic support. If the abnormalities are a result of DIC, patients will be at increased risk of organ failure and death [7]. When other mechanisms (impaired synthesis or permeability changes) predominate, the coagulation profile may be of limited clinical relevance.

It has been demonstrated that three important characteristics of DIC are present in severe Dengue virus infections: increased generation of thrombin, suppression of physiologic anticoagulant mechanisms and an impaired fibrinolytic system [4-6]. However, none of these studies combined a sufficient number of coagulation assays to determine DIC with reasonable certainty in a large group of Dengue virus infected individuals. Furthermore, the clinical significance in terms of predicting severe disease or death has also not been estimated. The aim of the present study was to evaluate Dengue virus associated coagulation abnormalities throughout time, to determine whether these coagulation abnormalities are associated with disease severity, and to assess whether these abnormalities are indeed a result of DIC.

PATIENTS AND METHODS

Patients and clinical procedures

The study was performed from February 2001 to December 2001 at the paediatric intensive care unit and the paediatric ward of the Dr. Kariadi Hospital in Semarang, Indonesia. Consecutive patients, aged 2 to 14 years, admitted to the hospital with a clinical diagnosis of suspected Dengue haemorrhagic fever or Dengue shock syndrome were included. Members of the study team recorded demographic data, medical history, physical examination findings and subsequent progress for each patient on a standard data form. Blood samples for coagulation studies were obtained on day of

admission (day 0), the following two days (day 1 and day 2), seven days after admission (day 7) and at a 1-month follow-up visit (day 30). The controls were healthy school-aged Javanese children from 6 to 13 years of age (median age 10 years) who originated from the same geographical area as the cases. The ethics committee of the Dr. Kariadi Hospital approved all clinical and laboratory aspects of this study. Blood samples were only taken from patients and controls provided that a parent or legal guardian gave informed consent.

Laboratory methods

All blood samples were centrifuged within 1-2 hours after retrieval at 15°C for 20 minutes at 1600*g. Plasma was separated, stored at -80°C and assayed batch-wise in The Netherlands after transportation on dry ice. Coagulation screening tests and the measurement of anticoagulant factors were performed on serial samples of included patients. Coagulation screening tests included activated partial thromboplastin time (PTT), prothrombin time (PT), D-dimer, prothrombin fragments 1 and 2 (F1+2) and fibrinogen. PT and PTT were performed on an MDA-180 haemostasis autoanalyser from bioMérieux (Durham, North Carolina, USA). In the PTT assay, an abnormal biphasic waveform profile can be determined by a decrease of light transmission at 580 nm immediately after addition of calcium but before initiation of clot formation. To quantify this effect, the slope of the initial phase of the light transmission was recorded, which is being proposed as a highly specific means to identify patients with sepsis and DIC [8]. Fibrinogen and antithrombin were quantitatively determined on a Behring Coagulation System using reagents from Dade Behring (Marburg, Germany). D-dimer level was measured by a latex-enhanced photometric immunoassay (MDA D-dimer, bioMérieux). Protein C was determined using the Coamatic protein C activity kit from Chromogenix (Mölndal, Sweden). Total protein S antigen levels were measured by ELISA employing antibodies from DAKO (Glostrup, Denmark). Free protein S antigen levels were measured by precipitating the C4b-binding protein-bound fraction with polyethylene glycol 8000 and measuring the concentration of free protein S in the supernatant. C4b-binding protein antigen was determined by enzyme-linked immunosorbent assay with a monoclonal antibody (8C11) as catching antibody and a monoclonal antibody as detecting antibody (9H10-peroxidase labelled) [9]. Reference values were obtained from the healthy control group and are given in tables 3 and 4. Patient's test results were considered abnormal when values fell outside the range measured in this group.

Determination of overt DIC status

The presence of overt DIC was established using a diagnostic algorithm proposed by the Scientific Subcommittee on DIC of the International Society on Thrombosis and Haemostasis (ISTH) [10]. Following this algorithm, a DIC score can be calculated with a score equal or more than 5 being compatible with overt DIC. A number of routine laboratory tests contribute to the DIC score: platelet count (a score of 2 for a platelet count <50.000 cells/mm^3 and a score of 1 for a platelet count between 50.000 and 100.000 cells/mm^3), PT (a score of 2 for a prolonged PT >6 seconds and a score of 1 for a prolonged PT between 3 and 6 seconds), fibrinogen (a score of 1 for fibrinogen

levels <1 g/l) and fibrin-related markers [10]. D-dimer levels were used as marker of fibrin cleavage. The ISTH overt DIC definition proposed a score of 2 for a moderate increase and a score of 3 for a strong increase in fibrin related markers, without providing a numerical cut-off for these points. A cut-off value of 4 mg/ml for D-dimer was used as threshold value for strong increases in fibrin related markers. Values between the upper level of normal for healthy controls and 4 mg/ml were defined as moderate increases.

Diagnostic procedures

The presence of Dengue was objectively confirmed by serological assays. A commercially available capture and indirect ELISA (Focus Technologies, Cypress, Calif., USA) was used for the detection of Dengue specific IgM and IgG antibodies according to the procedures described by the manufacturer. The sensitivity and specificity of the assays has been described elsewhere [11]. For some patients, a definitive serodiagnosis was not possible either because no convalescent sample was obtained or because the interval between two serologic samples was insufficient. Detection of Dengue antigen and RNA was attempted in these cases using a dot blot immunoassay and a Dengue serotype revere transcriptase PCR respectively [12]. Patients with serologic evidence of acute Dengue infection, a positive dot blot and/or positive PCR were considered to have confirmed Dengue. Those with definite negative serology and/or a well-substantiated alternative clinical diagnosis were classified as not Dengue. In the absence of a well-substantiated alternative clinical diagnosis and with inconclusive serology patients were classified as indeterminate.

Statistical analysis

Plasma levels of the analytes measured were compared between patients with circulatory failure during admission and those remaining hemodynamically stable. Circulatory failure was defined as hypotension for age (systolic pressure <90 mmHg for those >5 years of age and <80 mmHg for those <5 years of age) or narrow pulse pressure (<20 mmHg) [3]. Data are presented as medians with corresponding interquartile ranges or as numbers with percentage. Continuous variables were compared by the Kruskal Wallis test, since data were not normally distributed. For categorical variables, the chi-square or Fisher's exact tests were applied. Odds ratios and the corresponding 95% confidence intervals (95% CI) for the development of circulatory failure in the presence of overt DIC on admission or at any point in time during follow up were estimated by cross-tabulation. Two-sided P-values less than 0.05 were considered to indicate statistical significance. Analyses were performed with use of SPSS software, version 11.0.1.

RESULTS

A total of 108 patients were initially enrolled in this study. Parents from five patients withdrew informed consent during follow up, whereas 1 patient appeared not to suffer from Dengue during follow up but from measles. Of the remaining 102 patients, 39 had evidence of circulatory failure: 27 (26%) had shock on admission and 12 (12%) went on to develop shock during hospital admission. Five patients (5%) died.

The presence of Dengue was objectively confirmed in 88 patients (86%). Fourteen patients (14%) had inconclusive serology and were therefore categorised as indeterminate. Patient baseline characteristics are summarised in Table 1.

Table 1. Patient characteristics at admission°

Variable	Patients with confirmed Dengue	Patients classified as Indeterminate
Age (years), median (IQR)	7 (5-10)	8 (7-10.3)
Male sex, N (%)	44 (50)	5 (36)
Duration of illness (days), median (IQR)	4 (3-4)	3 (2.8-3)
Haemorrhagic tendency, N (%)	79 (90)	13 (93)
Positive tourniquet test, N (%)	63 (72)	11 (79)
Spontaneous haemorrhage, N (%)	52 (59)	8 (57)
Hepatomegaly, N (%)	59 (67)	9 (64)
Systolic blood pressure (mmHg), median (IQR)	90 (80-100)	100 (95-105)
Hypotension for age, N (%) †	26 (30)	1 (7)
Pulse pressure <20 mmHg, N (%)	7 (8)	1 (7)
Pulse rate (beats/min), median (IQR)	120 (104-128)	112 (100-122)
Presence of pleural effusion, N (%) ‡	50 (57)	5 (36)
Haematocrit (%), median (IQR)	41% (35-46)	40% (35-45)
Platelet count (cells/mm^3), median (IQR)	59.000 (36.000-90.000)	135.000 (86.000-176.000)
Platelet count ≤100.000 cells/mm^3, N (%)	73 (83)	4 (29)

° IQR, denotes Inter Quartile Range; N, denotes number
† hypotension is defined to be a systolic pressure of <80 mmHg for those <5 years of age, or <90 mmHg for those ³5 years [3]
‡ the presence of pleural effusion was assessed through a chest X-ray

COAGULATION SCREENING TESTS

The majority of patients had abnormal coagulation screening test results on the day of admission and on the first two subsequent days. Abnormal test results were found more frequently among patients with circulatory failure, except for F1+2 and D-dimer test results. F1+2 levels were elevated in 48 out of 52 hemodynamically stable patients and in all patients with circulatory failure (P=0.12). Elevated D-dimer levels were found in 42 out of 52 hemodynamically stable patients and in 31 out of 35 patients with circulatory failure (P=0.39). Low fibrinogen levels were found in 42 out of 52 hemodynamically stable patients and in all patients with circulatory failure (P<0.01). Prolonged PT values were found in 28 out of 51 hemodynamically stable patients and in 29 out of 36 patients with circulatory failure (P=0.02). Prolonged PTT values were found in 38 out of 52 hemodynamically stable patients and 33 out of 36 patients circulatory failure (P=0.05). The PTT waveform was abnormal during admission in 32% of the patients (abnormal test results in 12 out of 52 hemodynamically stable patients and in 16 out of 36 patients with circulatory failure; P=0.04).

Table 2. Results of analysis of coagulation screening test in 88 patients with confirmed Dengue infection and different disease severity*

Assays	Control	Patients	Hemodynamically stable		Circulatory failure		P-value
			N	Median (IQR)	N	Median (IQR)	
PTT (seconds)	27.3-41.5	On admission	46	45.8 (41.1-52.8)	34	57.7 (49.4-69.2)	<0.01
		Peak value	52	48.2 (41.3-54.6)	36	62.2 (51.8-75.5)	<0.01
WAVE (%T/sec)	> -0.074	On admission	46	-0.016 (-0.037,-0.012)	35	-0.044 (-0.087,-0.011)	<0.01
		Lowest value	52	-0.026 (-0.072,-0.005)	36	-0.068 (-0.093,-0.033)	<0.01
PT (seconds)	10.5-13.5	On admission	47	13.6 (12.5-15.1)	34	15.3 (13.5-16.8)	<0.01
		Peak value	51	13.6 (12.7-15.9)	36	16.0 (14.6-19.3)	<0.01
Fibrinogen (g/l)	2.4-4.6	On admission	51	2.1 (1.3-2.6)	36	1.0 (0.8-1.2)	<0.01
		Lowest value	52	1.8 (1.2-2.2)	36	0.9 (0.7-1.0)	<0.01
F1+2 (nmol/l)	0.5-1.1	On admission	47	1.4 (1.0-2.1)	35	1.6 (1.3-1.9)	0.26
		Peak value	52	2.0 (1.4-3.1)	36	2.0 (1.7-2.5)	0.81
D-dimer (µg/ml)	0.2-1.1	On admission	45	1.3 (0.9-1.9)	34	1.1 (0.7-1.6)	0.53
		Peak value	52	1.9 (1.3-2.7)	35	2.6 (1.4-3.9)	0.04

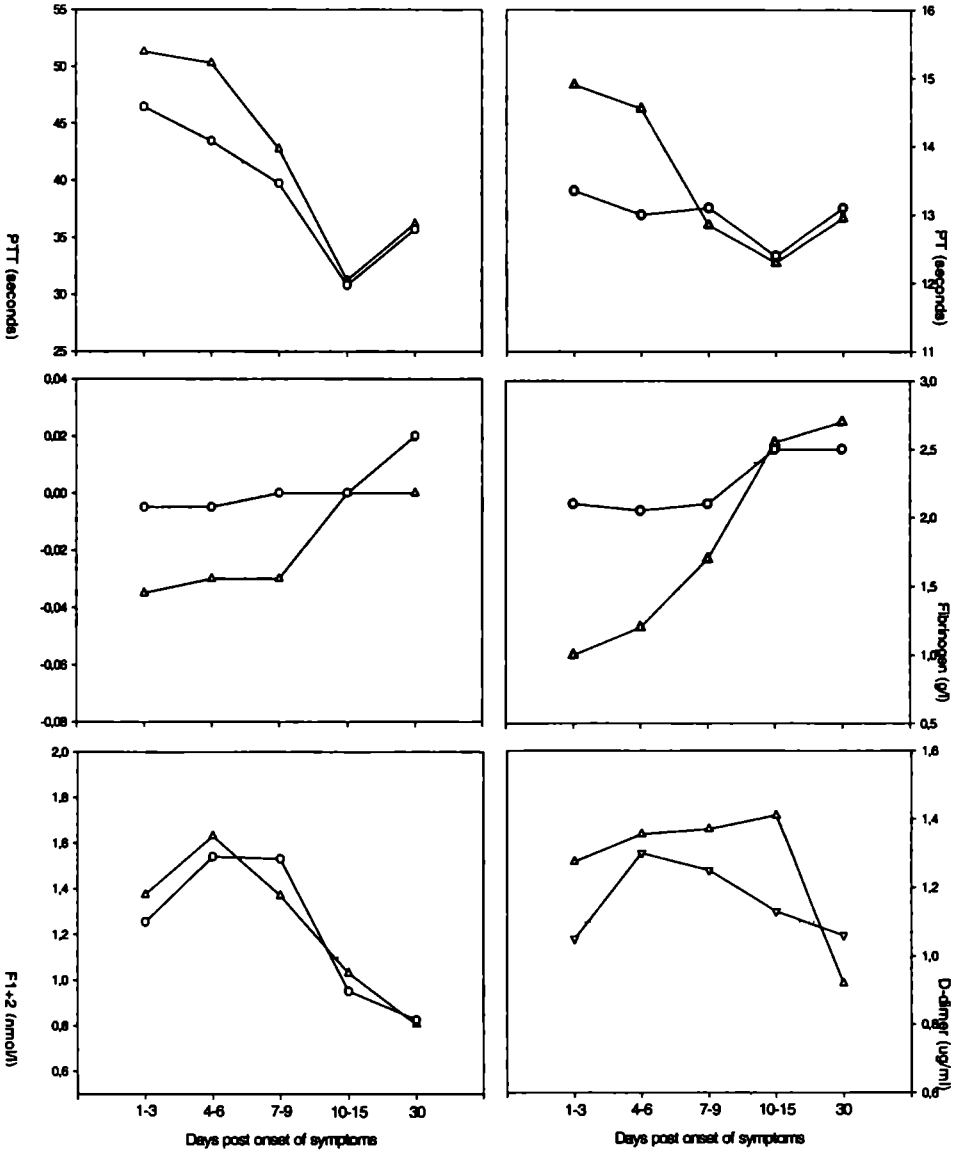
* Circulatory failure was defined as hypotension for age (systolic pressure <90 mmHg for those ≥5 years of age and <80 mmHg for those <5 years of age) or narrow pulse pressure (<20 mmHg) [3]. N denotes number of patients; IQR denotes interquartile ranges. P values are for the comparison of patients with circulatory failure and those remaining hemodynamically stable.

