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OPTIMISATION OF DENGUE DIAGNOSTIC TOOLS IN ORDER TO INCREASE THE KNOWLEDGE OF THE PATHOGENESIS

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Cover illustration: A dengue virus particle (49,000 x magnification).

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ABSTRACT

Dengue fever (DF) is the most common global viral mosquito-borne infection, with 100 million estimated cases annually in tropical and subtropical areas. The dengue viruses (*genus Flavivirus, Flaviviridae*) occur in 4 serotypes (DENV-1 to DENV-4). Dengue diseases range from a mild febrile disease to severe hemorrhagic fever. Infection with one serotype induces a life-long immunity, but does not elicit cross-protective antibodies to the other serotypes. Re-infection with a second serotype is associated with a more severe disease and is also a risk factor for dengue hemorrhagic fever. Each year the Swedish Institute for Infectious Disease and Control receives serum samples from several hundred Swedish travellers, with a suspected acute or past DF after travelling to dengue endemic areas. The aims of this thesis were to introduce methods suitable for the different phases of viremia and antibody development in the early and the late phases of disease, respectively and to determine the optimal methods in relation to the onset and sampling dates. In paper I, dengue IgG immunofluorescence assay (IFA) negative acute serum samples from 57 previously defined Swedish dengue patients (1997-2002), were investigated by different PCR assays and by dengue IgM ELISA. Only samples collected until day 5 post onset were found positive by PCR: in 73% (35/48) of the samples, dengue RNA of serotypes 1, 2 or 3 was detected. The number of genomes/ml varied between 10^3 and 10^8 , with a gradual decline over time. Dengue-specific IgM antibodies were found in 35% (20/57) of the samples. By a combination of the PCR assays and the IgM ELISA, a dengue diagnoses could be determined in as many as 84% (48/57) of early single samples. When the analyses were consecutively performed on the samples from 2002, 100% (13/13) of the samples were positive either by PCR or by IgM ELISA. In paper II, a dengue micro-NT (m-NT) for detection and serotyping of neutralising antibodies was developed and evaluated. Early convalescent samples (<6 weeks), complemented by late convalescent samples (>5 years) from 20

patients, previously serotyped by PCR were included in the study. The correct serotype was determined in 80% (16/20) of the late convalescent samples, while the serotype could not be determined in 4 patients. One patient did not produce any neutralising antibodies, another patient had most probably had two dengue infections with equally high titres of neutralising antibodies against both. In two patients a significant difference between the serotypes could not be determined. We found no correlation between dengue IFA IgG titres and m-NT titres in samples collected 5-10 years post onset. We have demonstrated that the m-NT is a reliable diagnostic tool for detection and serotyping of neutralising antibodies in late convalescent serum samples of primary dengue cases.

LIST OF PUBLICATIONS

- I. Lindegren, G., Vene, S., Lundkvist, Å., and Falk, K.I. (2005). Optimized diagnosis of acute dengue fever in Swedish travelers by a combination of reverse transcription-PCR and immunoglobulin M detection. *Journal of Clinical Microbiology* **43**: 2850-5.
- II. Lindegren, G., Vene, S., Falk, K.I., and Lundkvist, Å. (2008) Detection of Dengue Serotype-Specific Antibodies in Swedish Travellers by Micro-Neutralisation Test (m-NT). Submitted.

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LIST OF ABBREVIATIONS

ADE	Antibody- dependent enhancement
Ae.	Aedes
Arbovirus	Arthropod-borne virus
CDC	Centers for Disease Control and Prevention
cDNA	Complementary deoxy ribonucleic acid
DENV-1	Dengue virus serotype 1
DENV-2	Dengue virus serotype 2
DENV-3	Dengue virus serotype 3
DENV-4	Dengue virus serotype 4
DF	Dengue fever
DHF	Dengue hemorrhagic fever
DSS	Dengue shock syndrome
E	Envelope protein
ELISA	Enzyme-linked immuno sorbent assay
HAI	Hemagglutination inhibition assay
IFA	Immunofluorescence assay
IgG	Immunoglobulin G
IgM	Immunoglobulin M
JEV	Japanese encephalitis virus
kb	Kilo bases
m-NT	Micro-neutralisation test
NS	Non-structural
NS1	Non-structural protein 1
NT	Neutralisation test
prM	Precursor membrane-associated protein
rER	Rough endoplasmatic reticulum
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SMI	Swedish Institute for Infectious Disease Control
ss	Single stranded
TBEV	Tick-borne encephalitis virus
WHO	World Health Organization
WNV	West Nile virus
YFV	Yellow fever virus

INTRODUCTION

The word “dengue” probably originates from the Swahili phrase “Ki-dinga Pepo”, which means *a disease characterised by a sudden cramp-like seizure, caused by an evil spirit* (Carey 1971, Etymologia 2006).

1. Important years (A.D.) in the dengue history

265-420. Dengue fever (DF) or illnesses with similar symptoms has been known for a very long time. A disease somehow connected with flying insects and water, “water poison”, was described already in a Chinese medical encyclopaedia during Chin Dynasty (Gubler 1998).

1779-80. The first reported epidemics of probable dengue occurred simultaneously in Asia, Africa and North America. It has been questioned if all these epidemics actually were caused by the dengue viruses, or by the clinically similar chikungunya virus (Carey 1971); however, during the Philadelphia outbreak 1780, Benjamin Rush described a disease he called *break-bone fever*, which is believed to be the first definitive clinical report on dengue.

1823. The term “dinga” (“dyenga”) was for the first time used to designate a disease in Zanzibar (Carey 1971).

1906. Thomas Lane Bancroft suggested that the *Aedes aegypti* mosquito was involved in the transmission of dengue (Bancroft 1906).

1907. Percy Ashburn and Charles Franklin Craig were the first to prove that the etiological agent of dengue was a “filterable virus”, the second human viral disease after yellow fever to be identified (Carey 1971).

1939-1945. During World War II the ecologic disruption facilitated increased transmission of mosquito-borne diseases, and multiple dengue virus strains became

endemic (hyperendemicity) in Southeast Asia and the Pacific, also an increased incidence of complicated dengue emerged (Henchal and Putnak 1990).

1944-45. Albert B Sabin and co-workers isolated a number of dengue virus strains by inoculating infectious sera into human volunteers (Sabin 1952).

1953-54. The first epidemics of dengue hemorrhagic fever (DHF) occurred in Manila, Philippines.

2000- In the past 50 years the dengue incidence has increased dramatically, and at present dengue is endemic in more than 100 countries. Dengue is now the most common arboviral disease in the world. Between 2.5 to 3 billion people living in urban areas of tropical and subtropical regions are at risk, and it is estimated that 100 million cases of DF, a half of million cases of DHF and 25,000 fatal cases occur each year. The global population growth, unplanned and uncontrolled urbanisation, ineffective mosquito control, and increased air travel are factors that are associated with the increased incidence of dengue (Gubler 1998, Malavige et al. 2004, Figure 1).

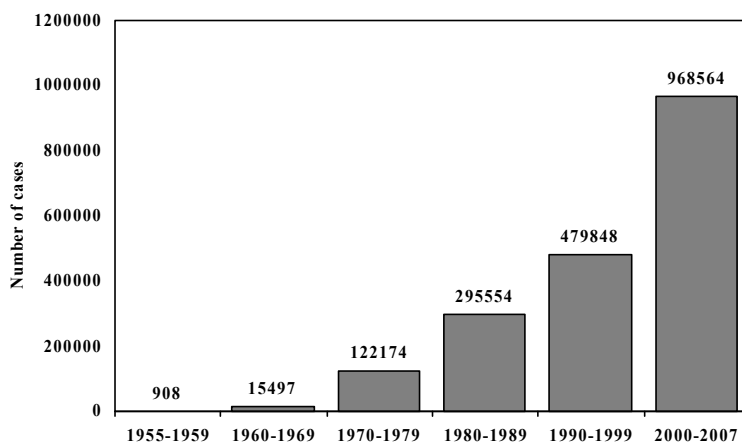


Figure 1. Average annual number of DF/DHF cases reported to the WHO.

