Dengue virus infection:
Diagnostics and molecular epidemiology

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Nothing in biology makes sense except in the light of evolution.

-Theodosius Dobzhansky
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<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arbovirus</td>
<td>arthropod-borne virus</td>
</tr>
<tr>
<td>ATCC</td>
<td>American type culture collection</td>
</tr>
<tr>
<td>BHQ1</td>
<td>black hole quencher 1</td>
</tr>
<tr>
<td>BLAST</td>
<td>basic local alignment tool</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CPE</td>
<td>cytopathic effect</td>
</tr>
<tr>
<td>ct</td>
<td>cycle threshold</td>
</tr>
<tr>
<td>DENV</td>
<td>dengue virus</td>
</tr>
<tr>
<td>DF</td>
<td>dengue fever</td>
</tr>
<tr>
<td>DHF</td>
<td>dengue haemorrhagic fever</td>
</tr>
<tr>
<td>DSS</td>
<td>dengue shock syndrome</td>
</tr>
<tr>
<td>dsDNA</td>
<td>double-stranded DNA</td>
</tr>
<tr>
<td>EIA</td>
<td>enzyme immunoassay</td>
</tr>
<tr>
<td>FAM</td>
<td>5 carboxy-fluorescein</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>IFA</td>
<td>immunofluorescence assay</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>IgM</td>
<td>immunoglobulin M</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>MAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>MEM</td>
<td>minimal essential medium</td>
</tr>
<tr>
<td>NS1</td>
<td>non-structural protein 1</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>preM</td>
<td>premembrane protein</td>
</tr>
<tr>
<td>PRNT</td>
<td>plaque reduction neutralization test</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute medium</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcription</td>
</tr>
<tr>
<td>Pfu</td>
<td>plaque forming unit</td>
</tr>
</tbody>
</table>
Abstract

Dengue is a mosquito-borne viral disease caused by the four dengue virus serotypes (DENV-1-4) and is currently considered as the most important arthropod-borne viral disease in the world. Nearly half of the human population lives in risk areas, and 50-100 million infections occur yearly according to the estimates by the World Health Organization. The disease can vary from a mild febrile disease to severe haemorrhagic fever and shock. A secondary infection with heterologous serotype increases the risk for severe disease outcome. During the last three decades the impact of dengue has dramatically increased in the endemic areas including the tropics and subtropics of the world. The current situation with massive epidemics of severe disease forms in urban environments has been associated with socio-ecological changes that have increased the transmission and enabled the co-circulation of different serotypes. Consequently, an increase of dengue has also been observed in travelers visiting these areas.

In travelers dengue is rarely a life threatening disease, however disease outcomes considered atypical have been reported. In the current study, a fatality caused by a brain hemorrhage was documented in a young Finnish patient experiencing a prolonged primary dengue infection. The increasing importance of dengue also triggered other studies that are compiled in this thesis. The aims were to provide information of dengue virus strains through virus characterization and molecular epidemiological studies of dengue virus isolates from Finnish travelers and from patients from an endemic country, Venezuela, and to develop a novel diagnostic tool for the detection of the DENV genome from patient samples. To accomplish these objectives classic virological methods including virus isolation and different antibody-based assays were used, in addition to antigen detection, RT-PCR-based methods, sequencing and sequence analysis.

For improving the laboratory diagnostics of dengue from early time-point patient samples, a novel real-time RT-PCR method was developed for the detection of DENV-1-4 RNA based on TaqMan chemistry. The method was shown
to be sensitive and specific for detecting DENV RNA and suitable for diagnostic use. The newly developed real-time RT-PCR was compared to other available early diagnostic methods including IgM and NS1 antigen detection using a panel of selected patient samples. The results suggest that the best diagnostic rates are achieved by a combination of IgM with RNA or NS1 detection.