Table 3. Results of analysis of anticoagulant factors in 88 patients with confirmed Dengue infection and different disease severity*

Assays	Control	Patients	Hemodynamically stable		Circulatory failure		P-value
			N	Median (IQR)	N	Median (IQR)	
Protein C (%)	57-130	On admission	51	60 (53-76)	36	43 (34-55)	<0.01
		Lowest value	52	58 (45-54)	36	40 (29-54)	<0.01
AT III (%)	94-123	On admission	51	99 (82-112)	36	72 (60-81)	<0.01
		Lowest value	52	97 (81-110)	36	65 (52-81)	<0.01
Tot prot S (%)	66-116	On admission	51	63 (53-73)	36	40 (33-45)	<0.01
		Lowest value	52	58 (49-67)	36	37 (30-44)	<0.01
Free prot S (%)	24-56	On admission	51	23 (19-29)	36	18 (14-24)	<0.01
		Lowest value	52	20 (17-25)	36	17 (14-20)	<0.01
Ratio free-to-total	31-51	On admission	51	40 (31-46)	36	48 (41-53)	<0.01
Protein S (%)		Highest value	52	41 (35-51)	36	52 (48-61)	<0.01

* Circulatory failure was defined as hypotension for age (systolic pressure <90 mmHg for those ≥5 years of age and <80 mmHg for those <5 years of age) or narrow pulse pressure (<20 mmHg) [3]. N denotes number of patients; IQR denotes interquartile ranges. P values are for the comparison of patients with circulatory failure and those remaining hemodynamically stable.

Figure 1. Coagulation screening test



Kinetic changes of median values of the results of coagulation screening tests, by days after onset of symptoms, in patients with circulatory failure (triangle) and hemodynamically stable patients (circle).

In Figure 1 the kinetic changes in coagulation test results are shown per days after onset of symptoms. Prolonged PTT and PT and decreased fibrinogen levels were present in the first six days after onset of symptoms, returning to normal values thereafter. Elevated D-dimer and F1+2 levels were generally present for a period of 10 to 14 days after onset of symptoms. The PTT waveform changed over time but median values remained within normal limits. Table 2 summarises the results of coagulation screening tests on admission and most abnormal values during follow up in relation to the occurrence of circulatory failure or not. Median values of PTT and PT were higher in patients with circulatory failure. Median values of PTT waveform on admission and during follow up were lower in patients with circulatory failure compared to patients who were hemodynamically stable. Values, however, still fell within the normal range. No statistical significant differences were observed for D-dimer and F1+2 levels on admission and peak values during admission. Median fibrinogen values on admission and lowest values during admission were significantly lower in patients with circulatory failure.

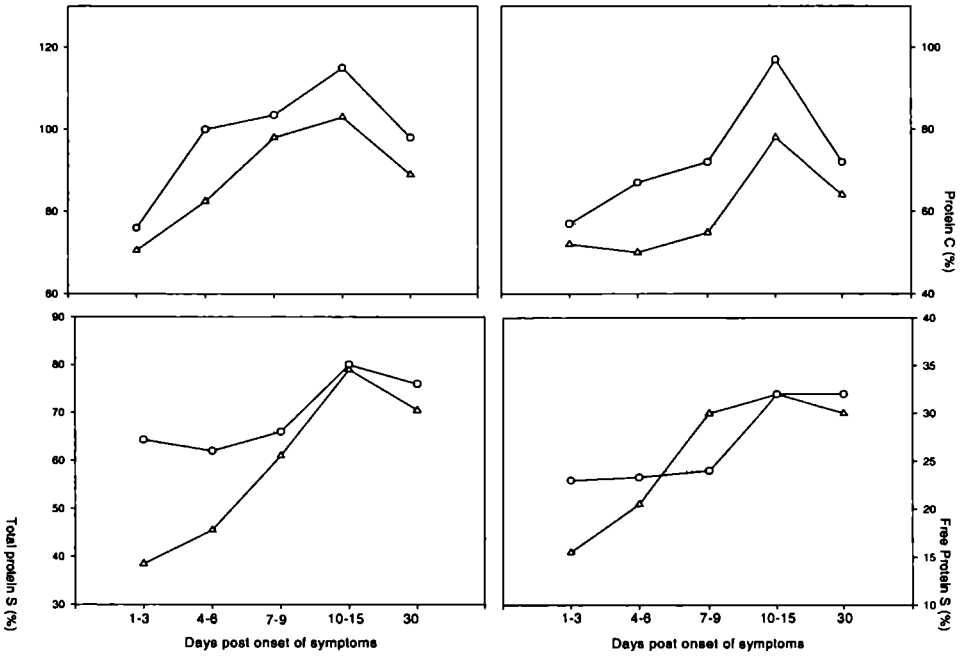
ANTICOAGULANT FACTORS

Levels of natural anticoagulant proteins were also affected. Antithrombin and protein C levels on admission were decreased in 18 out of 51 hemodynamically stable patients and 29 out of 36 patients with circulatory failure ($P<0.01$). Decreased free and total protein S levels on admission were also more frequent in patients with circulatory failure. Total protein S levels were decreased in 32 out of 51 hemodynamically stable patients and in all patients with circulatory failure ($P<0.01$). Twenty-six out of 51 hemodynamically stable patients and 27 out of 36 patients with circulatory failure had decreased free protein S levels ($P=0.03$).

In Figure 2 the kinetic changes of levels of anticoagulant proteins are shown per day after onset of symptoms. Levels of antithrombin and protein C, free and total protein S were not decreased or slightly decreased in the first week after onset of symptoms in hemodynamically stable patients. Low levels of anticoagulants, particularly total protein S, were observed in patients with circulatory failure. Table 3 summarises the results of the measurement of anticoagulant proteins on admission and most abnormal values during follow up in relation to the occurrence of circulatory failure. Particularly, free protein S levels were significantly decreased in patients with circulatory failure, whereas the ratio free-to-total protein S on admission and during follow up was higher in patients with circulatory failure. C4b-binding protein levels could only be measured in a subset of our patients (5 hemodynamically stable patients and 7 patients with circulatory failure) because of insufficient material left. C4b-binding protein values tended to be lower in patients with circulatory failure compared to values found in hemodynamically stable patients (respectively 23% (IQR 17-42) and 41% (IQR 38-50); $P=0.12$). It is thus likely that low free protein S and a high free-to-total protein S ratio are explained by low levels of total protein S.

On admission overt DIC was present in 12 out of 75 patients (16%) from whom all necessary test results were available. Fewer patients were diagnosed with overt DIC on the following days (8 out of 64 patients (13%) on day 1; 10 out of 79 patients (13%) on day 2). None of the patients had evidence of overt DIC on day 7 after admission. A total of 24 patients (28%) had evidence of overt DIC during follow up. Overt DIC occurred more frequently in patients with circulatory failure on and during admission compared to hemodynamically stable patients. Nine out of 32 patients with circulatory failure had overt DIC on admission compared to 3 out of 43 hemodynamically stable patients (OR 5.2, 95%CI 1.3-21.2; P=0.02). During follow up 16 out of 35 patients with circulatory failure had evidence of overt DIC compared to 8 out of 59 hemodynamically stable patients (OR 4.4, 95%CI 1.6-12.1; P<0.01).

Figure 2. Anticoagulant factors



Kinetic changes of median values of the results of the measurement of anticoagulant factors, by days after onset of symptoms, in patients with circulatory failure (triangle) and hemodynamically stable patients (circle).

DISCUSSION

Our prospective study shows that abnormal coagulation screening tests and alterations in levels of anticoagulant factors occur early in the course of disease in Dengue virus infected patients and that they are associated with disease severity. A considerable number of our patients had a combination of test results that is usually sufficient to establish a diagnosis of DIC with reasonable certainty [7]. When the severity of DIC was scored according to criteria proposed by the ISTH we found that the presence of overt DIC on admission or within the first two days after admission was significantly associated with circulatory failure [10]. Based on the presence of an increased level of thrombin production (F1+2), fibrin related markers (D-dimers), prolonged clotting times and reduced natural anticoagulants, there are sufficient arguments in favour of DIC. However, the coagulant and anticoagulant patterns are different from other types of DIC on the basis of the following arguments.

First, a number of studies on bacterial sepsis have shown that DIC causes severe deficiencies of anticoagulant factors, with protein C levels varying between undetectable levels and 40%, and antithrombin levels of 60% or less [13-18]. We found reduced protein C and antithrombin levels in 54% of our patients with median values being higher than those found in patients suffering from DIC associated with bacterial infections. We also found decreased total and free protein S levels in respectively 78% and 61% of our patients and low C4b-binding protein levels in patients with circulatory failure. In DIC, total protein S levels are usually only marginally affected or not affected at all whereas free protein S, the functionally active form of protein S, may be decreased as a result of elevated levels of C4b-binding protein [15;16;18-20]. Second, we found low fibrinogen plasma levels early in the course of disease in nearly all patients, including those with less severe disease. Because fibrinogen acts as an acute-phase reactant in patients with DIC associated with bacterial infections, plasma levels can remain well within the normal range for a long period of time. Typically, low fibrinogen plasma levels are only seen in very severe cases of DIC [14;21]. Third, the slope of the initial phase of the PTT waveform remained within the normal range in the majority of our patients. Recently, this test proved to be particularly useful in excluding overt DIC due to a high specificity, and our findings are therefore not indicative of overt DIC [8]. However, a normal waveform may still be compatible with a non-overt, or mild form of DIC [22]. Furthermore, the molecular mechanism contributing to this waveform is based on complex formation between very low density lipoproteins, C-reactive protein and calcium [23]. Since C-reactive protein is only marginally elevated in Dengue virus infected patients, the possibility exists that the formation of a complex between C-reactive protein and very low density lipoproteins remains inadequate [24;25].

In addition to DIC, alternative mechanisms are involved that may influence blood coagulation during infection with Dengue viruses. In view of the increased vascular permeability that characterises DHF and DSS it has been suggested that coagulation factors with a molecular weight close to that of albumin (M_r 69 kDa) diffuse into the extravascular compartment due to increased vessel permeability [6]. This phenomenon could explain the observed low levels of antithrombin (M_r 62 kDa), protein C (M_r 62 kDa) and protein S (M_r 69 kDa) in DHF and DSS patients. However,

low levels of anticoagulant proteins were found in patients who had no evidence of increased vascular permeability and it may be questioned whether the considerably larger fibrinogen (M_r 340 kDa) is also lost from the vascular compartment. In a study of cytokine profiles, no association was found between levels of proteins C and S and an increase in haematocrit, which is a marker of vascular permeability in patients suffering from DHF or DSS [26]. Decreased levels of proteins C and S were correlated with serum aminotransferases, suggesting that injury to hepatocytes may be involved in Dengue virus associated coagulation abnormalities. Our findings of low C4b-binding protein levels in DSS patients compared to non-shock patients in combination with decreased total and free protein S levels are in line with observations made in patients with liver disease or those taking oral anticoagulant therapy [19;20]. A failure to produce coagulation factors must therefore be taken into account.

In conclusion, our study demonstrates that Dengue virus infections may cause clinically significant coagulation abnormalities that are at least in part attributable to DIC. The presence of overt DIC at admission and during follow up was associated with an increased risk of circulatory failure. The degree and pattern of DIC caused by Dengue viruses are quite different from DIC associated with bacterial sepsis and it is likely that additional mechanisms like loss of coagulation factors from the vascular compartment due to increased vascular permeability or a failure to produce coagulation factors contribute to the observed abnormalities. The study of Dengue virus associated coagulation abnormalities may give important insights in how viruses influence the complex coagulation cascade different from bacterial pathogens. In addition, monitoring of laboratory markers of DIC may be helpful in identifying those patients at greatest risk of severe disease.

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ABSTRACT
The mechanisms contributing to bleeding complications in Dengue hemorrhagic fever (DHF), were studied by investigating the pattern of activation of the coagulation and fibrinolytic systems in 50 children with severe DHF. Thirteen patients died (26%), and activation of coagulation was most pronounced in the deceased group. Fibrinolysis was also activated, but this activation was relatively weak compared with that of coagulation as a result of persistently high plasminogen-activator-inhibitor (PAI) levels. High PAI levels also prevented a switch from the procoagulant to the profibrinolytic state in lethal DHF. The present study is the first to demonstrate such a mechanism in a viral infection. This imbalance between coagulation and fibrinolysis may be used as a prognostic marker, but it may also be used as a target for future therapeutic intervention.

INTRODUCTION

Dengue fever (DF) is the most prevalent viral disease transmitted by arthropod vectors worldwide [1]. The clinical picture varies from mild, uncomplicated DF to Dengue hemorrhagic fever (DHF) and Dengue shock syndrome (DSS). Dengue virus infections form a major and growing health care problem worldwide, especially in endemic areas such as South East Asia and Latin America. Each year millions of people are infected, and about 250.000–500.000 patients develop severe DHF/DSS, accompanied by a high mortality rate of 1-10% [1-3].

Limited data suggest that activation of coagulation and fibrinolysis play a role in the pathogenesis of DHF and DSS [4-7]. However, the pathogenetic mechanism underlying the bleeding complications and multiorgan failure (MOF) has not yet been elucidated. Systemic infections in general may influence haemostasis, leading to thrombohemorrhagic complications and disseminated intravascular coagulation (DIC), haemolytic uremic syndrome, thrombotic thrombocytopenic purpura, or vasculitis. Bleeding or thrombosis, or both, may be dominant symptoms. DIC may contribute to MOF and is associated with a high mortality [8-10]. However, current insights into the relationship between infectious diseases and the coagulation system are based mainly on data obtained from clinical and experimental studies of bacterial sepsis and little is known about the relevance of this system in severe viral infections [11;12].