2. Pathogenic flaviviruses

The dengue viruses belong to the flaviviruses, in which the yellow fever virus (YFV) is the prototype (*flavus* is the Latin word for yellow). There are more than 70 flaviviruses, and approximately 30 of them are pathogenic for humans. The majority of the viruses are arthropod-borne, and often restricted to a specific vector. Most arboviral infections are asymptomatic, or present with an influenza-like illness. However, several flaviviruses are important pathogens that may cause central nervous system disease, coma or death (Japanese encephalitis and tick-borne encephalitis viruses) (Calisher and Gould 2003, Weaver and Barrett 2004, Gould and Solomon 2008, Table 1).

Table 1. Some of the most important flaviviruses that are pathogenic for humans, by designation, principal vectors, principal hosts, and geographic distribution.

Virus	Vector	Hosts	Distribution
Yellow fever (YFV)	<i>Aedes</i> mosquitoes	Primates, humans	Africa, South America
Dengue (DENV1-4)	<i>Aedes</i> mosquitoes	Humans, primates	Tropics, subtropics
Japanese encephalitis (JEV)	<i>Culex</i> mosquitoes	Pigs, birds	India, China, Japan, Asia
West Nile (WNV)	mosquitoes ticks	Birds	Africa, Middle East, Europe, North America
St. Louis encephalitis (SLEV)	<i>Culex</i> mosquitoes	Birds	Americas
Murray Valley encephalitis (MVEV)	<i>Culex</i> mosquitoes	Birds	Australia
Tick-borne encephalitis (TBEV)	<i>Ixodes</i> ticks	Rodents	Europe, Asia

3. Classification and structure

The dengue viruses, (genus *Flavivirus*, family *Flaviviridae*) exist as four antigenically distinct serotypes, dengue 1 to dengue 4 (DENV-1 to DENV-4), which diverge ~ 30% across their polyprotein. DENV-1 and DENV-3 are the most closely related, followed by DENV-2 and DENV-4. Variation within the serotypes is common and several phylogenetic genotypes of different geographical origins occur (Holmes and Twiddy 2003, Holmes 2007). The flaviviruses have a single-stranded positive-sense RNA genome, approximately 11 kb long, and the complete genome has been sequenced for isolates for all four dengue serotypes (McKee et al. 1987, Kinney et al. 1997, Peyrefitte et al. 2003, Vasilakis et al. 2007). The genome consists of three structural protein genes, encoding the nucleocapsid or the core protein (C), a membrane-associated protein (M) and its precursor (prM) and an envelope protein (E), as well as seven non-structural (NS) protein genes. The E protein is associated with neutralisation, fusion and interactions with virus receptors. The non-structural proteins are addressed as NS1 to NS5. The NS1 glycoprotein, expressed on the surface of infected cells, is secreted extracellularly and detected in high titres in patients during the viremic phase. The NS2 region (NS2A and B) is supposed to play a role in the polyprotein processing, NS3 has viral protease functions in the cytosol, and the NS4 region (NS4A and B) is involved in RNA replication. The NS5 is the most conserved flavivirus protein, and is assumed to be the virus encoded RNA dependent RNA polymerase (Figure 2).

Structural				Non-structural					
C	pr M	E	NS1	NS 2A	NS 2B	NS3	NS 4A	NS 4B	NS5

Figure 2. Flavivirus polyprotein genes.

The genome is surrounded by an icosahedral nucleocapsid covered by a lipid bilayer envelope onto which M and E proteins are inserted. The virion is spherical and 40-50 nm in diameter. The flaviviruses share common antigenic determinants, and serological cross-reactions constitute a well-known diagnostic problem (Chambers et al. 1990, Gubler 1998, Henchal and Putnak 1990).

4. Dengue virus replication cycle

Dengue viruses attach to susceptible cells via interactions between the surface glycoprotein and cellular receptor(s). The virus particles are internalised by receptor-mediated endocytosis, and via a low pH-dependent membrane fusion the viral genome is released from the nucleocapsid into the cytoplasm. Viral RNA is translated into a single 3000 amino acid polyprotein, which is cleaved by host and viral proteases. The viral RNA replication begins, and the positive-sense RNA is transcribed into a negative strand intermediate that serves as a template of multiple positive strand progeny. Newly formed viral RNA interacts with the C protein to form the nucleocapsid, and the virus particles acquire their envelope by budding through the endoplasmic reticulum membranes. Mature virus particles are transported via secretory vesicles and released at the cell surface by exocytosis (Chambers et al. 1990, Gubler 1998, www.who.int/tdr/diseases/dengue/viral_rep.htm, Figure 3).

In addition, virus particles and non-neutralising IgG antibodies, from a previous dengue virus infection or maternal antibodies, may form antigen-antibody complexes. The complexes attach to monocytes/macrophages via Fc receptors found on cell surfaces, are internalised, and the virus can then replicate. Through this process called antibody-dependent enhancement (ADE), the virus can enter a larger amount of cells and increase virus production, and thereby contribute to the pathogenesis of

DHF/Dengue Shock Syndrome (DSS) (Henchal and Putnak 1990, www.cdc.gov/ncidod/dvbid/dengue/slideset, Gubler 1998).

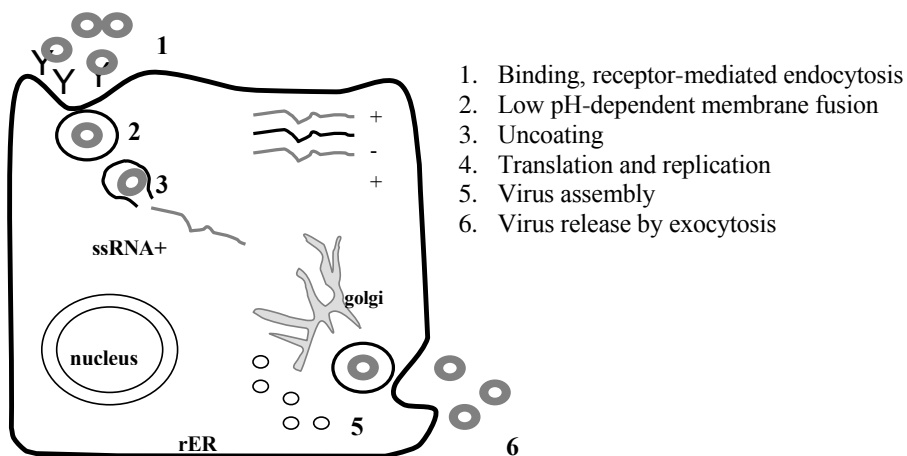


Figure 3. The replication cycle of the dengue viruses.

5. The mosquito vectors and virus transmission cycles

Dengue viruses are mainly transmitted from person to person during periodic epidemics in urban cycles, by *Aedes (Ae.)* mosquitoes. *Ae. aegypti*, the most important epidemic vector (also known as the vector of yellow fever and chikungunya viruses) is found in most subtropical and tropical regions of the world. The mosquito is highly domesticated and breeds almost exclusively in man-made water containers like flower vases, buckets and old automobile tires. Natural larval habitats are less common, but may include tree holes, and coconut shells. Embryonated eggs can withstand long periods of desiccation, which facilitates the survival of the species. The adult mosquitoes have an increased biting activity in the

mornings and afternoons. In case of interrupted feeding the mosquitoes may bite repeatedly, also several people - a behaviour that increases transmission efficacy. Compared with uninfected mosquitoes, infected ones take longer to complete a blood meal (Platt et al. 1997). Virus is transmitted to humans in female mosquito saliva, and replicates in target organs, e.g. local lymph nodes and the liver during an intrinsic period. Subsequently white blood cells and lymphatic tissue is infected, followed by virus release and circulation in the blood. A second mosquito acquires the dengue virus during feeding, the virus replicates in various mosquito organs and later infects the salivary glands, during an extrinsic incubation period of 8-12 days, and the transmission cycle continues. Female mosquitoes may also infect their offspring via vertical transmission of the virus (Lee and Rohani 2005). The *Ae. albopictus* mosquito is also a potential emerging vector of epidemic dengue, and has recently been found in several European countries (Benedict et al. 2007). Humans are the main amplifying host of the virus, but sylvatic dengue viruses are transmitted in an enzootic cycle between lower primates and *Ae.* mosquitoes in Southeast Asia and West Africa. Sylvatic mosquitoes may be important in maintaining dengue virus in an enzootic transmission cycle, but are not considered to have been important for the global re-emergence of DF/DHF so far. However, a possibility of cross-species transmission in the future is not considered unlikely (Gubler 1998, Vasilakis et al. 2007, Lai and Putnak 2007).