The dengue virus strains studied here included 11 DENV strains isolated from serum samples of Finnish travelers collected in 2000-2005. The results of sequence analysis demonstrated that the isolates presented a global sample of DENV strains from different areas including Asia, Africa and South America. The strains included all four dengue virus serotypes and several genotypes, which manifested as dengue fever in Finnish travelers. In the present study sequence analysis was also carried out for a collection of 23 novel DENV-2 isolates from Venezuelan patients in 1999-2005. A global sample of different DENV-2 genotypes and all the available DENV-2 sequences from Venezuela until 2008 were included in the analysis. The results showed that unlike the pattern of several other countries in the region, Venezuelan DENV-2 exclusively represented the American-Asian genotype, suggesting that no foreign DENV-2 lineages have recently been introduced to the country. The results suggest that the DENV-2 viruses have been maintained in the area where they have evolved into several phylogenetic groups with characteristic amino-acid changes in their envelope genes.

Studying the characteristics of the circulating dengue viruses in the endemic areas is important for surveillance of circulating strains and their involvement in local epidemics. The active surveillance is not currently performed in many countries mainly because of the lack of financial resources. Infected people are playing a major role in introducing dengue virus to novel areas, but also provide a way to study these viruses from endemic areas. To our knowledge, the DENV-2 isolate from a Finnish traveler returning from Ghana, Africa, was the first documentation of dengue in the country, demonstrating the potential of travelers as sentinels for dengue in the areas lacking surveillance. The up-to-date information of circulating DENV strains combined to the available epidemiological information aids in better understanding of the nature of the epidemics in the endemic areas.
1. Review of the literature

1.1 Preface

Viruses are extremely small biological agents that share several properties with cellular life. Viruses reproduce and have genomes that undergo evolution enabling them to adapt to changes in their environment. Viruses are obligatory intracellular parasites that use the host cell machineries, components and energy for production of progeny virions. Viruses have co-evolved closely with their host organisms, and all the three domains of life, Eukarya, Prokarya and Archaea are parasitized by their specific viruses (1).

1.2 Introduction to flaviviruses

Dengue virus belongs to the Flaviviridae family, genus Flavivirus. The virus family Flaviviridae consists of small (40-60 nm in diameter) enveloped animal viruses that have a genome of a single molecule of positive sense RNA. The family is divided into three genera: Hepacivirus, Pestivirus and Flavivirus. Only the genus Flavivirus includes arthropod-borne members (arboviruses) (2). Arboviruses demonstrate biological flexibility that enables them to infect and replicate in different host species, in arthropod and vertebrate cells (3). This strategy has been advantageous by ensuring effective transmission, spreading, survival and maintenance of arboviruses in nature.
1. Review of the literature

1.2.1. Taxonomic classification of the Genus Flavivirus

The name of the genus *Flavivirus* (referred to as flaviviruses) comes from *flavus*, the Latin word for yellow. The type species of the genus is Yellow fever virus and currently nearly 70 flavivirus species are known. Flaviviruses can be categorized based on their vector associations; viruses transmitted by mosquitoes (39 species), viruses transmitted by ticks (13 species) and viruses for which no arthropod vector has been identified, “no known vector viruses” (16 species) (1). In addition, several tentative species have been associated with the genus, including genetically divergent lineages isolated from bats, named Tamana bat virus (TABV) (4) and from mosquito cells, named Cell fusing Agent virus (CAV) (5). Flaviviruses can be antigenically classified into 8 complexes that correlate to the differences observed in the life cycles and host range (6). These include 2 separate antigenic groups of tick-borne viruses, associated with rodent or seabird hosts. The 4 clusters of mosquito-borne viruses include one antigenic group of viruses associated with birds and *Culex* spp. mosquito vectors and 3 groups of viruses associated with mammal hosts and *Aedes* spp. vectors. The no-known vector viruses have been divided into 2 antigenic groups, both including viruses isolated from bats and rodents (7). The host, vector and antigenic classifications also resemble the grouping of these viruses in phylogenetic trees (8-11) (Figure 1). However, for some viruses phylogenetically associated with mosquito-borne viruses there are no vectors currently known (Entebbe bat virus, Yokose virus) and it has been hypothesized that they have lost their ability to be vector borne (9).
Figure 1. Neighbor-joining phylogenetic tree based on complete open reading frame nucleotide sequences of 31 flavivirus species including representatives of mosquito-borne, tick-borne and no known vector viruses.* Currently no vectors are known for Entebbe bat and Yokose viruses despite their phylogenetic grouping among mosquito-borne flaviviruses. 1000 bootstrap replications were conducted, the support values above 70% are indicated. Bar represents nucleotide substitutions/site. Virus species: Japanese encephalitis virus (JEV), Usutu virus (USUV), Alfuy virus (ALFV), West Nile virus (WNV), St Louis encephalitis virus (SLEV), Rocio virus (ROCV), Bagaza virus (BAGV), Kokobera virus (KOKV), Bussaquara virus (BSQV), Iguape virus (IGUV), Kedougou virus (KEDV), Zika virus (ZIKV), dengue virus (DENV), Entebbe bat virus (ENTV), Yokose virus (YOKV), Sepik virus (SEPV), yellow fever virus (YFV), Rio Bravo virus (RBV), Montana myotis leukoencephalitis virus (MMLV), Modoc virus (MODV), Apoi virus (APOIV), Meaban virus (MEAV), Tyuleni virus (TYUV), Kyasanur forest disease virus (KFDV), Langat virus (LGTV), Louping ill virus (LIV), Tick-borne encephalitis virus (TBEV) and Cell fusing agent virus (CFAV).
1.2.2 Flaviviruses as human pathogens