A curative treatment or vaccine for DHF/DSS and other viral hemorrhagic pathogens is not available, which underlines the need to understand the pathogenesis of these infections. In the present prospective longitudinal study we investigated the balance between the activation patterns of the coagulation and fibrinolytic systems in the course of severe DHF.

METHODS

Study setting

The study was undertaken at the Dr. Kariadi University Hospital of the University of Diponegoro in Semarang, Central Java, Indonesia, where Dengue is endemic and is known to cause yearly outbreaks.

Patients and monitoring

Consecutive patients (children) admitted to the paediatric intensive care unit, with a clinical diagnosis of severe DHF (grade III to IV) according to WHO criteria [13], were included. In brief, the clinical diagnosis of DHF grade III was based on the presence of fever, a hemorrhagic tendency, plasma leakage manifested by pleural effusion, and/or a rise in haematocrit ($\geq$ 20%), thrombocytopenia ($< 100x10^9/L$), and evidence of circulatory failure manifested by a narrowing pulse pressure ($\leq$ 20 mmHg) or clinical signs of shock with the presence of a cold clammy skin. Those with profound shock were diagnosed as DHF grade IV.

To evaluate coagulation activity, the following tests were carried out: activated partial thromboplastin time (aPTT), prothrombin time (PT), fibrinogen, prothrombin fragment 1+2 (F1+2), thrombin-anti-thrombin complexes (TAT), protein C, and protein S. Fibrinolytic activity was evaluated by the measurement of tissue type plasminogen

activator (tPA), plasmin-antiplasmin complexes (PAP), plasminogen activator inhibitor (PAI) and D-dimers All samples were obtained during hospitalization, on three consecutive days, starting on the day of admission, with a fourth and final sample on day 7 or the day of discharge (survivors) or the day before death (non-survivors)

The study protocol received approval from the institutional Review Board of the University Hospital of Diponegoro University in Semarang and informed consent was obtained from children's parents or guardians before inclusion in the study

Diagnosis

The presence of Dengue was objectively confirmed by serological assays A capture and indirect enzyme-linked immunosorbent assay (ELISA) detected Dengue specific IgM and IgG antibodies in serum samples [14] Dengue infections were defined as primary when the ratio of Dengue-specific IgM to IgG serum antibodies was >1 When the ratio of Dengue specific IgM to IgG serum antibodies was <1, the infections were defined as secondary Dengue cases [13] Blood cultures were obtained in all patients to exclude bacterial infections

Blood collection

Venous blood (9 vol.) for measurement of the aPTT, PT, fibrinogen, F1+2, TAT complexes, protein C, protein S and D-dimers was drawn into Vacutainer tubes containing 0 105 M sodium citrate (1 vol) (Becton Dickinson, UK) For measurement of PAP complexes, tPA and PAI, blood was collected in siliconized Vacutainer tubes (Becton Dickinson) containing Polybrene (Janssen Chimica, Belgium) and EDTA (0.05%, w/v, and 10 mM, respectively, final concentrations) to prevent in vitro complex formation.

All blood samples were immediately immersed in melting ice and subsequently centrifuged at 4°C for 20 min. at 1600*g* Plasma samples were stored at –70°C until assayed. Routine laboratory tests (haematology and chemistry) were done in Indonesia. Research assays were carried out on samples transported to the Netherlands on dry ice.

Coagulation assays

Fibrinogen concentrations (Clauss method), aPTT and PT were measured by means of a KC 10 (Sigma Aldrich, Germany) mechanical coagulometer, using bovine thrombin (Fibrinomat, bioMérieux, France), actin FS (Dade Behring, Germany) and Thrombomat (bioMérieux, France), respectively F1+2 and TAT levels were determined by ELISA kits available commercially, according to the manufacturer's instructions (Enzygnost F1+2 micro and TAT micro, Dade Behring, Marburg Germany) Protein C antigen and free and total protein S antigen (with and without precipitation with PEG 25) were assessed with sandwich-type ELISA kits according to the manufacturer's instructions (Stago Boehringer, Ingelheim, Germany and DAKO-ITK Diagnostics)

Fibrinolysis

For the measurement of D-dimer levels, the TintElize D-dimer ELISA from Biopool (Sweden) was used, according to the manufacturer's instructions. TPA and PAI were measured with sandwich ELISA kits using specific monoclonal antibodies as described by de Boer et al. [15]. PAP complexes were measured with a radioimmunoassay, as described by Levi et al. [16].

Statistical analysis

The plasma levels of the analytes measured are presented as median values with their corresponding interquartile range (IQR). The Mann-Whitney-U test was used to compare the respective plasma levels of patients who died during the study (non-survivors) with those who recovered (survivors). The composition of the group changed and the number of patients became limited, due to mortality. The level of statistical significance of these data: two-tailed p-values of < 0.05 were considered to indicate statistical significance. Analyses were performed using statistical software (SPSS 9.0).

RESULTS

Patients

A total of 50 consecutive children with a clinical diagnosis of DHF were enrolled in the study. The base-line characteristics of the subgroups, i.e. survivors and non-survivors, were similar (Table 1). All patients were of Javanese origin, excluding racial differences.

Table 1. Baseline characteristics of the patients classified as DHF grade III and DHF grade IV*

	Survivors (n=37)	Non-survivors (n=13)
Age (yr)	6 8 (2 8)	6 0 (2 8)
Female sex (%)	46%	69%
Admission day	4 3 (0 9)	4 1 (1 4)
Clinical diagnosis of DHF		
DHF III (n)	34	9
DHF IV (n)	3	4
Thrombocytes (x 10^9/L)	63 9 (35 7)	48 7 (12 9)
Haematocrit (highest) (%)	38 (6 4)	36 (7 4)
White blood cell count (x10^9/L)	7 5 (4 3)	10 2 (5 1)
CRP	22 6 (33 5)	13 6 (18 8)
Pleural effusion right-sided (n)	12	3
Pleural effusion Bilateral (n)	2	6
Lowest systolic blood pressure (mmHg)	92 (11)	83 (11)
Lowest diastolic blood pressure (mmHg)	59 (24)	45 (31)
Clammy skin (n)	3	6
Cold extremities (n)	35	13

* Values are reported as means with their corresponding standard deviation or numbers (n). Patients were clinically diagnosed as DHF grade III or DHF grade IV (DSS), according the criteria of the WHO [13]. Normal values for Haematocrit 33 to 40%; Leucocytes 4-11x 10^9/L; Thrombocytes 150-400 x 10^9/L.

The serological assay confirmed Dengue virus infections in all patients, either by an IgM response or by a fourfold rise in IgG titres. Antibody profiles were typical for secondary Dengue virus infection, as only IgM to IgG ratios of <1 were found.

Thirteen patients (26%) died during follow up on the intensive care unit with laboratory-proven DIC and complications of shock. In all patients, blood cultures showed no bacterial growth.

Coagulation

Activation of the coagulation system was more pronounced in the deceased group than among survivors (Table 2). On admission, non-survivors had a more pronounced prolongation of APTT and PT than survivors, suggesting activation of the intrinsic pathway and the extrinsic or tissue factor pathway. This activation pattern is clearly manifested by an early increase in markers of thrombin generation i.e. F1+2 fragments, TAT complexes as well as diminished fibrinogen level. During follow up, these activation parameters tended to improve in the survivor group but not in the deceased group (Table 3). In accordance with this activation pattern, the coagulation inhibitor proteins C and S were lower among the deceased than among survivors, also reflecting pronounced activation of coagulation in the deceased group.

Fibrinolysis

The fibrinolytic system was activated as reflected by a rise in tPA, PAP and D-dimers in both groups on day of admission (Table 2). TPA and D-dimer levels were increased to a similar degree in both groups and PAP complexes were found to be lower in the non-survivor group compared with the survivor group. Levels of PAI, an inhibitor of fibrinolysis, were initially (Table 2) and persistently (Table 3) elevated in the deceased group during the course of the disease, whereas there was a slow decrease in PAI levels in the survivor group (Table 3).

These data show that the fibrinolytic system, when compared with the degree of activation of the coagulation system, was relatively impaired in the non-survivor group, resulting in an ongoing procoagulant state in this group, as reflected by a high TAT/PAP ratio, and lower levels of circulating PAP complexes. In the survivor group, there was a switch to a profibrinolytic ratio during the course of disease, reflected by a TAT-PAP ratio of <1 on the final day of follow up.

Table 2. Markers of coagulation and fibrinolysis on day of admission in patients with DHF grade III and DHF grade IV*°

	All Patients (n=50)	Non-survivors (n=13)	Survivors (n=37)	Normal	P-value†
aPTT (seconds)	52.0 (43.4-64.1)	71.1 (52.6-97.4)	47.6 (40.1-59.7)	24-36	0.001
PT (seconds)	13.2 (11.9-15.4)	16.3 (15.0-25.8)	12.6 (11.4-13.9)	10.5-13.5	0.001
F1+2 (nmol/l)	3.2 (2.0-5.0)	4.9 (2.7-7.2)	2.8 (1.9-4.0)	< 1.1	0.008
TAT (mg/l)	27.2 (13.3-65.7)	50.9 (28.5-117.5)	21.0 (10.9-52.0)	< 4.1	0.004
Fibrinogen (g/l)	1.6 (1.3-1.9)	1.3 (0.8-1.6)	1.7 (1.5-2.1)	1.7-4.0	0.005
Protein C (%)	52.5 (36.8-66.3)	38.0 (10.0-63.5)	56.0 (48.0-68.0)	100%	0.04
Protein S activity (%)	51.0 (39.0-64.3)	34.0 (30.5-48.0)	54 (42.5-66.5)	100%	0.002
Protein S free (%)	23.0 (19.0-28.0)	18.0 (14.3-24.8)	23.0 (20.0-28.0)	100%	0.02
TPA (ng/ml)	52.5 (41.0-61.0)	51.0 (25.0-69.0)	54.0 (41.0-61.0)	< 10	0.93
PAP (nMol)	8.6 (6.3-12.0)	6.4 (4.1-8.5)	10.0 (7.1-13.0)	< 7	0.03
PAI (ng/ml)	183.0 (86.8-392.5)	394.0 (270.0-1573.0)	130.0 (65.0-280.5)	30-60	0.001
D dimer (ng/ml)	241.0 (197.3-761.0)	301.0 (205.0-1303.0)	234.0 (179.5-715.5)	< 39	0.23
TAT/PAP	2.8 (1.3-6.5)	7.8 (3.3-23.7)	2.3 (1.1-5.8)	-	0.03

* Values of markers of coagulation and fibrinolysis on day of admission of all children (n=50) with DHF grade III and DHF grade IV. Values of the subgroup survivors (n=37) and non-survivors (n=13) are also given. The reported values are medians with their 25th and 75th interquartile ranges (IQR).
† Denotes the resulted P-value after comparison of survivors and non-survivors, using the Mann-Whitney-U-test.

Table 3. Markers of coagulation and fibrinolysis during follow up in patients with DHF grade III and DHF grade IV*°

		Day 1	Day 2	Day 3	Final sample	Normal
TAT (mg/l)	Survivors	21.0 (10.9-52.0)	14.0 (6.2-26.6)	13.1 (6.2-59.9)	6.6 (4.0-18.1)	<4.1
	Non-survivors	50.9 (28.5-117.5)	23.8 (14.1-79.8)	39.2 (22.7-51.2)	57.3 (17.5-97.1)	
PAP (nMol)	Survivors	10.0 (7.1-13.0)	7.8 (5.7-12.0)	10.0 (7.4-13.0)	12.0 (9.0-14.0)	< 7
	Non-survivors	6.4 (4.1-8.5)	7.0 (4.4-10.6)	7.9 (6.7-23.8)	33.4 (6.8-60.0)	
PAI	Survivors	130.0 (65.0-280.5)	145.0 (59.5-597.0)	93.0 (56.0-150.0)	44.0 (23.0-74.0)	30-60
(ng/ml)	Non-survivors	394.0 (270.0-1573.0)	1235.0 (230.5-3057.0)	469.0 (195.5-835.8)	200.0 (55.0-345.0)	
TAT/PAP	Survivors	2.3 (1.1-5.8)	1.6 (0.9-3.1)	1.6 (0.5-5.0)	0.6 (0.3-1.7)	
	Non-survivors	7.8 (3.3-23.7)	4.1 (1.6-13.6)	4.2 (1.1-7.7)	2.1 (1.6-2.6)	

* Values of markers of coagulation and fibrinolysis during hospital stay on three consecutive days and a fourth and final sample on day 7 or day of discharge (survivors) or day before death (non-survivors). All patients were diagnosed with DHF grade III and DHF grade IV. Number of survivors is 37 and number of non-survivors is 13 on day of admission, 8 on day 2, 6 on day 3 and 2 on day 7. Reported values are medians with their 25th and 75th interquartile ranges (IQR).

DISCUSSION

In the present study, it was demonstrated that DHF/DSS is characterized by activation of the coagulation and fibrinolytic systems, reflected by both a rise in circulating levels of markers of thrombin generation and markers of activation of fibrinolysis. However, during the initial phase of infection these activation processes were not in balance since TAT/PAP ratios were increased (ratio > 1), yielding a procoagulant state. In survivors this procoagulant state was counteracted by an activated protein C system, an activated protein S system and ongoing fibrinolysis, resulting in a net anticoagulant state, as reflected by an inversed TAT/PAP ratio (ratio < 1). In the non-survivor group such a change from the procoagulant to the anticoagulant state did not occur. Quite remarkably, PAI levels remained high during the course of the disease in the deceased group, contributing to an ongoing procoagulant state by a relative inhibition of fibrinolysis. The acquired protein C deficiency observed in our patients may contribute to a further enhancement of this procoagulant state and hence to DIC and/or MOF.