6. Clinical symptoms of dengue

Dengue is known by a number of local nick-names, referring to the symptoms: break-bone fever, dandy fever, bouquet fever, giraffe fever, polka fever, or the 5-day or 7-day fever (Henchal and Putnak 1990). The clinical presentation in dengue depends on the virus strain, as well as the age and immune status of the host. Infection with one

dengue serotype provides a life-long immunity against the infecting serotype, with various periods of cross-protective immunity to one or several of the other serotypes. Infection with a second serotype has been associated with more severe forms of disease and is a significant risk-factor to DHF (Guzman and Kouri 2003 and 2004, Henchal and Putnak 1990). There is also higher risk of DHF at locations with a simultaneous high level circulation of two or more serotypes (hyperendemicity).

Dengue virus infections can be classified as four different syndromes:

1. Undifferentiated fever
2. Classical dengue fever (DF)
3. Dengue hemorrhagic disease (DHF)
4. Dengue shock syndrome (DSS)

6.1. Undifferentiated fever

In several prospective studies it has been demonstrated that "silent" dengue transmission may be the most common manifestation. In one study (Burke et al. 1988) it was found that 87% of the participants who became infected by dengue virus were either asymptomatic or minimally symptomatic. Although the patients do not experience symptoms at the time of the acute infection, the immunity that results increases the risk for DHF during a subsequent infection (Chen et al. 1996, Endy et al. 2002, Poblaj et al. 2006, www.cdc.gov/ncidod/dvbid/dengue/index.htm).

6.2. Classical dengue fever (DF)

DF is an acute viral disease primarily of older children and adults. After a bite by an infective mosquito there is an incubation period of 3 to 15 days (average 4 to 7 days) and the disease begins by a sudden onset of high fever ($>39^{\circ}\text{C}$). The fever, which may continue for 2 to 7 days may be biphasic (saddleback fever), is accompanied by a variety of unspecific symptoms, including malaise, frontal headache, retro-orbital pain, joint pains, nausea, and vomiting. Viremia usually coincides with the fever period

(Waughn et al. 1997). Maculopapular, petechial, or erythematous rash may be present at different stages of the disease. Hemorrhagic manifestations of different severity are not uncommon. Most frequent are skin hemorrhages, gingival and nose bleeding, menorrhagia and gastrointestinal bleeding. Thrombocytopenia and leukopenia is also common. DF is generally a self-limiting disease, but convalescence may be prolonged and associated with fatigue and depression

(www.who.int/csr/resources/publications/dengue/012-23.pdf. Clinical diagnosis).

6.2.1. Quotation from a case report

The onset of dengue is very abrupt, which is illustrated by the following quotation from an early case report; *“Onset Oct. 28, 1922. Headache and general malaise on the morning of the attack. In the afternoon of the same day patient was stricken on the street and was taken home in a dazed condition”* (Harris and Duval 1924).

6.3. Dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS)

DHF has become a leading cause of hospitalisation and death among children under the age of 15 years in several countries in Asia. DHF is characterised by an abrupt onset of fever and symptoms resembling DF. During the early phase of the illness it is difficult to discriminate DHF from DF and other diseases; differential diagnoses include malaria, measles, rubella, influenza, typhoid, leptospirosis, and other diseases found in tropical areas. The critical stage in DHF is at the time when the febrile phase ends. A rapid fall in temperature is often accompanied by signs of circulatory disturbance of varying severity: sweating, cool extremities, changes in pulse rate and blood pressure. Severe cases with a critical loss of plasma may progress rapidly to profound shock and death. It has been hypothesised that the infected monocytes release vasoactive mediators, resulting in the increased vascular permeability and hemorrhagic manifestations that characterise DHF or DSS

(www.cdc.gov/ncidod/dvbid/dengue/index.htm).

6.3.1. Risk factors associated with DHF

1. Pre-existing non-neutralising antibodies, either acquired through a previous infection or as maternal antibodies
2. Virus strain (genotype): DHF can occur in a primary disease
3. Host genetics
4. Age; children below the age of 15 years and elderly people
5. Secondary disease and subsequent infections (Gibbons et al. 2007)
6. Higher risk in locations with more than one circulating serotype

6.3.2. Four criteria for DHF that have to be fulfilled (stated by WHO)

1. Fever, or recent history of acute fever
2. Hemorrhagic manifestations
3. Low platelet count (100,000 mm³ or less)
4. Objective evidence of plasma leakage caused by increased vascular permeability

6.3.3. Four grades of DHF (grades III and IV are considered to be DSS)

1. Grade I, fever and non-specific constitutional symptoms are present and the only hemorrhagic manifestation is a positive tourniquet test*
2. Grade II, in addition to the Grade I manifestations, there is spontaneous bleeding
3. Grade III, circulatory failure manifested by a rapid, weak pulse and presence of cold, clammy skin and restlessness
4. Grade IV, profound shock, with undetectable pulse and blood pressure

(www.cdc.gov/ncidod/dvbid/dengue/index.htm)

* The tourniquet test determines blood capillary fragility or platelet dysfunction. The test is done by inflating a blood pressure cuff to a point between the systolic and diastolic blood pressures for five minutes. The test is positive if there are more than 20 petechiae per square inch.

7. Dengue in travellers

Dengue is endemic in most tropical and subtropical areas of the world, of which several are popular tourist destinations, but the risk of infection for the average European tourist is low compared to that of the local populations. Most dengue virus infections in travellers are acquired in Asia, followed by the Americas with only a small proportion in Africa (Jelinek et al. 2002, Wichmann et al. 2003, and our unpublished data). However, according to several studies, dengue infection is the second most frequent cause (after malaria) of hospitalisation and/or fever in returning travellers (O'Brien et al. 2001, Stephenson et al. 2003 and Wichmann et al. 2007). The true incidence of dengue is probably underestimated, mainly due to under-diagnostics, but also to the fact that travellers who acquire dengue abroad are not reported (Wilder-Smith and Schwartz 2005). Most patients with imported dengue disease suffer from a mild to a moderately severe disease (Laferl et al. 2005), although fatal cases have recently been reported from both Finland and Norway (Huhtamo et al. 2006, Jensenius et al. 2007). Approximately 50 to 100 cases of acute dengue fever are laboratory diagnosed annually in returning travellers in Sweden (Figure 4).

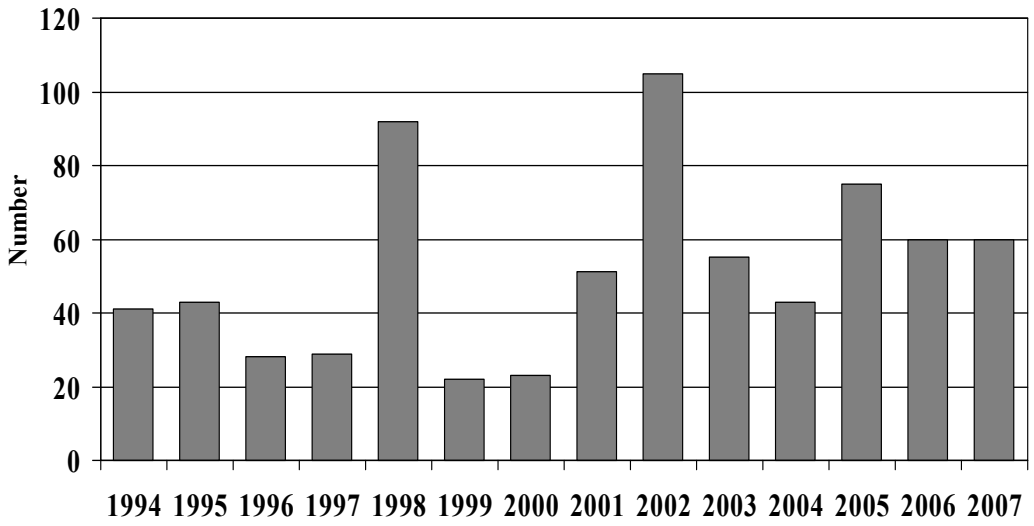


Figure 4. Number of imported dengue cases to Sweden (1994-2007) diagnosed by serological methods (IgG IFA, since 2002 complemented by IgM ELISA).