In flavivirus infections, the clinically ill patients represent “the tip of the iceberg” as most infections lead to subclinical infection or to mild disease with unspecific flu-like symptoms. The clinical symptoms caused by flaviviruses include fever and rash, which are also associated with other arboviral diseases. Currently, at least 29 species of flaviviruses are associated with human disease (Table 1) (3,12). The tick-borne flaviviruses are known to cause both encephalitic and haemorrhagic disease. However, for the mosquito-borne flaviviruses disease associations seem to correlate with their vector mosquito species. The mosquito-borne flaviviruses transmitted by Culex spp. mosquitoes, such as West Nile virus, are generally associated with the encephalitic type of disease whereas the Aedes spp. transmitted viruses include causative agents for febrile diseases with haemorrhagic symptoms such as dengue and yellow fever viruses (8).

In terms of global disease burden the most important flaviviral disease is dengue, which is caused by four closely related mosquito-borne dengue viruses (DENV-1-4) that account for an estimated 50-100 million infections each year in the tropics and subtropics of the world. The other important mosquito-borne flaviviruses include Yellow fever virus (YFV) (endemic in South America and Africa), causing severe hepatitis and haemorrhagic disease (13) and Japanese encephalitis virus (JEV), the leading cause of viral encephalitis in Asia (14). Additionally, West Nile virus (WNV) is a mosquito-borne flavivirus that has spread from its origins in Africa to Europe (15,16) and Asia and recently also to the New World (17,18) causing febrile or encephalitic disease (19). Another flavivirus related to WNV, Usutu (USUV), has also spread from Africa to Europe (20) although there is limited information on the pathogenicity to humans (21).

Tick-borne encephalitis virus (TBEV) is endemic in a large area spanning from Europe to Asia, causing encephalitic disease (22). Vaccines against flavivirus infections are currently available against YFV, JEV and TBEV (12). Patients usually recover fully from flavivirus infections with mild symptoms, but permanent
paralysis, prolonged neurological symptoms and persistent infections have also been reported from infections caused by TBEV (23), WNV (24) and JEV (25).

Table 1. Flaviviruses causing human disease; globally most important viruses are marked in bold (Modified from Gould & Solomon, 2008) (12).