Persistently high circulating PAI levels and increased TAT/PAP ratios were associated with an adverse clinical outcome in our study. This phenomenon has also been observed in patients with systemic bacterial infections [17-20]. Studies of experimental bacterial sepsis models demonstrated that specific cytokines mediate the derangement of coagulation and fibrinolysis [21]. Injection of endotoxin or tumor necrosis factor (TNF) into healthy volunteers resulted in early activation of fibrinolysis, as reflected by an early rise in TPA, which was rapidly shut off by increasing levels of PAI [22]. Coagulation was also activated early in these models and remained activated for hours [23]. Hence, administration of endotoxin or TNF induced a procoagulant state, characterized by an increased TAT/PAP ratio [22]. A similar procoagulant state has been described in experimental studies of baboons with lethal sepsis [15]. In these experimental models, coagulation and fibrinolysis appeared to be activated independently, which may lead to an imbalance between fibrin formation and breakdown [24]. Whether such a procoagulant state occurs in viral infections has not been investigated. This study is the first to demonstrate such a mechanism in patients with a severe viral infection.

Several issues of the present study merit further comment. First, only patients with the severest form of DHF were investigated. Hence, the findings are applicable only to patients with this spectrum of disease. Huang et al. [5] studied 25 patients with DF and DHF. These investigators also found a deranged fibrinolytic system, as demonstrated by elevated levels of tPA and PAI, although the levels of PAI were 4- to 10-fold higher in our series. Whether this is a result of differences in patient baseline characteristics or the inclusion predominantly of patients with mild DHF remains to be determined. Further studies are required to investigate this phenomenon in patients with milder DF. Second, all the enrolled patients who were classified clinically as having DHF on the basis of widely accepted clinical criteria had serologically confirmed Dengue virus infection. This occurred despite the inclusion of consecutive patients in order to avoid selection bias and explains the lack of a non-Dengue control group in this study.

Allotypic variations in the promotor region of the PAI gene may contribute to interindividual variations in PAI responses [25]. In patients with meningococcal disease, these allotypic variations are associated with an enhanced risk for septic shock after infection with Neisseria meningitides [26]. It is speculated that these allotypic variations of the PAI gene may also be associated with the severity of DHF/DSS. In addition, other genetic polymorphisms, such as those involved in the production and release of cytokines, may contribute to the outcome of Dengue virus infections [27;28]. The role of genetic factors in the risk of developing severe DHF is the subject of present prospective studies.

In conclusion, severe DHF is associated with a relative impaired fibrinolytic response as a result of elevated circulating PAI levels, which may contribute to a high mortality. In addition, high PAI levels and elevated TAT/PAP ratios may be used as prognostic markers in daily management. The procoagulant state demonstrated may be a target for future intervention among patients with this disease.

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Chapter 8

INCREASED PAI-1 PLASMA LEVELS AND RISK OF DEATH FROM DENGUE: NO ASSOCIATION WITH THE 4G/5G PROMOTER POLYMORPHISM.

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ABSTRACT
Dengue virus infected patients have high plasminogen activator inhibitor type I (PAI-1) plasma concentrations. Whether the insertion/deletion (4G/5G) polymorphism in the promotor region of the PAI-1 gene is associated with increased PAI-1 plasma concentrations and with death from Dengue is unknown. We, therefore, investigated the relationship between the 4G/5G polymorphism and PAI-1 plasma concentrations in Dengue patients and risk of death from Dengue. A total of 194 patients admitted to the Dr. Kariadi Hospital in Semarang, Indonesia, with clinical suspected severe Dengue virus infection were enrolled. Blood samples were obtained on day of admission, days 1, 2 and 7 after admission and at a 1-month follow-up visit. Plasma concentrations of PAI-1 were measured using a sandwich ELISA kit. The PAI-1 4G/5G polymorphism was typed by allele-specific PCR analysis. Concentrations of PAI-1 on admission and peak values of PAI-1 during admission were higher than the values measured in healthy controls. Survival was significantly worse in patients with PAI-1 concentrations in the highest tertile (at admission: OR 4.7 [95% CI 0.9-23.8], peak value during admission: OR 6.3 [95%CI 1.3-30.8]). No association was found between the PAI-1 4G/5G polymorphism, and PAI-1 plasma concentrations, Dengue disease severity and mortality from Dengue. These data suggest that the 4G/5G polymorphism has no significant influence on PAI-1 concentrations in Dengue virus infected patients and is not associated with the risk of death from Dengue. Other factors contributing to the variability of PAI-1 plasma concentrations in patients with Dengue need to be explored.

INTRODUCTION

Dengue is the most prevalent viral disease transmitted by arthropod vectors worldwide [1]. An estimated 50-100 million cases of Dengue fever and 500.000 cases of Dengue haemorrhagic fever resulting in around 24.000 deaths occur annually depending on epidemic activity [1;2]. At present, almost 30% of the world population is at risk for Dengue virus infection and it is expected that this number will increase substantially as transmission spreads to other yet unaffected geographic regions [3]. The viruses are transmitted to humans through infected mosquitoes, and may induce clinical manifestations ranging from a mild, uncomplicated febrile illness to the more severe Dengue haemorrhagic fever and Dengue shock syndrome. Increased vascular permeability is thought to be central in the pathogenesis of Dengue haemorrhagic fever and Dengue shock syndrome, since it results in the loss of plasma from the vascular compartment, which may give rise to shock in severe cases. Bleeding manifestations are believed to result from thrombocytopenia and thrombocytopathy, but increasing evidence suggests an important role for other abnormalities in the coagulation and fibrinolytic systems [4].

Nearly all patients suffering from Dengue haemorrhagic fever show some evidence of a deranged coagulation system, but coagulation abnormalities are most marked in Dengue shock syndrome patients [5]. The activity of the fibrinolytic system is amongst others regulated by plasminogen activator inhibitor type I (PAI-1), of which levels may greatly increase during acute phase reactions. Indeed, levels of PAI-1 are high in particular in Dengue shock syndrome patients with an adverse clinical outcome [6;7]. A single base pair insertion/deletion (4G/5G) polymorphism in the promotor region of the PAI-1 gene has been associated with increased plasma concentrations of PAI-1 and with the development of shock and death after infection with Neisseria meningitides [8;9]. We therefore investigated whether in Dengue virus infected patients increased PAI-1 levels are associated with a greater risk of death from Dengue and whether the 4G/4G genotype contributes to these higher levels.

PATIENTS AND METHODS

Patients and clinical procedures

Patients were selected from two observational studies conducted in the Dr. Kariadi Hospital in Semarang, Indonesia. In the first study, performed from 1996 to 1997 at the paediatric intensive care unit, a total of 50 patients with a clinical diagnosis of suspected Dengue shock syndrome were studied. Blood samples were obtained from these patients on day of admission and on days 1, 2 and 7 after admission. The study protocol and the results of the measurement of PAI-1 plasma levels have been described previously [6]. The second study was performed from 2001 to 2003 at the paediatric intensive care unit and the paediatric ward. Patients, aged 3 to 14 years, admitted to the hospital with a clinical diagnosis of suspected Dengue shock syndrome or with a clinical diagnosis of suspected Dengue haemorrhagic fever were included. Demographic data, medical history, physical examination findings and subsequent progress for each patient were recorded on a standard data form. Blood samples were obtained on day of admission, days 1, 2 and 7 after admission and at a 1-month follow-up visit. A formal classification, according to WHO criteria [2],

using all available clinical and laboratory data, was done after completion of both studies. If Dengue virus infected patients did not meet the criteria for Dengue haemorrhagic fever or Dengue shock syndrome, they were considered to suffer from Dengue fever. The controls were healthy school-aged children who had no history of Dengue haemorrhagic fever or Dengue shock syndrome and originated from the same geographical area as the cases.

Laboratory methods

All blood samples were centrifuged within 1-2 hours after retrieval at 15°C for 20 minutes at 1600*g. Plasma was separated, stored at -80°C and assayed batch-wise in the Netherlands after transportation on dry ice. Since coagulation test results are affected by poor sample quality, only test results of samples with no visible haemolysis or clot formation were included in this analysis. Plasma concentrations of PAI-1 were measured using two separate assays. A sandwich ELISA kit that has been described by de Boer et al [10], was used in the 1996-1997 study. In the second study a commercially available sandwich ELISA kit (Imulyse, Biopool, Sweden) was used. Genomic DNA from patients was prepared either from EDTA whole blood by means of a salting out method as described elsewhere or in case only plasma samples were available with use of the Invisorb® Spin Cell Mini Kit (Invitek GmbH, Berlin, Germany) [11]. The insertion/deletion (4G/5G) polymorphism in the promotor region of the PAI-1 gene was typed by allele-specific PCR and RFLP analysis [11]. The ethics committee of the Dr. Kariadi Hospital approved all clinical and laboratory aspects of both studies. Blood samples were taken from patients and controls provided that a parent or legal guardian gave informed consent.

Diagnostic procedures

Paired blood samples from both studies were tested for serologic evidence of acute Dengue infection. A commercially available capture and indirect ELISA (Focus Technologies, Cypress, Calif., USA) was used for the detection of Dengue virus specific IgM and IgG antibodies respectively. This was performed according to the procedures described by the manufacturer [12]. For some patients, a definitive serodiagnosis was not possible because no convalescent sample was obtained. Detection of Dengue antigen and RNA was attempted in these cases using a dot blot immunoassay and a Dengue serotype specific revere transcriptase PCR respectively [13]. Patients with serologic evidence of acute Dengue infection, a positive dot blot and/or positive PCR were considered to have confirmed Dengue virus infection. Those with definite negative serology and/or a well-substantiated alternative clinical diagnosis were classified as not Dengue. In the absence of a well-substantiated alternative clinical diagnosis and with inconclusive serology patients were classified as indeterminate.

Statistical analysis

Categorical data are expressed as numbers and frequencies, and were compared by means of χ^2 analysis. Continuous data are expressed as medians with corresponding interquartile ranges and were compared by means of the Kruskal Wallis test. Since PAI-1 plasma levels were measured using two different assays, the nonparametric

regression procedure of Passing and **Bablok** was used to compare these two methods and to validate recalculation of one of the datasets to pool the data [14]. For this purpose, 39 samples were analysed with both assays. The Passing and Bablok regression equation resulting from this comparison is given in the *Results* section, together with the 95% confidence intervals for the estimates of slope and intercept. PAI-1 plasma levels obtained from the 1996-1997 cohort were converted using the regression equation. We subsequently divided PAI-1 plasma levels on admission and peak PAI-1 plasma levels during admission into tertiles of similar size. Odds ratios and the corresponding 95% confidence intervals (95% CI) were estimated by cross-tabulation using the lowest tertile as reference category. To determine the association between PAI-1 concentration and mortality independent of age, sex, year project and plasminogen activator inhhibitor-1 polymorphism, we used **logistic** regression analyses. A P-value ≤ 0.05 was considered to indicate statistical significance. Analyses were performed using SPSS 11.0.1. Method comparison was performed using Analyse-it Clinical Laboratory statistics module version 1.62 for Microsoft Excel.

RESULTS

Of 233 enrolled patients with suspected Dengue haemorrhagic fever or Dengue shock syndrome admitted to the paediatric intensive care unit and the paediatric ward of the Dr. Kariadi Hospital, 202 (87%) were confirmed to have acute Dengue, 3 (1%) were categorised as definitely not Dengue and 28 (12%) as indeterminate. When a formal classification using the criteria set by the WHO was performed, 106 (52%) were classified as having Dengue shock syndrome, 76 (38%) as having Dengue haemorrhagic fever and 20 (10%) as having Dengue fever. Nineteen of 202 patients with confirmed Dengue (9%) died during follow up.

Genomic DNA was obtained from 194 patients. The remaining patients could not be typed because of insufficient volume of blood left or low yield of DNA. Clinical features and basic laboratory investigations on admission of the 194 patients are summarised in Table 1. The numbers of 4G/4G, 4G/5G and 5G/5G PAI-1 genotypes among patients in relation to mortality are summarised in Table 2. Of 192 control samples tested, 45 patients (23%) were 4G/4G homozygous, 83 (43%) were 4G/5G heterozygous, and 64 (33%) were 5G/5G homozygous. The frequencies of the PAI-1 promoter genotypes 4G/4G, 4G/5G, and 5G/5G did not differ significantly between the 1996-1997 project, the 2001-2003 project and the control group (P=0.520). The proportion of deaths among patients with the 4G/4G, 4G/5G and 5G/5G genotype did not differ significantly (1996-1997 project: P=0.979; 2001-2003 project: P=0.986; two projects combined: P=0.781). The genotype frequencies among patients with respect to final clinical diagnosis according to the criteria set by the WHO are summarised in Table 3. Results indicate that the PAI-1 promoter genotypes are not associated with Dengue disease severity (P=0.508).

Table 1. Clinical characteristics and laboratory findings

Characteristic	
Age, median (IQR), years	6 (4-10)
Male sex, n (%)	92 (47)
Duration of symptoms, median (range), days	4 (1-7)
Systolic blood pressure, median (IQR), mmHg	90 (80-100)
Pulse pressure <20 mmHg, n (%)	22 (11)
Spontaneous bleeding, n (%)	118 (62)
Haematocrit, median (IQR), %	41 (36-45)
Platelet count, median (IQR), cells $*10^3/mm^3$	58 (37-85)

° IQR denotes 25th and 75th interquartile range, n denotes number of patients.