8. Dengue transmission without a mosquito vector

Although dengue infections are asymptomatic to a large extent, the virus will still be present in the blood of the individual at some point after infection as it is in symptomatic dengue patients during the viremic phase, which precedes the symptoms. Virus transmission without the involvement of a mosquito vector is rare, however, cases of neonatal dengue (Chye et al. 1997, Petdachai et al. 2004), dengue infection after needle stick injury (Wagner et al. 2004), organ transplantation (Tan et al. 2005) and blood transfusion (Chuang et al 2008) have been documented. In two large studies, where plasma samples from dengue asymptomatic blood donors from various populations were analysed by different diagnostic methods, the presence of both dengue viral RNA and dengue virus after culture could be confirmed at a range from 0.04 to ~1 % (Mohammed et al. 2008, Linnen et al. 2008). After several reports of transmission of the closely related West Nile virus from blood donors (CDC 2002, Busch et al. 2006), a national blood donor screening was implemented in the United States in 2003.

9. Diagnostics

Different diagnostic methods are required for the various phases of the disease. In the acute viremic phase, isolation of the virus (mosquitoes, culture) or detection of viral RNA (molecular assays) which provides a more sensitive and rapid detection also for inactivated viruses (Kao et al. 2005) are the methods of choice. Dengue viruses can be detected in different clinical specimens, i.e. serum, plasma, blood cells and different cells of the immune system, and also in post mortem specimens such as liver, lung, spleen, lymph nodes, thymus, and cerebrospinal fluid (www.cdc.gov/ncidod/dvbid/dengue/slideset). Mosquito inoculation is the most sensitive technique for virus isolation, but also a wide range of cell lines of mammalian

and insect origin can be used for virus propagation. Cytopathic effects, a result of virus infection, vary on different cell types, virus serotype and strain, and the passage history of the virus (Henchal and Putnak 1990, Huhtamo 2006). After virus isolation, the presence of dengue virus has to be confirmed, by serological or by molecular methods. In persons that never have been infected with a flavivirus or immunised with a flaviviral vaccine (JEV, YFV, TBEV), the primary antibody response is characterised by rising levels of IgM antibodies 3-5 days post onset of disease, followed by production of IgG antibodies and neutralising antibodies (Figure 5). The IgM antibodies are generally detectable for 2-3 months after infection. The IgG and the neutralising antibodies are probably maintained for life (Papaevangelou and Halstead 1977, Guzman et al. 2000, Alvarez et al. 2006). In a secondary disease, the antibody response is characterised by a rapid rise of IgG antibodies, generally followed by a low level of IgM antibodies (Figure 6). In practice, serological diagnoses (HAI, ELISA, IFA) are in general less specific than virus isolation or RNA-detection because of extensive cross-reactions between flaviviruses and especially among the four dengue serotypes (Teles et al. 2005). Neutralisation tests (NT) are considered to be the most specific and sensitive serological assays, but a specific diagnosis is often only possible in primary disease. In subsequent flavivirus infections, antibodies with a greater affinity for the first flavivirus than for the current one are produced, a phenomenon called “original antigenic sin” (Halstead et al. 1983). Alternative tests to PCR and IgM assays for early detection of DF in single samples are the recently developed commercial (Bio-Rad Laboratories and PanBio Diagnostics) Dengue NS1 capture ELISAs. The NS1 protein is detectable as early as the first day of fever, and typically peaks around day 3-5 post onset, and subsides around day 9 (Bessoff et al. 2008, Figure 6). NS1 has also been shown to directly correlate with viremia in DENV-2 infected individuals, and moreover the NS1 levels have also been shown to be higher in patients with DHF.

Diagnoses by NS1 detection may be a valuable tool for prediction of disease severity (Libraty et al. 2002).

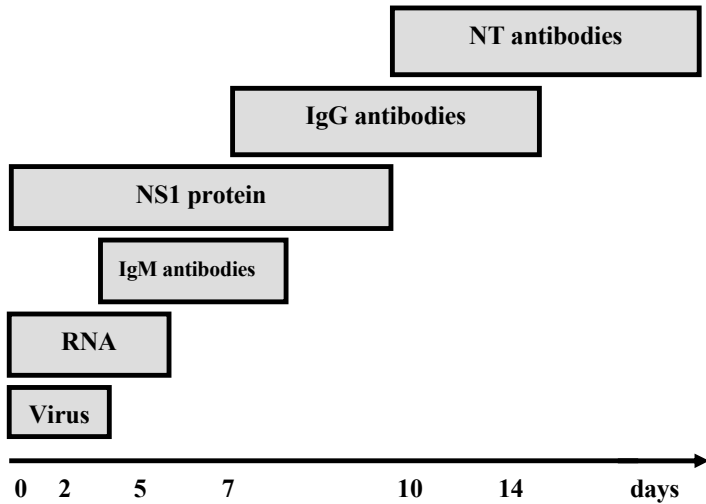


Figure 5. A schematic overview of the approximate different time points (days post onset of disease), for detection of virus, RNA and secreted NS1 protein, and for the beginning of antibody rise in a primary dengue infection.

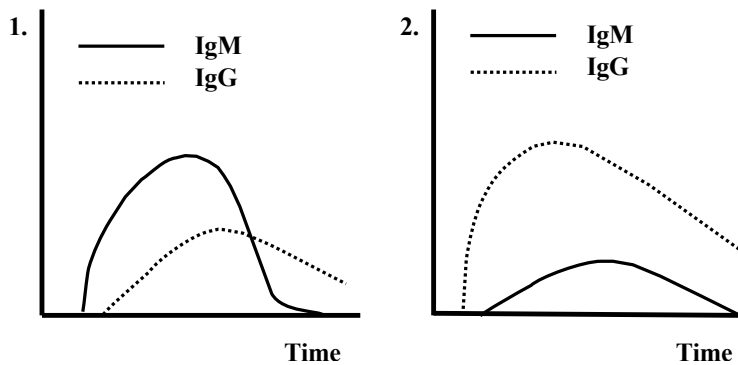


Figure 6. Different antibody profiles in a primary (1.) and in a secondary (2.) dengue virus infection.

10. Control and prevention

10.1. Vaccines

In spite of intensive research, the progress in vaccine development has been slow, and there are still no dengue vaccines sufficiently immunogenic for routine use available.

As there is no cross-protection between the four dengue serotypes, and because of the risk of ADE, a tetravalent vaccine most probably has to be developed to minimise the risk for severe disease and DHF. Monovalent lots of vaccines have shown adequate immunogenicities, but a combination into a tetravalent vaccine has failed so far.

(www.who.int/vaccine_research/diseases/vector/en/index.html).

10.2. Vector control

In the absence of vaccines and no specific drugs for treatment, the control of DF/DHF mainly depends on the control of the vector *Ae. Aegypti* (gwallior.nic.in):

- Personal protection: avoiding man-vector contact, by protective clothing, use of mosquito repellents, and the use of household insecticidal products.
- Environmental methods: by covering or emptying water storages and regular inspection and cleaning of air conditioners etc.
- Biological control: by use of larvivorous fish, and use of endotoxin-producing bacteria as effective mosquito control agents in large water containers.
- Chemical control: used in periods of high risk of outbreaks.

10.2.1. The effects of discontinued vector control in the Americas

After eradication of the *Ae. aegypti* mosquito from most of Central and South America epidemic dengue was rare in the 1950s, 1960s and most of the 1970s.

Twenty years after discontinuation of the eradication program the mosquito had almost reinvaded all the countries from which it had been eradicated and major epidemics of dengue followed. New dengue virus strains and serotypes were introduced and epidemic DHF emerged. A dramatic increase in incidence is in line with the previous situation in Southeast Asia (Gubler 1987).

AIMS

- The principal aim was to introduce adequate and reliable methods in the dengue diagnostics of Swedish travellers.
- The specific aim was to increase our knowledge of how to interpret the test results during the different phases of viremia and antibody responses.

MATERIALS AND METHODS

Paper I: Optimized diagnosis of acute dengue fever in Swedish travelers by a combination of reverse transcription-PCR and immunoglobulin M detection.

Serum samples. Acute serum samples, with dengue IgG negative (<10) or with low, (≤ 40) non-diagnostic (<320) IFA titres, from a total of 57 patients with a previously defined dengue infection were included in the study. Paired serum samples (acute and early convalescent) had previously (1997-2002) been analysed by indirect dengue IFA (Vene et al. 1995) in the routine diagnostics. A seroconversion or a significant (four-fold) rise in antibody titres had been verified in these samples. The majority of the patients were Swedish travellers returning from Southeast Asia (dengue endemic areas) with symptoms compatible with DF.