<table>
<thead>
<tr>
<th>Flavivirus</th>
<th>Vector association</th>
<th>Animal host association</th>
<th>Geographical distribution</th>
<th>Human disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkhurma</td>
<td>Tick</td>
<td>Human, sheep, camels</td>
<td>Arabian Peninsula</td>
<td>Haemorrhagic fever</td>
</tr>
<tr>
<td>Kyasanur Forest Disease</td>
<td>Tick</td>
<td>Monkeys</td>
<td>India</td>
<td>Haemorrhagic fever</td>
</tr>
<tr>
<td>Langat</td>
<td>Tick</td>
<td>Unknown</td>
<td>Malaysia, Thailand, Siberia</td>
<td>Encephalitis</td>
</tr>
<tr>
<td>Louping ill</td>
<td>Tick</td>
<td>Sheep, grouse, hares</td>
<td>UK, Ireland</td>
<td>Encephalitis</td>
</tr>
<tr>
<td>Omsk haemorrhagic fever</td>
<td>Tick</td>
<td>Muskrats, rodents?</td>
<td>Western Siberia</td>
<td>Haemorrhagic fever</td>
</tr>
<tr>
<td>Powassan</td>
<td>Tick</td>
<td>Small mammals</td>
<td>Russia, USA, Canada</td>
<td>Encephalitis</td>
</tr>
<tr>
<td>Tick-borne encephalitis</td>
<td>Tick</td>
<td>Rodents</td>
<td>Europe, Asia</td>
<td>Encephalitis</td>
</tr>
<tr>
<td>Bagaza</td>
<td>Mosquito (Culex spp.)</td>
<td>Unknown</td>
<td>Africa</td>
<td>Fever</td>
</tr>
<tr>
<td>Banzi</td>
<td>Mosquito (Culex spp.)</td>
<td>Unknown</td>
<td>Africa</td>
<td>Fever</td>
</tr>
<tr>
<td>Bussuquara</td>
<td>Mosquito (Culex spp.)</td>
<td>Unknown</td>
<td>Brazil</td>
<td>Fever</td>
</tr>
<tr>
<td>Ilheus</td>
<td>Mosquito (Culex spp.)</td>
<td>Birds</td>
<td>South and Central Africa</td>
<td>Fever</td>
</tr>
<tr>
<td>Japanese encephalitis</td>
<td>Mosquito (Culex trita eniorhynchus)</td>
<td>Birds, pigs</td>
<td>Asia</td>
<td>Encephalitis</td>
</tr>
<tr>
<td>Murray Valley encephalitis</td>
<td>Mosquito (Culex annulirostris)</td>
<td>Birds</td>
<td>Australia</td>
<td>Encephalitis</td>
</tr>
<tr>
<td>Rocio</td>
<td>Mosquito (Culex spp.)</td>
<td>Birds</td>
<td>Brazil</td>
<td>Encephalitis</td>
</tr>
<tr>
<td>St Louis encephalitis</td>
<td>Mosquito (Culex spp.)</td>
<td>Birds</td>
<td>USA, South and Central America</td>
<td>Encephalitis</td>
</tr>
<tr>
<td>West Nile</td>
<td>Mosquito (mainly Culex spp.) and ticks</td>
<td>Birds, horses</td>
<td>Worldwide?</td>
<td>Encephalitis</td>
</tr>
<tr>
<td>Usutu virus</td>
<td>Mosquito (Culex spp.)</td>
<td>Birds</td>
<td>Africa, Central Europe</td>
<td>Fever, rash</td>
</tr>
<tr>
<td>Disease</td>
<td>Vector</td>
<td>Hosts</td>
<td>Affected Areas</td>
<td>Symptoms</td>
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<tr>
<td>--------------</td>
<td>----------------------------</td>
<td>--------------------------------------------</td>
<td>---------------------------</td>
<td>----------------------------</td>
</tr>
<tr>
<td>Koutango</td>
<td>Unknown</td>
<td>Rodents?</td>
<td>Senegal</td>
<td>Fever, rash</td>
</tr>
<tr>
<td>Ntaya</td>
<td>Mosquito</td>
<td>Unknown</td>
<td>Africa,</td>
<td>Fever</td>
</tr>
<tr>
<td>Dengue 1-4</td>
<td>Mosquito (Aedes spp.)</td>
<td>Human, monkeys</td>
<td>Tropics and subtropics</td>
<td>Fever, haemorrhagic fever</td>
</tr>
<tr>
<td>Yellow fever</td>
<td>Mosquito (Aedes spp., Haemagogus spp.)</td>
<td>Human, monkeys</td>
<td>Africa, South America</td>
<td>Haemorrhagic fever, hepatitis</td>
</tr>
<tr>
<td>Sepik</td>
<td>Mosquito</td>
<td>Unknown</td>
<td>New Guinea</td>
<td>fever</td>
</tr>
<tr>
<td>Zika</td>
<td>Mosquito (Aedes spp.)</td>
<td>Monkeys?</td>
<td>Africa, Asia</td>
<td>Fever, rash</td>
</tr>
<tr>
<td>Wesselsbron</td>
<td>Mosquito (Aedes spp.)</td>
<td>Unknown</td>
<td>Africa, Asia</td>
<td>Fever?</td>
</tr>
<tr>
<td>Spondweni</td>
<td>Mosquito (Aedes circumluteolus)</td>
<td>Unknown</td>
<td>Africa</td>
<td>Fever</td>
</tr>
<tr>
<td>Modoc</td>
<td>Unknown</td>
<td>Rodent?</td>
<td>USA</td>
<td>Encephalitis</td>
</tr>
<tr>
<td>Dakar bat</td>
<td>Unknown</td>
<td>Bats?</td>
<td>Africa</td>
<td>Fever</td>
</tr>
<tr>
<td>Rio Bravo</td>
<td>Unknown</td>
<td>Bats</td>
<td>USA, Mexico</td>
<td>Fever</td>
</tr>
<tr>
<td>Apoi</td>
<td>Unknown</td>
<td>Rodents?</td>
<td>Japan</td>
<td>Encephalitis</td>
</tr>
</tbody>
</table>
1.3 Dengue virus