Table 2. Clinical outcome of Dengue virus infected patients classified by PAI-1 genotype

	1996-1997			2001-2003		
Genotype	All patients	Survivors	Non-survivors	All patients	Survivors	Non-survivors
G4/G4	8 (18%)	6 (14%)	2 (5%)	33 (22%)	32 (21%)	1 (1%)
G4/G5	15 (34%)	11 (25%)	4 (9%)	62 (41%)	60 (40%)	2 (1%)
G5/G5	21 (48%)	15 (34%)	6 (14%)	55 (37%)	53 (35%)	2 (1%)
Total	44 (100%)	32 (73%)	12 (27%)	150 (100%)	145 (97%)	5 (3%)

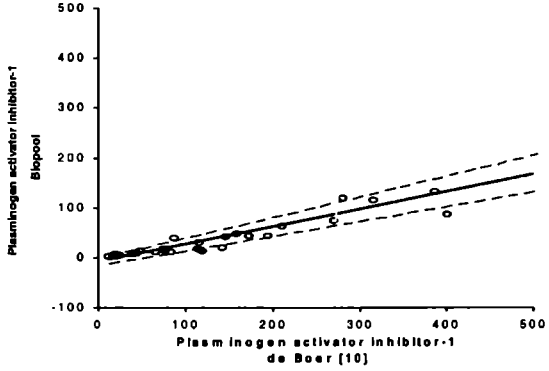
Table 3. Dengue disease severity and PAI-1 genotype

Genotype	DF	DHF	DSS
G4/G4	6 (3%)	13 (7%)	22 (11%)
G4/G5	6 (3%)	35 (18%)	36 (19%)
G5/G5	8 (4%)	27 (14%)	41 (21%)
Total	20 (10%)	75 (39%)	99 (51%)

PAI-1 plasma levels were measured in 124 patients from whom sufficient frozen plasma samples was available with the use of two separate ELISA's. Thirty-nine random plasma samples were used for the comparison of the two assays. PAI-1 level measurements are known to be dependent on the assay employed, since not all antibodies used have the same specificity with respect to the various molecular forms of PAI-1 in blood (active PAI-1, PAI-1 complexed with vitronectin, inactive or latent PAI-1 and PAI-1 complexed with its target proteases tissue-type and urokinase-type plasminogen activator) [15]. Passing & Bablok regression analysis (Figure 1), however,

clearly showed that the two assays employed in this study have a straightforward linear relationship between their outcomes. This allowed us to convert the PAI-1 levels measured in the 1996-1997 cohort to levels that would have been obtained with the assay used in the second cohort, using the Passing and Bablok regression equation (an intercept of -7.42 ng/ml (95% CI, -16.39 to -3.24 ng/ml) and slope of 0.35 (95% CI, 0.30 to 0.42)). Concentrations of PAI-1 on admission (71 ng/ml [42-118]) and peak values during admission (96 ng/ml [64-199]) were higher than the concentrations measured in the healthy control group (30-60 ng/ml). Patients included in the 2001-2003 project and diagnosed with DSS had higher PAI-1 plasma levels on admission (P=0.002) and during admission (P<0.001) than those diagnosed with DF or DHF (Table 4).

Figure 1. Comparison of two PAI-1 assays



Comparison of PAI-1 plasma levels obtained by a Biopool assay and an assay previously described by de Boer and colleagues [10]). The Passing & Bablok regression equation is: y = 0,3505x - 7,4227 ng/ml; n=39. *Solid line*, regression line; *dashed lines*, 95% CI for the regression line.

Table 4. Relation between PAI-1 plasma levels and disease severity°

Project	PAI-1	DF	DHF	DSS
	(ng/ml)	Median (IQR)	Median (IQR)	Median (IQR)
1996-1997 †	Admission	-	-	56.7 (33.0-130.2)
	Peak values	-	-	91.6 (37.7-343.6)
2001-2003	Admission	77.0 (69.0-99.0)	60.0 (42.3-82.5)	107.0 (52.0-670.0)
	Peak values	82.0 (69.0-129.0)	78 (59.3-118.3)	264.0 (117.0-844.0)
Total	Admission	77.0 (69.0-99.0)	60.0 (42.3-82.5)	87.0 (35.0-180.8)
	Peak values	82.0 (69.0-129.0)	78 (59.3-118.3)	130.7 (53.7-531.1)

° IQR denotes 25th and 75th interquartile range.
† Only DSS patients were included in the 1996 project.

However, no statistical significant difference was present between patients with different Dengue disease severities when both projects were combined (PAI-1 plasma levels on admission: P=0.212 and during admission: P=0.089). Survival was significantly worse in patients with PAI-1 concentrations in the highest tertile (Table 5). As shown by multiple logistic regression analysis, the odds ratio for the risk of death -adjusted for age, sex, year project and plasminogen activator inhibitor-1 genotype- of PAI-1 levels at admission in the highest tertile was 7.57 (95%CI, 1.16-49.30). The adjusted odds ratio for the risk of death of peak PAI-1 levels during admission in the highest tertile was 7.41 (95%CI, 1.30-42.26). Figure 2 illustrates the relation between PAI-1 plasma concentrations and the PAI-1 genotypes. Differences were not statistically significant (values at admission: P=0.919; peak values during admission: P=0.470).

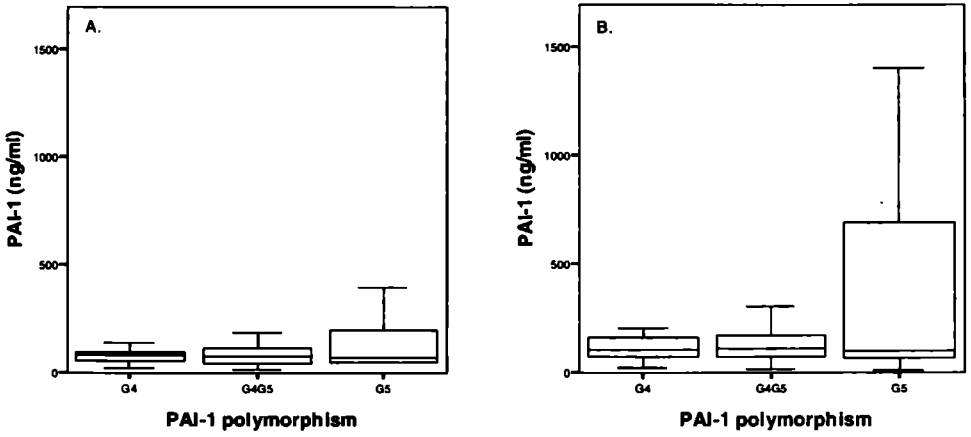
Table 5. Mortality risk for plasminogen activator-1 plasma levels°

PAI-1	ng/ml	Number of patients	Number of deaths	OR (95% CI)	OR_{adj} (95% CI) †
Value at admission	< 50	41	2	1	1
	50-94	40	3	1.6 (0.3-10.0)	3.3 (0.4-25.0)
	> 94	41	8	4.7 (0.9-23.8)	7.6 (1.2-49.3)
Maximum value	< 72	41	2	1	1
during admission	72-151	42	3	1.5 (0.2-9.5)	2.1 (0.2-18.2)
	> 151	41	10	6.3 (1.3-30.8)	7.4 (1.3-42.3)

° CI, denotes confidence interval; OR, denotes odds ratio; OR_{adj} denotes adjusted odds ratio

† Risk of death adjusted for age, sex, year project and plasminogen activator inhhibitor-1 polymorphism. Lowest tertile was used as reference category.

Figure 2. Relation between PAI-1 plasma concentrations and PAI-1 genotype in Dengue virus infected patients



Box-and-whisker plots of PAI-1 plasma concentrations on admission (A.) and peak values during admission (B.). The central line in the box plot represents the median, the boxed areas represent the interquartile ranges. Whiskers at the ends of the box show the distance from the end of the box to the largest and smallest observed values that are less than 1.5 box lengths from either end of the box.

DISCUSSION

This study was undertaken to investigate the relationship between PAI-1 plasma concentrations and clinical outcome of Dengue virus infections, and to establish whether PAI-1 plasma concentrations in Dengue virus infected individuals are associated with the 4G/5G promotor polymorphism in the PAI-1 gene. We and others have previously found increased PAI-1 plasma concentrations in patients with severe Dengue in particular in those with a poor clinical outcome [5;7]. Since a genetic predisposition to produce high PAI-1 plasma concentrations appears to be associated with poor clinical outcome in Neisseria meningitides infections [8;9], we hypothesised that Dengue virus infected individuals carrying the 4G/4G genotype have higher PAI-1 plasma concentrations and are therefore at increased risk of death. Consistent with previous studies, we found increased PAI-1 concentrations in Dengue virus infected individuals. However, PAI-1 plasma concentrations were not related to Dengue disease severity, but were significantly associated with death from Dengue. No significant association between PAI-1 plasma concentrations and carriage of the 4G/4G genotype was observed. The frequencies of the three genotypes between survivors and non-survivors, and between patients with different disease severities were not different. These findings suggest that increased PAI-1 plasma concentrations, and Dengue disease severity and mortality are not dependent on the 4G

polymorphism in the PAI-1 gene in this population.

An increased risk of death in Dengue virus infected patients with high PAI-1 plasma concentrations adds to findings of PAI-1 levels being able to predict lethality in patients with bacterial sepsis in a very sensitive way [16-20]. One of the primary roles of PAI-1 *in vivo* is to inhibit tissue-type plasminogen activator, the major proteolytic activator of plasminogen [21;22]. By inhibiting fibrinolytic activity, increased PAI-1 concentrations may contribute to a procoagulant state leading to an increased deposition of fibrin and formation of microthrombi with subsequent multiorgan failure and death. A variety of cells, including endothelial cells, hepatocytes and platelets, synthesize and secrete PAI-1 in response to inflammatory stimuli such as interleukin-1 and tumour necrosis factor [10;22-24]. The release of these inflammatory mediators by monocytes and T lymphocytes activated by Dengue virus may well contribute to the over-production of this inhibitor of fibrinolysis [25-27]. Dawson and colleagues showed that the common insertion/deletion (4G/5G) polymorphism in the promotor region of the PAI-1 gene affects the response of the gene to acute phase stimuli [23]. The 4G allele produced six times more mRNA than the 5G allele in response to interleukin-1 [23]. Eriksson and colleagues, however, were unable to reproduce these findings and based on their study results they concluded that the insertion/deletion (4G/5G) polymorphism is not related to an allele-specific response to interleukin-1 [28]. Instead, they found that the insertion/deletion (4G/5G) polymorphism influences basal PAI-1 transcription only [28].

Apparently other underlying mechanisms not related to the 4G/5G polymorphism must be involved in the increase in PAI-1 levels found in Dengue virus infected individuals. This might include clearance impairment rather than or in addition to stimulation of synthesis. It is interesting to note that PAI-1 is cleared from the circulation by the liver [29]. Indeed in patients with severe liver disease, PAI-1 has been shown to be increased as a result of a decrease in hepatic clearance [30;31]. Since hepatic dysfunction is a relatively common finding in severe Dengue virus infections, it is possible that a less efficient clearance contributes to increased PAI-1 levels in Dengue virus infected individuals [32-34]. Previous studies investigated factors that could potentially influence PAI-1 levels, including environmental factors, metabolic determinants, ethnicity and a variety of other polymorphisms within the PAI-1 gene [35-38]. It remains unclear whether and to what extent these factors contribute to the variability in PAI-1 levels in Dengue virus infected individuals. Previously studied individuals were either healthy, were patients with coronary artery disease, or were patients with diabetes mellitus. Clearly these study populations cannot be compared to patients suffering from a severe infectious disease that is characterised by an overwhelming inflammatory response.

Several potential limitations of the present study should be noted. The 1996-1997 cohort was characterized with a high mortality rate of 27%. Although the exact reason for this high mortality remains to be determined, it is likely that it results from a combination of factors. Our study was performed in a Tertiary Hospital that serves a large part of Middle-Java. Patients may travel long distances to be treated in this hospital and it could well be that they are presented late in the course of disease. Initial fluid resuscitation according to WHO guidelines is generally insufficient in

these cases and patients usually end up in profound shock. Despite admission at the Paediatric Intensive Care Unit mortality rate is high. In addition, the 1996-1997 rainy season was characterised by high numbers of patients admitted to hospitals because of DHF/DSS and high number of non-survivors. It is believed that an unusually virulent virus circulated that year although microbiological sampling could not be performed at that time because of limited resources.

Study size is an important issue in the establishment of an association between the insertion/deletion (4G/5G) polymorphism in the PAI-1 gene and clinical outcome. Hermans and colleagues previously found an association between the homozygous 4G deletion polymorphism and mortality from Neisseria meningitides infection among 129 patients from two different cohort groups [8]. This association was also observed when the largest of the two cohort groups was studied separately, but was not seen in the smallest group in which only 37 patients were included. In order to obtain a sufficient number of patients, we therefore decided to combine the results of two different projects. This decision was based on the fact that these two projects included patients with the same ethnic background, used similar trial procedures and applied uniform diagnostic and clinical management procedures. Although mortality rates between the 1996-1997 cohort and the 2001-2003 cohort differed considerably, one must realise that in the 1996-1997 cohort only DSS patients were included. In the 2001-2003 cohort also included patients who had no evidence of circulatory failure were included. Mortality rate among DSS patients included in the 2001-2003 cohort was 18%. Our findings of similar frequencies of the PAI-1 genotypes within the 1996-1997 project and the 2001-2003 project support our decision to combine both groups.

In conclusion, this study demonstrates that high PAI-1 plasma levels are associated with an increased risk of death from Dengue without the 4G/5G polymorphism in the promotor of the gene for PAI-1 playing a role. Additional studies are needed to explore the possibility of other polymorphisms within the PAI-1 gene and factors, like ethnicity or environmental factors, contributing to the variability of PAI-1 plasma concentrations in patients with Dengue.

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IV. Vascular leakage and immunology :

Chapter 9

INFLAMMATORY GENE EXPRESSION CHANGES IN CHILDREN WITH SEVERE DENGUE VIRUS INFECTION

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Submitted

ABSTRACT
The host response to dengue virus infection is characterized by production of numerous cytokines, but the overall picture appears to be complex in vivo. In vitro data suggested that a state of immunoparalysis occurs. This study aimed to describe cytokine patterns in whole blood samples measuring mRNA levels from 50 genes encoding inflammatory proteins simultaneously. Whole blood mRNA from 56 Indonesian children with severe dengue virus infections was isolated on day 0, 1, 2, 7 and 30 after hospital admission. mRNA was analyzed in a single reaction by multiplex ligation-dependent probe amplification (MLPA). The gene profiles showed up-regulation during infection of 10 genes including interferon (IFN)-α, interleukin (IL)-12a, macrophage inhibitory factor (MIF) and toll like receptor (TLR)7. Concurrently, the nuclear factor (NF)κB pathway was down-regulated together with downstream effector genes encoding IL-β and IL-8. Among other associations, mortality in the study (n=4) was negatively correlated with IFN-γ synthesis. Together, these data suggest that the in vivo host response to severe dengue virus infections is characterized by a general antiviral response including up-regulation of IFN-γ and TLR7 synthesis whereas a state of immunoparalysis in circulating leukocytes characterized by down-regulation of the NFκB pathway is concurrently induced.

INTRODUCTION

Dengue virus infection is the most prevalent mosquito-borne disease worldwide. It is particularly common in tropical regions and affects an estimated 50-100 million people each year [1]. Clinical manifestations range from a mild febrile illness to more severe forms of disease called dengue haemorrhagic fever and to the potentially lethal dengue shock syndrome [2]. A hallmark of dengue hemorrhagic fever and dengue shock syndrome is increased vascular permeability that leads to bleeding manifestations and loss of fluid from the vascular to the extravascular compartment. The condition is treated by fluid replacement therapy and general supportive care.