Dengue IgM ELISA. The samples (n=44) from 1997-2001 were retrospectively analysed, while the samples (n=13) from 2002 were consecutively tested using a commercial Dengue IgM Capture ELISA (PanBio Diagnostics). The test results were presented as negative (-), equivocal (\pm) or positive (+) dengue IgM.

Multiplex dengue RT-PCR. As controls for the presence of viral RNA, Vero cells were inoculated with dengue viruses (DENV-1, strain Hawaii; DENV-2, strain New Guinea C; DENV-3, strain H-87; DENV-4, strain H-241) and the infected supernatants, as well as the acute samples, were extracted either by manual or automatic methods. The extracted RNA was tested by a previously described RT-PCR (Harris et al. 1998). Briefly, five primers targeting the capsid gene (C) were included in the assay, resulting in different size RT-PCR products of the dengue serotypes (DENV-1, 482 bp; DENV-2, 119 bp; DENV-3, 290 bp; DENV-4, 389 bp).

Modified dengue TaqMan PCR. Viral RNA controls were prepared as previously described, except that DENV-1, strain Hawaii was replaced by strain West Pac. A previously published TaqMan PCR (i.e. four serotype-specific DENV-1 to DENV-4 PCRs) (Callahan et al. 2001) was modified by a random primer synthesis of cDNA of the viral and the patient RNA, by reduction of the reaction volumes, by selection of new DENV-2 primers and also by construction of dengue serotype-specific plasmid standards. The primer pairs for DENV-2, DENV-3 and DENV-4 were located in the C-gene while the DENV-1 primer pair was targeting the NS5 region in the genome. The samples were analysed in the four specific dengue TaqMan assays, positive reactions were plotted against the standard curves and the genome contents/ml serum were calculated.

Paper II: Detection of Dengue Serotype-Specific Antibodies in Swedish Travellers by Micro-Neutralisation Test (m-NT).

Serum samples. Early convalescent samples (1997-2002) complemented by late convalescent samples (2007) from a total of 20 dengue patients were analysed for the presence of serotype-specific neutralising antibodies. The patients had previously been serotyped by the multiplex RT-PCR and/or by the TaqMan PCRs in the acute samples in study I.

Dengue micro-neutralisation test (m-NT). A previously described micro-neutralisation test based on the 96-well ELISA format (Vorndam and Beltran 2002) was introduced after a few alterations, among these the introduction of an alternative substrate system and a novel cut-off value calculation. The early and the late convalescent samples were serially diluted and analysed in triplicate.

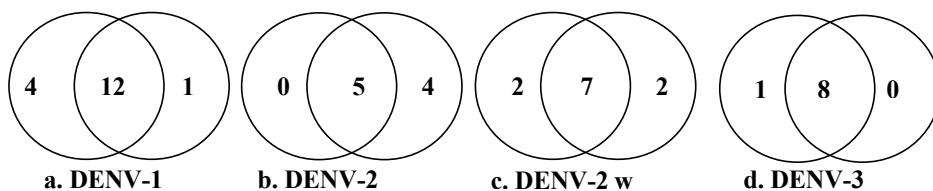
Dengue IgG IFA. The late convalescent samples were also analysed by the previously described dengue IgG IFA.

RESULTS AND DISCUSSION

Dengue tests are needed for diagnostics, case surveillance, epidemiology, and vaccine evaluation. Depending on the purpose, different specifications for the dengue tests must be fulfilled. For clinical use in non-endemic countries like Sweden, an early diagnosis, distinction between the serotypes and other flaviviruses are of high priority, while in dengue-endemic countries also a marker of severe disease and distinction between first and subsequent infections must also be added. In Sweden we receive several hundred patient serum samples annually from travellers returning from dengue endemic countries. Increasing travel has made it important to establish reliable diagnostic tools for identification of dengue viral infections, as well as discrimination of severe imported fevers of unknown origin. Today ELISA or rapid tests based on immuno chromatography (lateral flow assays) are the most common used methods for dengue IgM and IgG detection, and several commercial tests are available on the market. At Swedish Institute for Infectious Disease Control (SMI) a well documented indirect IFA IgG (Vene et al 1995) is used in the diagnostics, but as in most other serological dengue assays, cross reactions are frequent and thus antibodies to flaviviruses other than dengue may also be detected by the IFA. To perform reliable dengue diagnoses the patient's clinical symptoms and travel history, as well as the sampling data in relation to onset data always have to be considered together with the achieved IFA results. By the dengue IFA clinically significant titres (≥ 320) are not seen until at least 7 days post onset of disease, which have led to late diagnoses and under-diagnostics in case of early single samples. To optimise the dengue diagnostics, in the early phase of the disease as well as of past dengue infections, we found it necessary to introduce new methods as a complement to the indirect dengue IFA.

Results of the multiplex RT-PCR and the TaqMan PCRs. By the multiplex RT-PCR and/or by serotype-specific TaqMan PCRs dengue RNA of serotypes 1, 2 or 3 could be detected in 73% (35/48) of the samples collected until day 5 post onset of disease. The samples collected > 5 days post onset were all negative by the PCR assays, as well as all samples analysed in the routine diagnostics after the study (data not shown). Samples of dengue serotype 4 could not be detected in the study, or later by analyses routinely performed at SMI. Dengue 4 infections have been associated with hemorrhagic fever in secondary disease (Nisalak et al. 2003, Klungthong et al. 2004, Buchy et al. 2005), while most Swedish dengue patients have primary dengue infections. Comparison of the PCR results showed that 21% (10/48) of the samples yielded discrepant results, i.e. they were positive in only one of the two PCR systems. By the Multiplex PCR thirteen samples were of DENV-1 serotype, nine of DENV-2 serotype and eight of DENV-3 serotype as compared to the results of the TaqMan PCR, where sixteen samples were of DENV-1 serotype, five of DENV-2 serotype and nine of DENV-3 serotype. After construction of new DENV-2 wobbled primers, RNA could be detected in two additional samples. The DENV-1 TaqMan PCR was shown to be more sensitive as compared to the multiplex PCR, while the DENV-2 TaqMan PCR seemed to be more sensitive to sequence variations (Figures 7a-d). Due to the fact that the samples in the study originated from a period of several years, from travellers who had visited different geographical locations, significant strain variability could be expected. By the combination of the 2 different PCR systems the results were optimised, while a negative PCR did not exclude the presence of dengue RNA. The genomes/ml serum was also measured by quantitative PCR using serotype-specific plasmid standards. Quantification of RNA may serve as a disease severity marker, since a high viral load has been associated with DHF (Waughn et al 2000, Wang et al. 2002, Wang et al. 2006). The four dengue virus plasmids that were

produced showed almost identical detection limits (500 molecules/ml) and the efficiencies were almost 100%, i.e. a two-fold increase in generated DNA products in each PCR cycle. The number of genomes/ml in the acute samples varied between 1×10^3 and 5×10^8 , and the mean number of genomes/ml for each sampling day demonstrated a gradual decline over time. The mean number of genomes/ml in the samples collected 1997-2001 was 3×10^6 as compared to the mean of the samples from 2002 which was 8×10^7 . An additional explanation for the discrepant results in the TaqMan PCR may be the quality of the RNA, the samples collected 1997-2001 had been repeatedly freeze-thawed, while the samples from 2002 were consecutively analysed.



Figures 7a-d. Results of the dengue TaqMan PCRs are shown in the left circles and results by the multiplex RT-PCR are shown in the right circles. The overlapping area represents the common results of both PCR systems. In figure 7c the DENV-2 results are illustrated after re-analyses with wobbled (w) primers.

IgM results and comparison with the PCR results. IgM antibodies could be detected in 35% (20/57) of the acute phase samples by ELISA, and traces of IgM antibodies (equivocal test results) in an additional 5% (3/57). In the samples collected ≤ 5 days post onset IgM antibodies were detected in 17% (8/48) of the PCR negative samples and in 19% (9/48) of the PCR positive samples, IgM could not be detected in 54% (26/48) of the PCR positive samples and a total of 10% (5/48) of the samples were negative both by IgM ELISA and by the PCR assays, (Table 2). The majority of

the patients were IgM positive day 5 post onset of disease, but in some cases IgM antibodies were detected as early as day 2. The number of genomes/ml varied 1×10^4 – 6×10^6 in the IgM positive patient samples that also were quantified by TaqMan PCR.