A brief history of dengue

The name for dengue disease comes from the Swahili language, meaning a disease caused by an evil spirit. Although dengue-like illness was reported from China nearly 1000 year ago, the first major epidemics occurred in the 17th century in different parts of the world. The global shipping trade most likely mediated the introduction and spreading of the principal vector of dengue, an African origin mosquito Aedes aegypti, and also dengue viruses. During the early 1900s dengue was shown to be a mosquito-borne filterable agent. Dengue virus strains were first isolated in the 1940s, when research focused on studying the major causes of illness and morbidity of the troops in the Pacific and Asia (26).

1.3.1 Structure and replication

Genome

As for all flaviviruses, the dengue virus genome is a single RNA molecule of positive polarity (ss+RNA). The genomic RNA is the only viral mRNA, containing a single open reading frame. The genomic RNA as such is infectious and generates the production of virions when transported into a suitable host cell. The genome is approximately 11 Kb in length and it is flanked by conserved untranslated regions (UTR) which form secondary structures that mediate genome circularization and have important functions in genome replication (27). The UTRs are well conserved in sequence and structure. The dengue virus 5’ UTR is approximately 100 bp in length and has a type I cap (m7GpppAm) at the 5’ end. It has a 2-loop secondary structure where the larger 5’ terminal stem-loop harbors the most conserved sequence regions. This loop is separated by a poly U
sequence from the smaller loop that includes the AUG start codon. The 3’ UTR follows the stop codon of NS5 gene and is approx 380-470 bp in length, forming three domains. Unlike the cellular mRNAs, the flavivirus genome does not have a poly A tail at the 3’ end. The genome cyclization through interactions of 5’ and 3’ ends are required for replication involving several elements of the genomic RNA (28).

Structure

The flavivirus virion consists of host cell-derived lipid bilayer, three viral structural proteins; capsid, membrane and envelope protein; and the RNA genome (Figure 2). The genome is packed into an icosahedral capsid composed of viral capsid protein (C), which is a small basic dimer-forming protein also found to co-localize to the nucleus in infected cells (29). In immature virions found inside the infected cells, the envelope glycoprotein (E) is arranged in trimers that make the immature virion surface to have a spiky appearance. In this form, the precursor of the membrane protein (preM) prevents the premature fusogenic activity of the envelope protein. During the virion maturation, the pre-part is cleaved from M by furin, and the envelope protein is rearranged from trimers to head-to tail dimers that lie flat against the membrane making the mature virion surface appear smooth (30,31). In flavivirus infections, the main antibody responses are formed against the structural proteins E and preM, and in addition to these, the T-cell responses are also developed against C-protein (32).
**Virus encoded non-structural proteins**