The pathogenesis of hemorrhagic dengue disease is still poorly understood. It involves the development of coagulation abnormalities as well as activation of the immune system with production of numerous cytokines and chemokines [3, 4]. In dengue disease, the host defense strongly depends on interferon (IFN)-γ production, but other cytokines are also involved, as well as antibody production and T cell responses [4, 5]. Increased levels of inflammatory proteins including tumor necrosis factor (TNF)-α, soluble TNF receptors, soluble interleukin (IL)-2 receptor, IL-8, IL-13, IL-18, soluble CD8 and transforming growth factor (TGF) ß1 have been associated with disease severity [6-11]. Similarly, IL-1receptor antagonist (RA), IL-6, IL-8, IL10 and macrophage inhibitory factor (MIF) were associated with mortality [11-13]. Host defense against dengue disease is further characterized by a reduced capacity of circulating monocytes from dengue patients to produce TNF-α *in vitro* after stimulation with endotoxin or dengue antigen [12] [14]. This phenomenon suggests a so-called state of immunoparalysis [15, 16]. In general, cytokine production depends on recognition of pathogens via Toll-like receptors (TLRs). In viral disease, TLR3, TLR7, TLR8 and TLR9 are commonly involved, but specific knowledge about TLRs in dengue infections is limited [17-19].

The broad and complex array of inflammatory mediators that has been suggested to be involved in dengue disease and the multiple associations with disease severity and outcomes were based upon different studies with measurements of only a limited spectrum of mediators. The multiplicity and complexity of parameters that are altered in dengue infection, however, requests a more integral approach that incorporates the measurement of multiple parameters within one study. RNA-based gene profiling methods offer this opportunity to examine cytokine profiles in a larger context. We previously developed and validated a sensitive quantitative assay for the measurement of multiple mRNA levels in a single reaction, multiplex ligation-dependent probe amplification (MLPA) [20-23]. The MLPA panel is composed of target genes encoding various mediators of inflammation, including cytokines, chemokines, cytokine receptors, intracellular signalling molecules and Toll-like receptors.

In this study, we aimed to investigate factors involved in the host defense to dengue infection in whole blood samples from a group of children with severe dengue disease in Indonesia using MLPA technology, and searched to define predictors of disease severity and clinical outcome.

METHODS

Study design

Patients were selected from an observational study previously conducted in the Dr. Kariadi Hospital in Semarang in Indonesia [24]. Children, aged 3 to 14 years, admitted to the paediatric intensive care unit or paediatric ward with a clinical diagnosis of suspected dengue hemorrhagic fever or dengue shock syndrome were included. For the current analysis, all subjects for whom mRNA material was collected were selected. They were included in the study from February 2002 until March 2003. At admission and during follow-up, demographic and clinical data were collected using a standardized data collection form. Citrated blood samples were obtained on day of admission, days 1, 2 and 7 after admission and at a 1-month follow-up visit.

Diagnostic procedures

Paired blood samples were tested for serologic evidence of acute dengue infection. Commercially available enzyme-linked immunosorbent assays (Focus Technologies, Cypress, Ca, USA) were used for the detection of dengue virus specific IgG and IgM antibodies, according to the procedures described by the manufacturer. The sensitivity and specificity of these tests have been evaluated previously [25]. Cases were considered serologically-confirmed if the IgM ELISA was positive during the acute phase of disease (optical density of the sample higher than the optical density of the cut-off serum provided by the manufacturer) and/or if a four-fold increase in IgG titre was demonstrated in paired acute and convalescent sera. For some patients, a definitive serodiagnosis was not possible because no convalescent sample was obtained. Detection of dengue virus antigen and/or viral RNA was attempted in these cases using a dot blot immunoassay and a dengue serotype specific reverse transcription PCR respectively [26]. Patients with definitive serodiagnosis and/or positive dot blot and/or positive PCR were considered to have confirmed dengue virus infection.

mRNA analysis

Blood samples were centrifuged within 1-2 hours after retrieval at 15 °C for 20 minutes at 1600 x *g*. Subsequently, mRNA was stored at -80 °C using Red Blood Cell Lysis Buffer (Roche, Mannheim, Germany) according to the manufacture's protocol. The samples were transported to the Netherlands on dry ice and mRNA was isolated using High Pure RNA Isolation Kit (Roche, Mannheim, Germany). mRNA of multiple inflammatory molecules was analyzed by MLPA as described previously [20]. MLPA is insensitive to the total amount of mRNA that is included in the reaction; therefore, the profile is independent of the total white blood cell (WBC) count. All samples were tested with the same batches of reagents, and a negative and endotoxin-stimulated control sample were included on each plate. The final polymerase chain reaction (PCR) fragments amplified with carboxyfluorescein-labeled primers were separated by capillary electrophoresis on a 16-capillary ABI-Prism 3100 Genetic Analyzer (Applied Biosystems, Nieuwerkerk aan de IJssel, The Netherlands). Peak area and height were processed using GeneScan analysis software (Applied Biosystems). The levels of mRNA for each gene were expressed as a normalized ratio of the peak area divided by the peak area of a control gene, beta 2 microglobulin (B2M), resulting in the relative abundance of mRNAs of the genes of interest.

Statistical analysis

All calculations were carried out using SPSS version 13.0 (SPSS Inc., Chicago, IL, USA). The relative mRNA levels of subjects were compared for each day during admission (day 0, 1, 2 and 7) with normal baseline values at the 1-month follow up visit using Wilcoxon signed ranks test. Only detectable levels were analyzed. A subset of mRNA's, including GSTP1, IL4, IL12A, IFNG, LTA and MIP1B (for abbreviations, see table 2), with >25 % of values below detection limit was analyzed using Mann Whitney U test to correct for lack of paired samples. mRNA's with >80 % of values below the detection limit were excluded from analysis and considered not detectable. *P*-values for each time point were corrected for multiple comparisons according to the method of Benjamini and Hochman, with q=0.1 [27]. Correlations were calculated by Spearman's Rho test. A $P<0.05$ was considered statistically significant.

RESULTS

Patient characteristics

Samples for mRNA analysis were collected successfully from 56 children with confirmed dengue virus infections. The children were classified according to WHO criteria as having dengue fever (n=7; 13%), dengue hemorrhagic fever (n=29; 51%) or dengue shock syndrome (n=20; 36%). Further patient characteristics at admission are given in table 1. During stay at the hospital, 10 patients (18%) developed severe complications related to their dengue infections. Profound shock was noted in 7 children (13%) and recurrent shock in 5 children (9%). Other complications included disseminated intravascular coagulation (n=3; 5%), encephalopathy (n=3; 5%) or pulmonary oedema (n=1; 2%). Eventually, 4 patients (7%) died. These patients had all been classified as having DSS at admission. All surviving patients made a complete recovery within a maximum of 12 days of stay in the hospital.

Table 1. Clinical characteristics and laboratory findings.

	all (n=56)	DF (n=7)	DHF (n=29)	DSS (n=20)
Age, median (IQR), years	6 (5-9)	7 (5-8)	7 (5-10)	6 (5-7)
Male sex, n (%)	22 (39)	3 (43)	11 (38)	8 (40)
Duration of symptoms, median (IQR), days	4 (3-4)	3 (3-4)	4 (3-4)	4 (3-5)
Spontaneous bleeding, n (%)	31 (55)	2 (29)	12 (41)	11 (55)
Systolic blood pressure, median (IQR), mmHg	90 (80-100)	80 (70-100)	90 (90-100)	80 (60-90)
Pulse pressure ≤20 mmHg, n (%)	24 (43)	2 (29)	11 (38)	14 (70)
Heart frequency, median (IQR), min^{-1}	120 (112-132)	112 (100-132)	120 (112-131)	122 (120-132)
Hematocrit, median (IQR), %	41 (37-45)	38 (27-43)	42 (38-45)	42 (40-48)
Platelet count, median (IQR), cells * $10^3/mm^3$	57 (42-77)	70 (52-134)	54 (41-80)	57 (40-73)
Duration of hospital stay, median (IQR), days	5 (3-6)	5 (3-8)	4 (3-5)	6 (3-7)
Mortality, n (%)	4 (7)	0 (0)	0 (0)	4 (20)

Children from Indonesia were admitted to the hospital with severe dengue virus infections (n=56). A distinction was made between dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) according to WHO criteria. IQR, denotes InterQuartile Ranges; n, denotes number.

Gene expression profiling

Gene expression profiles of inflammatory genes during hospital stay were compared for each day to baseline values at a 1-month follow up visit (table 2). From the follow-up visits, 33 samples could be collected and analyzed. The profile showed up-regulation of 10 genes, down-regulation of 11 genes and no effect on 19 genes, whereas 9 genes were not detectable. In alphabetical order, up-regulation on consecutive days occurred for cyclin-dependent kinase inhibitor 1A (CDKN1A), glutathione S-transferase (GSTP1), IFNG, IL12A, MIF, protein-tyrosine phophatase nonreceptor-type 1 (PTPn1), Toll-like receptor 4 subunit 3 (TLR4R3) and TLR7. Furthermore, interleukin 15 subunit 1 (IL15R1) and polyadenylate-specific ribonuclease (PARN) were up-regulated at day 2 and day 1, respectively. Down-regulation of genes was observed at consecutive days for CD14, IL1b, IL8, nuclear factor kappa-B subunit 1 (NFκB1), NFκB2, TNFR1, TLR2 and TLR4R4. At admission, also phosphodiesterase 4B (PDE4B) and protein-tyrosine phophatase type 4a, 2 (PTP4a2) were down-regulated, and at the first day after admission TLR1. Other genes were not significantly affected or not detectable.

Correlations

Correlations with disease severity and clinical outcomes were calculated for each gene expression level at admission (table 3). The development of DSS correlated with PTPN1, but with no other inflammatory markers. Mortality was associated with down-regulation of IFNG and GSTP1, and up-regulation of TNFRS1, TLR4R1, TLR4R3, CD14, TLR1 and TLR5. Furthermore, IL15R1 correlated negatively with the occurrence of complications, and length of hospital stay correlated with MYC. The expression of MIF correlated significantly with CDKN1A ($r=0.415$; $p=0.01$).

Table 2. MLPA gene profile.

Gene transcription response	Gene symbol	Descriptive name	day 0	day 1	day 2	day 7
Up	CDKN1A	Cyclin-dependent kinase inhibitor, 1A	0.001	0.001	0.011	NS
	GSTP1	Glutathione S-transferase*	0.003	0 001	0.002	NS
	IFNG	Interferon, gamma*	0.016	0.005	0.027	0.021
	IL12A	Interleukin 12, subunit p35*	0.006	0.039	NS	NS
	IL15(1)	Interleukin 15, transcript variants 1 and 3	NS	NS	0.036	NS
	MIF	Macrophage migration inhibitory factor	0.000	0.000	0.001	0.004
	PARN	Polyadenylate-specific ribonuclease	NS	0.002	NS	NS
	PTPN1	Protein-tyrosine phophatase, nonreceptor-type 1	0.005	0.002	0.005	NS
	TLR4(3)	Toll-like receptor 4, transcript variant 3	0.007	0.027	0.006	NS
	TLR7	Toll-like receptor 7	0 002	0 000	NS	NS
Down	CD14	Monocyte differentiation antigen CD14	0 024	0 018	0 031	NS
	IL1B	Interleukin 1, beta	0 006	0 003	0 004	NS
	IL8	Interleukin 8	0 013	0 005	0.026	NS
	NFKB1	Nuclear factor kappa-B, subunit 1	0 009	0 009	NS	0 042
	NFKB2	Nuclear factor kappa-B, subunit 2	0 004	0.000	0.006	NS
	PDE4B	Phosphodiesterase 4B, cAMP specific	0 004	NS	NS	NS
	PTP4A2	Protein-tyrosine phophatase, type 4a, 2	0.002	NS	NS	NS
	TNFR1A	Tumor necrosis factor receptor superfamily 1a	0.000	0.000	0.001	0.049
	TLR1	Toll-like receptor 1	NS	0 010	NS	NS
	TLR2	Toll-like receptor 2	0.005	0 004	0.020	0.031
	TLR4(4)	Toll-like receptor 4, transcript variant 4	0.004	0 000	0.023	NS
Non significant	BMI1	BMI-1 oncogene homolog	NS	NS	NS	NS
	IL1RA	Interleukin 1 receptor antagonist	NS	NS	NS	NS
	IL4	Interleukin 4	NS	NS	NS	NS
	LTA	Lymphotoxin alfa	NS	NS	NS	NS
	MCP1	Monocyte chemotactic protein 1	NS	NS	NS	NS
	MD2	MD2 protein	NS	NS	NS	NS

Transcriptional changes of 50 inflammatory gene expression levels using multiplex ligation-dependent probe amplification in 56 patients with severe dengue virus infections. Blood samples were collected at hospital admission (day 0) and day 1, 2 and 7 after hospital admission. Levels at each time point were compared to baseline levels collected at a 1-month follow up visit. *P*-values are presented indicating up- or downregulation of gene transcription during infection. *P*-values were calculated by Wilcoxon's signed rank test, or Mann Whitney U test when >25% of values was below the detection limit (*), with multiple measurements correction by the method of Benjamini and Hochberg; q=0.1. A P-value<0.05 was considered statistically significant. NS= Not Significant. ND= Not Detectable.

Table 3. Correlations.

	mRNA	r	*P*-value
DSS	PTPN1	0.350	0.032
Mortality	IFNG	-0.547	0 021
	GSTP1	-0.374	0 042
	CD14	0.390	0 013
	TNFRS1	0.344	0 034
	TLR1	0.318	0 046
	TLR4R1	0.332	0.036
	TLR4R3	0.368	0.019
	TLR5	0.377	0.037
Complications	IL15R1	-0.374	0.029
Length of hospital stay	MYC	0 362	0 026

Associations of expression levels of inflammatory genes with disease severity and clinical outcomes in children with (haemorrhagic) dengue fever (n=36) and dengue shock syndrome (DSS, n=20). mRNA levels at admission were determined by MLPA. Correlations were calculated according to Spearman's Rho and expressed as correlation coefficient (r). A *P*-value <0.05 was considered statistically significant.