Table 2. Dengue IgM and PCR results in 48 acute patient samples collected ≤ 5 days post onset.

	IgM positive	IgM negative	Total
PCR positive	19% (9/48)	54% (26/48)	73% (35/48)
PCR negative	17% (8/48)	10% (5/48)	27% (13/48)
Total	~36% (17/48)	~64% (31/48)	100% (48/48)

m-NT results. A serotype-specific m-NT-titre was defined as at least a four-fold homologous titre as compared to the titres against any of the other serotypes. Seventy percent reduction of the mean OD value (~ 1) of the negative serum control in each ELISA was selected as the cut-off value, and the reciprocal of the highest serum dilution below the cut-off value was set as the end-point m-NT titre. Serum dilutions that were partially neutralised, and resulted in OD values in the range between the cut-off value and the negative control, were considered to be negative, i.e. not neutralising. The correct serotype, as compared to previous PCR analyses was determined in a total of 80% (16/20) of the patients that were analysed by the m-NT. In the early convalescent sera, collected within 3 weeks post onset, serotype-specific neutralising antibodies could be detected in 30% (6/20) of the samples, as compared to 80% (16/20) in the late convalescent samples, collected five to ten years post onset. In four cases serotyping by the m-NT was not possible. One patient lacked detectable

neutralising antibodies in spite of a previous dengue analysis that had resulted in detection of DENV-1 RNA by PCR, detection of IgM antibodies by ELISA and a seroconversion determined by IgG IFA. The antibody pattern of another patient, previously found to be DENV-2 positive by PCR, indicated that it most probably was a secondary dengue infection, as equally high m-NT titres against DENV-2 and DENV-3 were found by the m-NT. Secondary infections are rare in Swedish patients, but have been described (Lindback et al. 2003, Wittesjo et al. 1993). In two patients the neutralising antibodies were not serotype-specific. Various periods of cross-protective immunity to one or several of the other serotypes occur after a dengue infection (Endy et al. 2004), and to determine at which time point the majority of the patients had produced dengue serotype-specific antibodies, additional samples collected within the first year post onset, would have been valuable. A great advantage to performing a NT by the ELISA micro-format is the objective interpretation of the results, as in plaque assays, a plaque over-lap phenomenon, depressing neutralising titres might occur. Also the reduction of the quantity of reagents in a micro assay is favourable.

Dengue IFA results in the convalescent samples. IFA titres in single samples equal to or exceeding 320 are considered to be clinically significant in the early phase of the disease, while past dengue infections usually result in low titres that are difficult to interpret. The late convalescent samples were analysed by dengue IFA, and the titres were distributed as follow: <80 in 5 patients, 80 in 9 patients, and >80 in 6 patients, while the titres in the early convalescent samples ranged between 160 and $\geq 10,240$ (Figure 8). We found no correlation between m-NT titres and the IFA titres, except for one patient who was negative in both assays. However, we conclude that titres ≥ 80 are most likely to be a sign of past dengue disease.

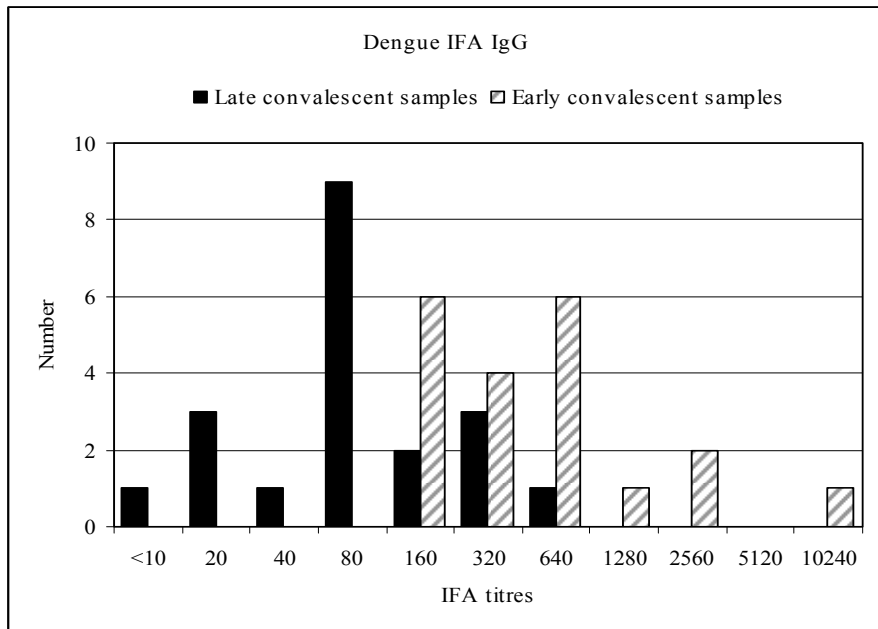


Figure 8. Dengue IFA IgG antibody distribution in the early and late convalescent samples of the 20 patients in the study.

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REFERENCES

- Alvarez, M., Rodriguez-Roche, R., Bernardo, L., Vázquez, S., Morier, L., Gonzalez, D., Castro, O., Kouri, G., Halstead, S.B., and Guzman, M.G. (2006). Dengue hemorrhagic fever caused by sequential dengue 1-3 virus infections over a long time interval: Havana epidemic, 2001-2002. *American Journal of Tropical Medicine and Hygiene* **75**(6), 1113-1117.
- Bancroft, T.L. (1906). On the aethology of dengue fever. *Australian Medical Gazette* **25**:17-18.
- Benedict, M. Q., Levine, R. S., Hawley, W. A., and Lounibos, L. P. (2007). Spread of the tiger: global risk of invasion by the mosquito *Aedes albopictus*. *Vector Borne and Zoonotic Diseases* **7**(1), 76-85.
- Bessoff, K., Delorey, M., Wellington, S., and Hunsperger, E. (2008). Comparison of 2 Commercially Available Dengue NS1 Capture Enzyme-Linked Immunosorbant Assays for Diagnosis of Acute DENV Infection with a Single Clinical Sample. *Clinical and Vaccine Immunology*, published online ahead of print on 6 August 2008.
- Buchy, P., Van Loung, V., Khanh Toan, B., Xuan Mai, T.T., Glaziou, P., Thu Ha, L.T., Viet Lo, L., and Trong Chien, B. (2005) Secondary dengue virus type 4 infections in Vietnam. *Southeast Asian Journal of Tropical Medicine Public Health* **36**(1):178-85.
- Burke, D. S., Nisalak, A., Johnson, D. E., and Scott, R. M. (1988). A prospective study of dengue infections in Bangkok. *American Journal of Tropical Medicine & Hygiene* **38**(1), 172-80.
- Busch, M. P., Wright, D. J., Custer, B., Tobler, L. H., Stramer, S. L., Kleinman, S. H., Prince, H. E., Bianco, C., Foster, G., Petersen, L. R., Nemo, G., and Glynn, S. A. (2006). West Nile virus infections projected from blood donor screening data, United States, 2003. *Emerging Infectious Diseases* **12**(3), 395-402.
- Calisher, C. H., and Gould, E. A. (2003). Taxonomy of the virus family Flaviviridae. *Advances in Virus Research* **59**, 1-19.
- Callahan, J. D., Wu, S. J., Dion-Schultz, A., Mangold, B. E., Peruski, L. F., Watts, D. M., Porter, K. R., Murphy, G. R., Suharyono, W., King, C. C., Hayes, C. G., and Temenak, J. J. (2001). Development and evaluation of serotype- and group-specific fluorogenic reverse transcriptase PCR (TaqMan) assays for dengue virus. *Journal of Clinical Microbiology* **39**(11), 4119-24.
- Carey, D. E. (1971). Chikungunya and dengue: a case of mistaken identity? *Journal of the History of Medicine and Allied Sciences* **26**(3), 243-62.
- CDC. (2002). West Nile Virus Activity -United States, October 10-16, 2002, and Update on West Nile Virus Infections in Recipients of Blood Transfusions. *MMWR Weekly*, **51**(41) 929-931.
- Chambers, T. J., Hahn, C. S., Galler, R., and Rice, C. M. (1990). Flavivirus genome organization, expression, and replication. *Annual Review of Microbiology* **44**, 649-88.
- Chen, W. J., Chen, S. L., Chien, L. J., Chen, C. C., King, C. C., Harn, M. R., Hwang, K. P., and Fang, J. H. (1996). Silent transmission of the dengue virus in southern Taiwan. *American Journal of Tropical Medicine and Hygiene* **55**(1), 12-6.