In flavivirus infected cells, in addition to the structural proteins, seven virus encoded non-structural (NS) proteins can be detected (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). The functions for all the individual NS-proteins are not well characterized; however, they are known to mediate the RNA replication and viral polyprotein processing (33) (Figure 3). The NS1 protein is a multimer-forming glycoprotein, which is secreted from infected cells. In dengue virus infection, patients have measurable levels of NS1 protein in the blood, which can be utilized as a diagnostic marker of the infection. NS1 protein has been shown to interact with the host immune system, but it also has functions in replication. The NS2A and NS2B are small membrane-associated proteins, NS2B functioning as a cofactor for the multifunctional NS3 protein, which has trypsin-like serine protease and helicase activities. The structure of NS3 is characterized in detail (34-37). The NS4A and NS4B are small membrane associated proteins; NS4A has been associated with membrane alterations (38) and NS4B has been associated with membrane structures involved in the replication. The NS5 protein is the viral RNA-dependent RNA polymerase mediating the genome replication. Also for NS5, the structure is known (39-41). Of the non-structural proteins, NS1 is known to raise antibody-responses. It is also known to evoke T-cell responses, in addition to NS3, NS4B and NS5 (32).
Life cycle in a cell

Currently, the host cell receptors and possible co-receptors of dengue viruses are not well characterized; however, several potential receptors or components of the dengue receptor complex have been identified including the laminin receptor (42,43), mannose receptor (44), dendritic-cell receptor (45) in human cells and heat shock proteins in mosquito cells (46).

Dengue virus binds to the cellular receptor via E-protein, which mediates the receptor-mediated uptake of the virion to the host cell by endocytosis. After the internalization the low pH of the endosome triggers a conformational change in the E-protein, which reveals a fusion peptide mediating the fusion between the virion and endosome membranes (47-49). As a result, the capsid is released from the viral envelope into the cell. The capsid dissociates and releases the viral genome into the host cell cytoplasm where it is translated as a single large polyprotein (Figure 3). The polyprotein is targeted to the endoplasmic reticulum (ER) where it is oriented by its signal sequences and membrane anchor domains, leaving it partly on the cytosolic and partly to the ER lumenal side. The processing by virus and host encoded proteases leads to the formation of individual viral proteins: three structural proteins; capsid (C), a precursor for the membrane protein (preM) and envelope protein (E); and the seven non-structural proteins that have functions mediating the replication, polyprotein processing and virion assembly (50).

The replication takes place in virus-induced vesicular membrane structures associated with the ER. The viral +sense RNA genome is replicated through a negative-sense intermediate, which is in turn used as a template in making more genomic positive strands. Through an unknown mechanism, the genomes are packed into capsids. The virions bud into the ER lumen as immature forms that have preM and E proteins in the surface (51). The immature virions are transported through the trans-Golgi network (52) where they are matured by a cleavage of pr/M creating infectious, mature virions that are transported out of the cell by exocytosis (2).
1. Review of the literature

Figure 2. Virion structure. Arrangement of envelope proteins in immature virion (trimers) and in mature virion (dimers).

Figure 3. Flavivirus genome and polyprotein processing. Modified from Bartenschlager & Miller 2008 (50).
1.3.2. Transmission

Vector mosquitoes

All the known vectors of DENV are mosquitoes belonging to *Aedes* genus. The species known to have the ability to become infected by DENV, to replicate it and transmit it include *Ae. aegypti*, *Ae. albopictus* and *Ae. polyniensis* of the subgenus *Stegomyia*. These species are at least partially domestic and anthropophilic. The life cycle of a mosquito includes four separate stages: egg, larva, pupa and adult (Figure 4), the first three stages requiring an aqueous environment. The duration of the developmental stages depend on the environment’s temperature and availability of food at the larval stage. For *Ae. aegypti* it takes roughly 8-10 days at room temperature to reach the adult stage (26).