DISCUSSION

The pathogenesis of severe, critical disease following dengue virus infection involves activation of multiple inflammatory pathways. To our knowledge, this is the first study to report high throughput gene expression profile changes of inflammatory genes in a well defined cohort of patients with severe dengue virus infections. The profile showed characteristics of a general antiviral response, with up-regulation of IFN-γ, an established major antiviral cytokine in dengue disease, together with up-regulation of IL-12, a potent stimulator of IFN-γ production [5, 28]. Most gene transcription profile changes were noted at admission, with a more or less gradual decline of changes during recovery, thereby validating an active role for the implicated genes in acute severe dengue infections.

The most prominent changes in this study were observed for elevated mRNA levels of MIF. This pro-inflammatory lymphokine is a major protein secreted after stimulation with bacterial endotoxin [29]. In a study of adult patients with dengue, elevated plasma levels of MIF could also be detected and were associated with mortality. Of note, MIF has been involved in induction of cyclin dependent kinase inhibitors, which can inhibit cell cycle progression [30]. Indeed in our study, CDKN1A gene transcription was strongly affected and correlated with MIF expression, which is consistent with an interaction between both mediators.

The activation of Toll like receptors was characterized by an increased expression of TLR7 and TLR4(3). TLR7 was previously identified as an endosomal pattern recognition receptor for single-stranded RNA viruses [18, 31]. This study suggests that TLR7 also plays a role in dengue virus infection. The role of TLR4 is not clear

from this study. Whereas transcript variant TLR4R3 was up-regulated, transcript variant TLR4R4 was down-regulated, and no effect was noted for the other transcript variants, TLR4R1 and TLR4R2. Furthermore, whereas the TLR4 co-factor CD14 was down-regulated, there was no effect for the TLR4 co-factor MD2. Other TLRs, including TLR1 and TLR2, were down-regulated, and therefore their role in dengue infection is probably limited. In a microarray based study, TLR3 was up-regulated *in vitro* after infection of endothelial cells with dengue virus [32]. However, in the present study, levels of TLR3 could not be detected.

The intracellular NFκB pathway is a major route for inflammatory stimuli to the release of most pro-inflammatory mediators, including TNF-α, IL-1ß and IL-8. Following *in vitro* stimulation with dengue, the NFκB pathway was stimulated [33] [34]. The current study, however, showed that *in vivo* gene expression levels of the NFκB pathway were inhibited in circulating leukocytes, together with IL-1ß and IL-8. This *in vivo* inhibition of gene transcription of NFκB, IL-1ß and IL-8 may reflect a state of immunoparalysis in these severely ill patients. A major feature of immunoparalysis is a reduced ability of isolated peripheral blood mononuclear cells to produce TNF-α after stimulation. Indeed, this has been observed for dengue patients in several studies [12] [14] [35]. The down-regulation of IL-1ß and IL-8 transcription in the current study further supports the concept of a functional inhibition of NFκB transcription in circulating leukocytes. The reduced transcription levels of IL-1ß and IL-8 are not reflected in the frequently reported elevated protein plasma levels of these cytokines, however [11] [12] [32] [36]. Apparently, the protein plasma levels of these cytokines result from a source different from circulating leukocytes. Together, the data indicate that immunoparalysis in severely ill children with dengue occurs in circulating leukocytes, and that other sources add to the release of NFκB dependent pro-inflammatory cytokines into the circulation.

A wide range of gene expression changes in this study was associated with various clinical outcomes. We previously reported an association between IL-1RA plasma levels and mortality, but these data could not be confirmed in the present study on the gene transcription level [12]. Interestingly, mortality in this study was negatively associated with IFN-γ gene transcription. Indeed, dengue virus has been shown *in vitro* to be able to inhibit IFN-γ synthesis [37]. IFN-γ is a central antiviral cytokine in dengue infection and is in vitro capable of directly inhibiting viral transcription [38]. Mice with null mutations of the IFN-γ receptor were more susceptible to dengue infection [5]. In patients with trauma, IFN-γ polymorphisms were associated with the development of sepsis [39]. These data suggest that defective IFN-γ production, either inherited by the host or caused by the pathogen, can be detrimental in dengue infection.

In summary, this study highlighted changes in inflammatory gene expression in circulating leukocytes following severe dengue infections. The profile showed up-regulation of 10 genes including IFN-γ, IL12a, MIF and TLR7. Down-regulation of the NFκB pathway and lack of effect on TNF-α transcription suggested a state of immunoparalysis. Mortality in the study was negatively correlated with IFN-γ synthesis. Together, these data suggest that immunoparalysis occurs in circulating leukocytes in severely ill children with dengue virus infection and may be a factor contributing to a poor prognosis.

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SUMMARY

Epidemics of dengue fever (DF) and dengue haemorrhagic fever (DHF) have become an important public health problem in Indonesia. Nowadays the disease is endemic in many large cities and small towns throughout Indonesia and has also spread to certain smaller villages. Data on dengue epidemiology which are described in the medical literature and which are obtainable from the World Health Organization have been gathered and reviewed in **chapter 1**. The numbers are worrisome: Dengue cases in Indonesia are increasing over time, the four dengue serotypes have spread over all 29 Indonesian provinces, and epidemics are characterised by an increase in absolute number of deaths. In the absence of an effective vaccine and with no existing specific therapeutic agent, dengue can be expected to continue to escalate in this region of the world. This emphasizes the necessity to study dengue virus infections. In this thesis we focused on three important factors of dengue virus infections: clinical aspects & management, coagulation & endothelium, and vascular leakage & immunology.

CLINICAL ASPECTS AND MANAGEMENT

In **chapter 3**, the diagnostic accuracies of the WHO classification system and modifications to this system to detect patients with circulatory failure are described. A total of 152 patients with confirmed dengue were evaluated. The results show that the WHO classification system has a sensitivity of 86% (95% CI 76-94) to detect patients with shock. Quite remarkable all modifications on the WHO classification system had a higher sensitivity ranging from 88%-99%. It was also demonstrated that the WHO classification system was in only modest agreement with the intuitive classification by treating physicians. Treating physicians were inclined to classify patients with evidence of plasma leakage as having dengue haemorrhagic fever even in the absence of both thrombocytopenia and a haemorrhagic tendency. If the purpose of classifying dengue disease severity is to identify the maximum number of patients with circulatory failure, then it would seem, that on the basis of these results, one of these modified classification systems is to be preferred.

A standardized classification system for the severity of dengue virus infection is crucial for optimal communication of scientific data to improve our understanding of the pathogenesis and treatment of the disease. Others have also argued that it is time for a reassessment and that we need a simple, reproducible and user-friendly classification system that is applicable throughout the health-care system of the countries where it is to be used (Deen et al. Lancet 2006; 368: 170–173 and Rigau-Pérez. Lancet Infectious Diseases 2006; 6: 297-302). For this, a large multicentre descriptive study is needed to obtain the evidence to establish a robust dengue classification scheme for use by clinicians, epidemiologists, public-health authorities, vaccine specialists, and scientists involved in dengue pathogenesis research.

In the absence of specific therapeutic options, treatment of severe dengue virus infections is limited to supportive measures and predominantly relies on adequate fluid replacement. Recommendations for fluid replacement strategies were developed

following key findings in Bangkok in the 1960s and evolved into the WHO guidelines of 1974, updated in 1986, 1994, and 1997 Until recently, only few studies compared different fluid replacement strategies in properly conducted clinical trials, showing variable results. The aim of the study described in **chapter 4** was to evaluate the effects of a colloid fluid regimen containing 6% hydroxyethyl starch (HES) of 200 kD molecular weight in comparison to currently by the WHO recommended treatment with Ringer's Lactate (RL) in an open-labeled, randomized clinical trial. Sixty Indonesian children admitted to the hospital with severe DSS were included Children were assigned initial fluid resuscitation for 10-30 minutes with HES (n=30) or RL (n=30) Several clinical and laboratory data were collected Treatment with HES significantly reduced mortality from 27% to 7%, which is a reduction of 74%. In addition, organ complication rates, shock recovery times and duration of vascular leakage were shortened in the intervention group, as well as duration of mechanical ventilation and length of stay at the PICU No adverse reactions were noted A recent study (N Wills et al. Engl J Med 2005; 353:877-889) demonstrated that crystalloid Ringer's lactate is as effective as either of the colloids for initial resuscitation in patients with moderately severe shock. The findings described in **chapter 4** suggest that a short, initial infusion of colloid fluids like HES in children with severe shock is safe and can improve mortality in a cost-effective way.

COAGULATION AND ENDOTHELIUM

The characteristic hemorrhagic manifestations, such as epistaxis, gingival bleeding, gastrointestinal bleeding, haematuria and menorrhagia, are primarily thought to be due to vasculopathy, thrombocytopenia and thrombocytopathy Defects in coagulation and fibrinolysis were felt not to lie central in the pathogenesis of severe disease. However, recent in vitro data suggest coagulopathy and disturbances in fibrinolysis to play a more pivotal role in the pathophysiology of dengue than currently perceived. If disturbances in coagulation and fibrinolysis are predictors of clinical outcome of dengue virus infections this could have important consequences for both diagnosis and treatment. In **chapter 5** the literature was critically reviewed to assess an association between coagulation and fibrinolysis activation, and clinical outcome of Dengue virus infections. In general, the selected studies demonstrated activation of both the coagulation and the fibrinolytic system in Dengue virus infection. This activation of coagulation and fibrinolysis was more pronounced in severe infections and in cases with ultimately a poor clinical outcome. However, the findings were inconsistent and comparisons between the studies could not be done adequately because of differences in patient characteristics, settings and assays used.

To clarify the role of disseminated intravascular coagulation in Dengue, we examined coagulation abnormalities in Dengue virus-infected patients throughout time (**chapter 6**). Eighty-eight patients with severe Dengue virus infection were enrolled Blood samples were obtained on day of admission, days 1, 2 and 7 after admission and at a 1-month follow-up visit Coagulation screening tests and measurement of anticoagulant factors were performed on serial samples of included patients. The majority of patients had coagulation abnormalities on admission and

on the first two days following admission. Abnormal coagulation screening tests and alterations in levels of anticoagulant factors were associated with disease severity. A diagnosis of overt disseminated intravascular coagulation was present on admission in 16% and during follow up in 28% of our patients. This was associated with an increased risk of severe disease (disseminated intravascular coagulation present on admission: OR 5.2, 95%CI 1.3-21.2; disseminated intravascular coagulation present during follow up: OR 4.4, 95%CI 1.6-12.1). Anticoagulant and fibrinogen patterns, however, were substantially different from bacterial associated disseminated intravascular coagulation and were rather in line with observations made in patients with liver disease. These data indicate that Dengue virus infections may cause gross coagulation abnormalities. Although the observed coagulation abnormalities lead to a positive overt disseminated intravascular coagulation score, they cannot be explained by disseminated intravascular coagulation alone. Additional mechanisms need to be taken into account.

In **chapters 7** and **8** we demonstrate that the fibrinolytic system is also activated in children with severe dengue virus infection, but that this activation is relatively weak compared with that of coagulation as a result of persistently high plasminogen-activator-inhibitor (PAI) levels. High PAI levels prevented a switch from the procoagulant to the profibrinolytic state in lethal dengue virus infection. Since a genetic predisposition to produce high PAI-1 plasma concentrations appears to be associated with poor clinical outcome in other infections, we hypothesized that Dengue virus infected individuals carrying the 4G/4G genotype have higher PAI-1 plasma concentrations and are therefore at increased risk of death. An association between PAI plasma concentrations in Dengue virus infected individuals and the 4G/5G promotor polymorphism in the PAI-1 gene, is studied and described in **chapter 8**. No significant association between PAI-1 plasma concentrations and carriage of the 4G/4G genotype was observed. The frequencies of the three genotypes between survivors and non-survivors, and between patients with different disease severities were not different. These findings suggest that increased PAI-1 plasma concentrations, and Dengue disease severity and mortality are not dependent on the 4G polymorphism in the PAI-1 gene in this population. Additional studies are needed to explore the possibility of other polymorphisms within the PAI-1 gene and factors, like ethnicity or environmental factors, contributing to the variability of PAI-1 plasma concentrations in patients with Dengue.

VASCULAR LEAKAGE AND IMMUNOLOGY

The host response to dengue virus infection is characterized by production of numerous cytokines, but the overall picture appears to be complex. In vitro data suggested that a state of immunoparalysis occurs. This study aimed to describe cytokine patterns in whole blood samples measuring mRNA levels from 50 genes encoding inflammatory proteins simultaneously. Whole blood mRNA from 56 Indonesian children with severe dengue virus infections was isolated on day 0, 1, 2, 7 and 30 after hospital admission. mRNA was analyzed in a previously developed and validated sensitive quantitative assay, multiplex ligation-dependent probe

amplification (MLPA). The gene profiles showed up-regulation during infection of 10 genes including interferon (IFN)-α, interleukin (IL)-12a, macrophage inhibitory factor (MIF) and toll like receptor (TLR)7. Concurrently, the nuclear factor (NF)kB pathway was down-regulated together with downstream effector genes encoding IL-1b and IL-8. Among other associations, mortality in the study (n=4) was negatively correlated with IFN-γ synthesis. Together, these data suggest that the in vivo host response to severe dengue virus infections is characterized by a general antiviral response including up-regulation of IFN-γ and TLR7 synthesis whereas a state of immunoparalysis in circulating leukocytes characterized by down-regulation of the NFkB pathway is concurrently induced.

CONCLUSIONS

In this thesis epidemiological, clinical and pathophysiological aspects of dengue virus infections are addressed that resulted in some new information. The data described herein add up to the limited number of studies performed thus far and in turn have raised additional questions, thereby giving direction to future studies. Our results indicate that coagulation abnormalities play a more pivotal role in dengue disease pathogenesis than currently perceived. Whether this may be influenced by a specific therapeutic strategy remains to be determined. One could speculate on the effects of desmopressin, a vasopressin analogue that increases the plasma levels of factor VIII and vWF, and that it may provide an opportunity to control the observed haemostatic disorders. This issue, as well as the WHO classification system and the complex interplay of inflammatory mediators, are the focus of current and future prospective follow up studies.