- Chuang, V.W.M., Wong, T.Y., Leung, Y.H., Law, Y.L., Tsang, O.T.Y., Chan, K.M., Tsang, I.H.L., Que, T.L., Yung, R.W.H., Liu, S.H. (2008). Review of dengue fever cases in Hong Kong during 1998 to 2005. *Hong Kong Medical Journal* **14**, 170-7.
- Chye, J. K., Lim, C. T., Ng, K. B., Lim, J. M., George, R., and Lam, S. K. (1997). Vertical transmission of dengue. *Clinical Infectious Diseases* **25**(6), 1374-7.
- Endy, T. P., Chunsuttiwat, S., Nisalak, A., Libraty, D. H., Green, S., Rothman, A. L., Vaughn, D. W., and Ennis, F. A. (2002). Epidemiology of inapparent and symptomatic acute dengue virus infection: a prospective study of primary school children in Kamphaeng Phet, Thailand. *American Journal of Epidemiology* **156**(1), 40-51.
- Endy, T. P., Nisalak, A., Chunsuttiwat, S., Vaughn, D. W., Green, S., Ennis, F. A., Rothman, A. L., and Libraty, D. H. (2004). Relationship of preexisting dengue virus (DV) neutralizing antibody levels to viremia and severity of disease in a prospective cohort study of DV infection in Thailand. *Journal of Infectious Diseases* **189**(6), 990-1000.
- Etymologia. (2006) *Emerging Infectious Diseases*, www.cdc.gov/eid. **12**(6), 893.
- Gibbons, R.V., Kalanarooj, S., Jarman, R.G., Nisalak, A., Vaughn, D.W., Endy, T. P., Mammen Jr, M.P., and Srikiatkachorn. (2007). Analysis of Repeat Hospital Admissions for Dengue to Estimate the Frequency of Third or Fourth Dengue Infections Resulting in Admissions and Dengue Hemorrhagic Fever, and Serotype Sequences. *American Journal of Medicine and Hygiene.*, **77**(5), 910-913.
- Gould, E. A., and Solomon, T. (2008). Pathogenic flaviviruses. *Lancet* **371**(9611), 500-9.
- Gubler, D. J. (1987). Dengue and dengue hemorrhagic fever in the Americas. *Puerto Rico Health Sciences Journal* **6**(2), 107-11.
- Gubler, D. J. (1998). Dengue and dengue hemorrhagic fever. *Clinical Microbiology Reviews* **11**(3), 480-96.
- Guzman, M. G., and Kouri, G. (2003). Dengue and dengue hemorrhagic fever in the Americas: lessons and challenges. *Journal of Clinical Virology* **27**(1), 1-13.
- Guzman, M. G., and Kouri, G. (2004). Dengue diagnosis, advances and challenges. *International Journal of Infectious Diseases* **8**(2), 69-80.
- Guzman, M. G., Kouri, G., Valdes, L., Bravo, J., Alvarez, M., Vazques, S., Delgado, I., and Halstead, S. B. (2000). Epidemiologic studies on Dengue in Santiago de Cuba, 1997.[see comment]. *American Journal of Epidemiology* **152**(9), 793-9; discussion 804.
- gwallior.nic.in/PREVENTION_AND_CONTROL_DENGU.pdf. Prevention and Control of Dengue and Dengue Haemorrhagic Fever
- Halstead, S. B., Rojanasuphot, S., and Sangkawibha, N. (1983). Original antigenic sin in dengue. *American Journal of Tropical Medicine and Hygiene* **32**(1), 154-6.
- Harris, E., Roberts, T. G., Smith, L., Selle, J., Kramer, L. D., Valle, S., Sandoval, E., and Balmaseda, A. (1998). Typing of dengue viruses in clinical specimens and mosquitoes by single-tube multiplex reverse transcriptase PCR. *Journal of Clinical Microbiology* **36**(9), 2634-9.
- Harris, W. H., and Duwal, C.W. (1924). Studies upon the etiology of dengue fever. 817-833. www.jem.org.

- Henchal, E. A., and Putnak, J. R. (1990). The dengue viruses. *Clinical Microbiology Reviews* **3**(4), 376-96.
- Holmes, E. C., and Twiddy, S. S. (2003). The origin, emergence and evolutionary genetics of dengue virus. *Infection, Genetics & Evolution* **3**(1), 19-28.
- Holmes, E. C. (2007) The evolution and epidemiology of dengue virus. *Salud Pública de México* **49**, 296-300.
- Huhtamo, E., Vuorinen, S., Uzcategui, N. Y., Vapalahti, O., Haapasalo, H., and Lumio, J. (2006). Fatal dengue virus infection in a Finnish traveler. *Journal of Clinical Virology* **37**(4), 323-6.
- Jelinek, T., Muhlberger, N., Harms, G., Corachan, M., Grobusch, M. P., Knobloch, J., Bronner, U., Laferl, H., Kapaun, A., Bisoffi, Z., Clerinx, J., Puente, S., Fry, G., Schulze, M., Hellgren, U., Gjorup, I., Chalupa, P., Hatz, C., Matteelli, A., Schmid, M., Nielsen, L. N., da Cunha, S., Atouguia, J., Myrvang, B., Fleischer, K., and European Network on Imported Infectious Disease, S. (2002). Epidemiology and clinical features of imported dengue fever in Europe: sentinel surveillance data from TropNetEurop. *Clinical Infectious Diseases* **35**(9), 1047-52.
- Jensenius, M., Berild, D., Ormaasen, V., Maehlen, J., Lindegren, G., and Falk, K. I. (2007). Fatal subarachnoidal haemorrhage in a Norwegian traveller with dengue virus infection. *Scandinavian Journal of Infectious Diseases* **39**(3), 272-4.
- Kao, C. L., King, C. C., Chao, D. Y., Wu, H. L., and Chang, G. J. (2005). Laboratory diagnosis of dengue virus infection: current and future perspectives in clinical diagnosis and public health. *Journal of Microbiology, Immunology & Infection* **38**(1), 5-16.
- Kinney, R. M., Butrapet, S., Chang, G. J., Tsuchiya, K. R., Roehrig, J. T., Bhamarapavati, N., and Gubler, D. J. (1997). Construction of infectious cDNA clones for dengue 2 virus: strain 16681 and its attenuated vaccine derivative, strain PDK-53. *Virology* **230**(2), 300-8.
- Klungthong, C., Zhang, C., Mammen, M. P., Jr., Ubol, S., and Holmes, E. C. (2004). The molecular epidemiology of dengue virus serotype 4 in Bangkok, Thailand. *Virology* **329**(1), 168-79.
- Laferl, H., Szell, M., Bischof, E., Wenisch, C. (2006). Imported dengue fever in Austria 1990-2005. *Travel Medicine and Infectious Disease*, **4**, 319-323.
- Lai, C-J, Putnak, R. (2007). Dengue and the Dengue Viruses. *Emerging Viruses in Human Populations, Edward Tabor (Editor)*, 269-298.
- Lee, H.L., and Rohani, A. (2005). Transovarial Transmission of Dengue Virus in *Aedes aegypti* and *Aedes albopictus* in Relation to Dengue Outbreak in an Urban Area in Malaysia. *Dengue Bulletin* **29**, 106-110.
- Libraty, D. H., Young, P. R., Pickering, D., Endy, T. P., Kalayanarooj, S., Green, S., Vaughn, D. W., Nisalak, A., Ennis, F. A., and Rothman, A. L. (2002). High circulating levels of the dengue virus nonstructural protein NS1 early in dengue illness correlate with the development of dengue hemorrhagic fever. *Journal of Infectious Diseases* **186**(8), 1165-8.
- Lindback, H., Lindback, J., Tegnell, A., Janzon, R., Vene, S., and Ekdahl, K. (2003). Dengue fever in travelers to the tropics, 1998 and 1999. *Emerging Infectious Diseases* **9**(4), 438-42.

- Linnen, J.M., Vinelli, E., Sabino, E.C., Tobler, L.H., Hyland, C., Lee, T-H., Kolk, D.P., Broulik, A.S., Collins, C.S., Lanciotti, R.S., and Busch, M.P. (2008). Dengue viremia in blood donors from Honduras, Brazil, and Australia. *Transfusion* **48**, 1355-1362.
- Malavige, G. N., Fernando, S., Fernando, D. J., and Seneviratne, S. L. (2004). Dengue viral infections. *Postgraduate Medical Journal* **80**(948), 588-601.
- McKee, K. T., Jr., Bancroft, W. H., Eckels, K. H., Redfield, R. R., Summers, P. L., and Russell, P. K. (1987). Lack of attenuation of a candidate dengue 1 vaccine (45AZ5) in human volunteers. *American Journal of Tropical Medicine and Hygiene* **36**(2), 435-42.
- Mohammed, H., Linnen, J.M., Muñoz-Jordán, J.L., Tomashek, K., Foster, G., Broulik, A.S., Petersen, L., Stramer, S.L. (2008). Dengue virus in blood donations, Puerto Rico, 2005. *Transfusion* **48**, 1348-1354.
- Nisalak, A., Endy, T. P., Nimmannitya, S., Kalayanarooj, S., Thisyakorn, U., Scott, R. M., Burke, D. S., Hoke, C. H., Innis, B. L., and Vaughn, D. W. (2003). Serotype-specific dengue virus circulation and dengue disease in Bangkok, Thailand from 1973 to 1999. *American Journal of Tropical Medicine and Hygiene* **68**(2), 191-202.
- O'Brien, D., Tobin, S., Brown, G. V., and Torresi, J. (2001). Fever in returned travelers: review of hospital admissions for a 3-year period. *Clinical Infectious Diseases* **33**(5), 603-9.
- Papaevangelou, G. and Halstead, H.B. (1977) Infections with two dengue viruses in Greece in the 20th century. Did dengue hemorrhagic fever occur in the 1928 epidemic? *Journal of Tropical Medicine* **80**, 46-51.
- Petdachai, W., Sila'on, J., Nimmannitya, S., and Nisalak, A. (2004). Neonatal dengue infection: report of dengue fever in a 1-day-old infant. *Southeast Asian Journal of Tropical Medicine and Public Health* **35**(2), 403-7.
- Peyrefitte, C. N., Couissinier-Paris, P., Mercier-Perennec, V., Bessaud, M., Martial, J., Kenane, N., Durand, J. P., and Tolou, H. J. (2003). Genetic characterization of newly reintroduced dengue virus type 3 in Martinique (French West Indies). *Journal of Clinical Microbiology* **41**(11), 5195-8.
- Platt, K. B., Linthicum, K. J., Myint, K. S., Innis, B. L., Lerdthusnee, K., and Vaughn, D. W. (1997). Impact of dengue virus infection on feeding behavior of *Aedes aegypti*. *American Journal of Tropical Medicine and Hygiene* **57**(2), 119-25.
- Poblap, T., Nitatpattana, N., Chaimarin, A., Barbazan, P., Chauvancy, G., Yoksan, S., and Gonzalez, J. P. (2006). Silent transmission of virus during a Dengue epidemic, Nakhon Pathom Province, Thailand 2001. *Southeast Asian Journal of Tropical Medicine and Public Health* **37**(5), 899-903.
- Sabin, A. B. (1952). Research on dengue during World War II. *American Journal of Tropical Medicine and Hygiene* **1**(1), 30-50.
- Stephenson, I., Roper, J., Fraser, M., Nicholson, K., Wiselka, M. (2003). *Travel Medicine and Infectious Disease* **1**, 89-93.
- Tan, F. L., Loh, D. L., Prabhakaran, K., Tambyah, P. A., and Yap, H. K. (2005). Dengue haemorrhagic fever after living donor renal transplantation.[erratum appears in *Nephrol Dial Transplant*. 2005 Jun;20(6):1281 Note: Tambyah, Paul Ananth [added]; Yap, Hui-Kim [added]]. *Nephrology Dialysis Transplantation* **20**(2), 447-8.

- Teles, F.R.R., Prazeres, D.M.F., and Lima-Filho, J.L. (2005) Trends in dengue diagnosis. *Reviews in Medical Virology* **15**, 287-302.
- Wagner, D., de With, K., Huzly, D., Hufert, F., Weidmann, M., Breisinger, S., Eppinger, S., Kern, W. V., and Bauer, T. M. (2004). Nosocomial acquisition of dengue.[see comment]. *Emerging Infectious Diseases* **10**(10), 1872-3.
- Vasilakis, N., Holmes, E. C., Fokam, E. B., Faye, O., Diallo, M., Sall, A. A., and Weaver, S. C. (2007). Evolutionary processes among sylvatic dengue type 2 viruses. *Journal of Virology* **81**(17), 9591-5.
- Wang, W-K., Chao, D-Y., Kao, C-L., Wu, H-C., Liu, Y-C., Li, C-M., Lin, S-C., Ho S-T., Huang, J-H., and King, C-C. (2002). High Levels of Plasma Dengue Viral Load during Defervescence in Patients with Dengue Hemorrhagic Fever: Implications for Pathogenesis. *Virology* **305**, 330-338.
- Wang, W-K., Chen, H-L., Yang, C-F., Hsieh, S-C., Juan, C-C., Chang, S-M., Yu, C-C., Lin, L-H., Huang, J-H., and King, C-C. (2006). Slower Rates of Clearance of Viral Load and Virus-Containing Immune Complexes in Patients with Dengue Hemorrhagic Fever. *Clinical Infectious Diseases* **43**, 1023-30.
- Waughn, D.W., Green, S., Kalayanaraj, S., Innis, B.L., Nimmanitya, S., Suntayakorn, S., Rothman, A.L., Ennis, F.A., and Nisalak, A. (1997). Dengue in the Early Febrile Phase: Viremia and Antibody Responses. *The Journal of Infectious Diseases* **176**, 322-30.
- Waughn, D.W., Green, S., Kalayanaraj, S., Innis, B.L., Nimmanitya, S., Suntayakorn, S., Endy, T.P., Raengsakulrach, B., Rothman, A.L., Ennis, F.A., and Nisalak, A. (2000) Dengue Viremia Titer, Antibody Response Pattern, and Virus Serotype Correlate with Disease Severity. *The Journal of Infectious Diseases* **181**, 2-9.
- Weaver, S. C., and Barrett, A. D. (2004). Transmission cycles, host range, evolution and emergence of arboviral disease. *Nature Reviews Microbiology* **2**(10), 789-801.
- Vene, S., Mangiafico, J., and Niklasson, B. (1995). Indirect immunofluorescence for serological diagnosis of dengue virus infections in Swedish patients. *Clinical and Diagnostic Virology* **4**, 43-50.
- Wichmann, O., Mühlberger, N., and Jelinek, T. (2003). Dengue – The Underestimated Risk in Travellers. *Dengue Bulletin* **27**, 126-136.
- Wichmann, O., Gascon, J., Schunk, M., Puente, S., Siikamaki, H., Gjorup, I., Lopez-Velez, R., Clerinx, J., Peyerl-Hoffmann, G., Sundoy, A., Genton, B., Kern, P., Calleri, G., de Gorgolas, M., Muhlberger, N., Jelinek, T., and European Network on Surveillance of Imported Infectious, D. (2007). Severe dengue virus infection in travelers: risk factors and laboratory indicators.[see comment]. *Journal of Infectious Diseases* **195**(8), 1089-96.
- Wilder-Smith, A., and Schwartz, E. (2005). Dengue in travelers.[see comment]. *New England Journal of Medicine* **353**(9), 924-32.
- Wittesjo, B., Eitrem, R., and Niklasson, B. (1993). Dengue fever among Swedish tourists. *Scandinavian Journal of Infectious Diseases* **25**(6), 699-704.
- Vorndam, V., and Beltran, M. (2002). Enzyme-linked immunosorbent assay-format microneutralization test for dengue viruses. *American Journal of Tropical Medicine & Hygiene* **66**(2), 208-12.
- www.cdc.gov/ncidod/dvbid/dengue/index.htm
- www.cdc.gov/ncidod/dvbid/dengue/slideset
- www.ncbi.nlm.nih.gov/sites/entrez

www.who.int/tdr/diseases/dengue/viral_rep.htm

www.who.int/csr/resources/publications/dengue/012-23.pdf. *Clinical diagnosis*

www.who.int/vaccine_research/diseases/vector/en/index.html