SAMENVATTING

Epidemieën met het Dengue virus zijn een belangrijk probleem voor de volksgezondheid in Indonesië. De ziekte is tegenwoordig endemisch in verschillende grote Indonesische steden en verspreidt zich ook naar de kleinere steden en dorpen. Gegevens over de epidemiologie van Dengue die beschreven zijn in de literatuur en die bekend zijn bij de Wereld Gezondheids Organisatie zijn verzameld en samengevat in **hoofdstuk 1**. De getallen zijn verontrustend: het aantal Dengue gevallen in Indonesië stijgt, de vier Dengue serotypes hebben zich verspreid over alle 29 Indonesische provincies, en epidemieën worden gekenmerkt door een toename van het absolute aantal patiënten, dat komt te overlijden. Bij afwezigheid van een effectief vaccin en zonder specifieke behandelingsmogelijkheden, is het de verwachting dat Dengue een toenemend probleem voor de volksgezondheid zal vormen. Dit benadrukt de noodzaak om infecties met het Dengue virus te bestuderen. In dit proefschrift wordt er aandacht besteed aan drie belangrijke factoren van infecties met het Dengue virus: klinische aspecten & behandeling, stolling & endotheel en vaatlekkage & immunologie.

KLINISCHE ASPECTEN EN BEHANDELING

In **hoofdstuk 3** worden de resultaten beschreven van een onderzoek waarbij de diagnostische nauwkeurigheid van het classificatie systeem van de Wereld Gezondheids Organisatie is getoetst op het herkennen van patiënten met circulatoir falen. In totaal werden 152 patiënten onderzocht. De resultaten tonen aan dat het classificatie systeem van de Wereld Gezondheids Organisatie een sensitiviteit heeft van 86% (95% BI 76-94) om patiënten met circulatoir falen te herkennen. Opmerkelijk is het feit dat verschillende modificaties op dit systeem een hogere sensitiviteit hebben variërend van 88% tot 99%. Het classificatie systeem van de Wereld Gezondheids Organisatie komt ook matig overeen met de intuïtieve classificatie uitgevoerd door de behandelaars. Patiënten met aanwijzingen voor vaatlekkage werden doorgaans geclassificeerd als hebbende Dengue haemorrhagic fever, zelfs als andere criteria zoals een laag aantal bloedplaatjes en/of bloedingneiging ontbraken. Op basis van deze bevindingen kan men zich af vragen of het systeem van de Wereld Gezondheids Organisatie in zijn huidige vorm gebruikt moet worden om de ernst van een Dengue virus infectie te classificeren, vooral omdat aangetoond is dat er onvoldoende overeenstemming blijkt te zijn met de classificatie van ernst van ziekte zoals dat in de dagelijkse praktijk gebeurt.

Een gestandaardiseerd classificatie systeem voor de ernst van Dengue is cruciaal voor het vergelijken en gebruiken van wetenschappelijke onderzoeksresultaten. De discussie hierover is recent aangewakkerd door publicaties waarin aangegeven werd dat het tijd is voor een herwaardering van het huidige systeem en dat een eenvoudig, reproduceerbaar en gebruikersvriendelijk classificatie systeem op dit moment noodzakelijk is (Deen et al. Lancet 2006; 368: 170–173 en Rigau-Pérez. Lancet Infectious Diseases 2006; 6: 297-302). Een multicentre studie zal de wetenschappelijke onderbouwing moeten leveren voor een nieuw, robuust Dengue classificatie systeem, dat gebruikt kan worden door clinici, epidemiologen, gezondheidszorg organisaties, vaccinatie specialisten en diegenen die betrokken zijn bij onderzoek naar de pathofysiologie van Dengue.

Bij afwezigheid van specifieke behandelopties voor ernstige Dengue virus infecties is men aangewezen op ondersteunende maatregelen die vooral bestaan uit adequate vochttoediening. Adviezen hieromtrent zijn al in de jaren 60 ontwikkeld en resulteerden in de eerste richtlijnen van de Wereld Gezondheids Organisatie van 1974, die later bijgewerkt is in 1986, 1994 en 1997. Er zijn tot op heden weinig studies verricht waarbij verschillende vocht transfusie strategieën met elkaar zijn vergeleken. Daarbij komt dat de studies die gedaan zijn uiteenlopende resultaten laten zien. Het doel van de openlabel, gerandomiseerde klinische trial beschreven in **hoofdstuk 4** was om de effecten van een ondersteunende strategie met 6% hydroxyethyl starch (HES) met een moleculair gewicht van 200 kD te vergeleken met het huidige door de Wereld Gezondheids Organisatie voorgestelde regiem met Ringer's Lactaat (RL). Zestig Indonesische kinderen met een ernstige Dengue virus infectie werden geïncludeerd. De kinderen kregen gedurende 10-30 minuten een initiële vochttoediening met HES (n=30)of met RL (n=30). Verschillende klinische en laboratorium gegevens werden verzameld. Behandeling met HES reduceerde de mortaliteit van 27% naar 7% (een reductie van 74%). Daarbij waren secundaire parameters zoals organ complication rate, shock recovery time en duur van vascular leakage, beademing en verblijf op de Pediatric Intensive Care Unit in de interventie groep aanzienlijk beperkt of verkort. Er deden zich geen nadelige bijwerkingen voor. Recent is beschreven dat Ringer's Lactaat even effectief is als elk ander colloïd oplossing voor de initiële behandeling van patiënten met een matig ernstige shock (N Wills et al. Engl J Med 2005; 353:877-889). De resultaten die beschreven worden in **hoofdstuk 4** suggereren dat bij kinderen met ernstige hemodynamische instabiliteit een korte, initiële behandeling met een colloïde oplossing veilig is en de mortaliteit op een kosteneffectieve manier kan verbeteren.

STOLLING EN ENDOTHEEL

Tot voor kort werd aangenomen dat de karakteristieke bloedingneigingen zoals epistaxis, tandvleesbloedingen, gastrointestinaal bloedverlies, hematurie en menorrhagie, een gevolg waren van vasculopathie, thrombocytopenie en thrombocytopathie. Van afwijkingen in de stolling en fibrinolyse werd aangenomen dat zij geen rol van betekenis speelden in de pathogenese van ernstige Dengue virus infecties. Recente in vitro studies hebben echter aangetoond dat afwijkingen in de stolling en fibrinolyse ook een belangrijke rol lijken te spelen in de pathogenese. Indien afwijkingen in de bloedstolling als marker kunnen fungeren voor ernst van ziekte en klinisch beloop, dan kan dit consequenties hebben voor zowel diagnostiek als behandeling. In **hoofdstuk 5** is de literatuur over stollingsafwijkingen bij Dengue samengevat en gekeken is of er een associatie bestaat tussen afwijkingen in de stolling en het beloop van infecties met het Dengue virus. De systematisch geselecteerde studies tonen aan dat zowel de stollingscascade als de fibrinolyse geactiveerd zijn tijdens infectie met het Dengue virus. Dit wordt vooral gezien bij ernstige infecties. Echter, de bevindingen waren niet eensluidend en vergelijkingen tussen de verschillende studies konden niet gemaakt worden vanwege verschillen in patiënt karakteristieken, setting van het onderzoek en de gebruikte testmethodes.

Om de rol van diffuse intravasale stolling bij Dengue te bepalen, onderzochten wij stollingsafwijkingen bij patiënten die geïnfecteerd waren met het Dengue virus (**hoofdstuk 6**). Achtentachtig patiënten met een ernstige infectie werden geïncludeerd. Bloedmonsters werden afgenomen bij opname, op dag 1, 2 en 7 na opname en bij een controle bezoek na 1 maand en werden gebruikt voor de bepaling van stollingstijden, stollingsactivatie markers en anticoagulante factoren. Het merendeel van de patiënten had bij opname en in de eerste 2 dagen daaropvolgend stollingsafwijkingen die geassocieerd waren met ernst van ziekte. De diagnose diffuse intravasale stolling kon bij opname gesteld worden in 16% van de gevallen en gedurende de opname in 28% van de gevallen. De aanwezigheid van diffuse intravasale stolling was geassocieerd met een verhoogde kans op een ernstiger beloop (diffuse intravasale stolling bij opname: OR 5.2, 95%BI 1.3-21.2; diffuse intravasale stolling tijdens follow up: OR 4.4, 95%BI 1.6-12.1). De gevonden patronen van anticoagulante factoren en fibrinogeen verschillen echter wezenlijk van de patronen zoals die gezien worden bij diffuse intravasale stolling ten gevolge van een bacteriële infectie en lijken meer op de patronen zoals die gezien worden bij patiënten met een leversynthese functiestoornis. Deze resultaten tonen aan dat een infectie met het Dengue virus uitgesproken afwijkingen kan geven in de stollingscascade. Ondanks het feit dat dit leidt tot een positieve diffuse intravasale stolling score moet er rekening mee gehouden worden dat andere factoren dan diffuse intravasale stolling deze afwijkingen veroorzaken.

In de **hoofdstukken 7** en **8** wordt er aangetoond dat de fibrinolyse ook geactiveerd is bij kinderen die geïnfecteerd zijn met het Dengue virus, maar dat deze activatie relatief zwak is ten opzichte van de stolling als gevolg van aanhoudende hoge concentraties van plasminogen-activator-inhibitor (PAI). Hoge PAI concentraties voorkomen een switch van de procoagulante naar een profibrinolytische (anti-coagulante) status bij een letale infectie. Aangezien een genetische aanleg om hoge PAI concentraties te maken geassocieerd is met een slechte klinische uitkomst, zoals aangetoond bij andere infecties, stelden wij ons de vraag of Dengue virus geïnfecteerde patiënten met het 4G/4G genotype hogere PAI concentraties hebben en of zij een verhoogd risico hebben op overlijden De resultaten van dit onderzoek worden beschreven in **hoofdstuk 8**. Er werd geen associatie gevonden tussen dragers van het 4G/4G genotype en PAI concentraties. De verdeling van de verschillende genotypen onder overlevenden en overledenen, en onder patiënten met een variërende ernst van ziekte was niet afwijkend. Deze bevindingen suggereren dat verhoogde PAI concentraties, en ernst van Dengue en mortaliteit onafhankelijk zijn van het 4G polymorfisme in de promotor regio van het PAI gen. Vervolg onderzoek zal moeten uitwijzen of andere polymorfismen in het PAI gen en factoren zoals etniciteit en/of omgevingsfactoren een bijdrage leveren aan de variabiliteit van PAI concentraties bij patiënten geïnfecteerd met het Dengue virus.

VAATLEKKAGE EN IMMUNOLOGIE

De reactie van de gastheer op een infectie met het Dengue virus wordt gekarakteriseerd door de productie van pro- en anti-inflammatoire cytokinen. In vitro gegevens suggereren dat een immunoparalyse optreedt. Het onderzoek beschreven in **hoofdstuk**

9 had als doel de verschillende patronen van cytokine activatie in kaart te brengen door het meten van mRNA concentraties van 50 genen die coderen voor ontstekingseiwitten. Van 56 Indonesische kinderen met een ernstige Dengue virus infectie werd mRNA geisoleerd uit volbloed van dag van opname en dag 1, 2, 7 en 30 na opname . Een recent ontwikkelde en gevalideerde kwantitatieve test, de multiplex ligation-dependent probe amplification (MLPA), werd hier voor gebruikt. Het genetisch profiel toonde een up-regulatie van 10 genen waaronder interferon (IFN)-α, interleukin (IL)-12a, macrophage inhibitory factor (MIF) en toll like receptor (TLR)7. De nuclear factor (NF)κB pathway werd daarentegen onderdrukt, tezamen met de genen die coderen voor IL-1b en IL-8. De mortaliteit in deze studie (n=4) was negatief gecorreleerd aan de productie van IFN-γ. Samenvattend tonen deze resultaten aan dat de gastheerrespons in reactie op een ernstige infectie met het Dengue virus gekarakteriseerd wordt door een antivirale reactie met up-regulatie van IFN-γ en TLR7 synthesis en een immunoparalyse van circulerende leucocyten door een onderdrukking van de NFκB pathway.

CONCLUSIE

In dit proefschrift worden epidemiologische, klinische en pathofysiologische aspecten van infecties met het Dengue virus besproken. Onze bevindingen laten zien dat afwijkingen in de stollingscascade een belangrijker rol in de pathogenese van ernstige ziekte spelen dan tot voor kort werd aangenomen. Of dit ruimte biedt aan eventuele therapeutische interventie zal nog nader onderzocht moeten worden. Men kan speculeren over de effecten van desmopressine, een vasopressine analoog dat vWF en factor VIII vrijmaakt. Dit zou mogelijk een gunstig en corrigerend effect kunnen hebben op de geobserveerde stollingsafwijkingen. Toekomstig onderzoek zal zich hierop richten.

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Erratum

Ondanks de zorgvuldigheid waarmee deze publicatie is samengesteld, zijn verschillende punten niet juist vermeld. Hiervoor excuses.

Erratum bij de kaft:
De subtitel is niet correct afgedrukt. Dit moet zijn "Clincial assessment, pathophysiology and management."

Erratum bij hoofdstuk 5:
Tabellen 3, 4 en 5 zijn verwisseld.
Tabel 3 laat de resultaten zien van de stollingsbepalingen. Dit hoort tabel 4 te zijn.
Tabel 4 laat de resultaten zien van de fibrinolysebepalingen. Dit hoort tabel 5 te zijn.
Tabel 5 laat de karakteristieken zien van de verschillende studies. Dit hoort tabel 3 te zijn.

Erratum bij acknowledgements:
In de tweede alinea is de naam Osterhause verkeer afgedrukt. Dit moet zijn Osterhaus.

Stellingen behorende bij het proefschrift

Dengue virus infection: Clinical assessment, pathophysiology and management

1. The epidemiology of DHF in Indonesia is changing from children to adolescents and adults (this thesis)
2. The WHO system for classification of Dengue disease severity needs to be evaluated (this thesis)
3. Initial aggressive fluid resuscitation with colloid has a pivotal role in the management of severe Dengue virus infections (thesis)
4. DIC in DHF is different from bacterial associated DIC (this thesis)
5. A state of immunoparalysis occurs in severe dengue virus infection (thesis)
6. Disturbances in coagulation and fibrinolysis in Dengue may be an aspect for intervention (this thesis)
7. PAI- 4G/5G polymorphism is not associated with the risk of death in patients with Dengue (this thesis)
8. Dengue virus infection eradication programs should involve the host, the agent, and the environment
9. Curiosity and enthusiasm are fundamental to be successful
10. Logic, ethics and esthetics are three principles I used for education, research and humanity

Tatty Ermin Setiati
Nijmegen, 7 December 2006