Figure 4. Mosquito life cycle.
Dengue virus transmission cycles in the wild and urban environments

Two transmission cycles are known for dengue viruses, one of them involving non-human primates (monkeys) and jungle mosquitoes, referred to as the sylvatic cycle, and the second being the human- to human epidemic transmission cycle occurring in urban environments (Figure 5.). The life cycle of DENV involves a replication step in both mosquito and primate hosts. Following an infectious blood meal from a viremic host, after a period of 8-12 days the mosquito can infect new primate hosts. In both cycles, from an infected mosquito female, the virus is transmitted to the progeny mosquitoes through transovarial transmission and possibly is also transmitted between mosquitoes sexually (26). The human-to human transmission presents a particular adaptation of dengue viruses, as for most other arboviruses humans are dead end hosts that are not able to produce sufficient levels of viremia to infect new mosquitoes (26). DENV can be transmitted from human to human by infectious blood of a viremic patient via needlestick or mucocutaneous exposure (53-55). In addition to DENV, human-to human transmission has also been reported for YFV and WNV (12). -

Figure 5. Sylvatic and urban transmission cycles of DENV.
1.3.3 Genetic diversity and evolution

As for all RNA viruses, the DENV RNA-dependent RNA polymerase makes errors during replication and creates variability in the virus genome. Consequently, dengue viruses exist as populations of genetic variants, also called “quasispecies” (56,57). Additional variability in DENV genomes is derived from recombination (58,59). The relative proportions of variants in virus populations change over time due to the effects of genetic drift and selective pressures. The selection targets virus phenotype based on its interactions with the environment. The phenotypic properties of DENV affecting the host interactions in primates and in mosquitoes, such as the infectivity, replication and transmission efficiencies are crucial. It has been reported that most of the mutations in DENV genomes are deleterious thus highlighting the importance of selection in DENV evolution (60,61).

Dengue viruses are highly adapted to their mosquito-hosts and presumably have their origins in mosquito-viruses that first adapted to sylvatic life cycles between jungle mosquitoes and non-human primates. Based on the phylogenetic analysis, it has been suggested that the DENVs involved in human epidemics originate from the sylvatic DENV strains. As the sylvatic transmission cycles have been demonstrated for all 4 serotypes, it is considered that the four serotypes have emerged independently from their ancestral sylvatic progenitors. From these sylvatic cycles the jump to human-to-human urban cycle was possible via mosquitoes feeding also on humans in rural areas. The sylvatic life cycles of DENV are found only in Africa for DENV-2, while they are found for all DENV types in the Malay peninsula, suggesting an Asiatic origin for dengue viruses (62).

The evolutionary rates reported for DENVs are approximately $10^4$ to $10^5$ substitutions/site/year (63-68). Based on these rates, it has been estimated that dengue viruses were separated from their progenitors approximately 1000 years ago, and that the zoonotic transfer from the sylvatic cycles to urban epidemic cycles occurred between 125 and 320 years ago (65). It has been estimated that
the genetic diversification of epidemic strains to the different genotypes occurred within the last 200 years, and coincided with the beginning of major human epidemics (63). The diversification was likely linked to the virus adaptation to human- to human transmission in various geographical regions (69).

The four dengue virus serotypes can co-circulate in the endemic areas because the immunity to one serotype does not protect from the infection by a heterologous serotype. This is likely a result of selection that was driven by the restrictive effects of the cross-protective antibodies raised against heterologous serotypes. The dengue virus strains, which were able to escape this neutralization, had a significant competing advantage and became the dominant lineages. This evolutionary adaptation not only enabled the co-circulation of the four serotypes but also had a great influence to their pathogenicity for humans (62).

**Variety of dengue viruses: genotypes within serotypes**

The four dengue virus types (DENV-1-4) form a phylogenetic group that is more closely related to one another than to other flaviviruses, also forming an antigenic complex of their own. Despite the close relationships between the four serotypes, they are considered separate flavivirus species based on their antigenic and genetic differences (1). Because of their antigenic differences, the four DENV types are also called dengue virus serotypes. Dengue viruses are diverse, the four DENV serotypes differ in nucleotide sequence approximately 25-35 %. From the four DENV serotypes, DENV-4 appears to be the most divergent, followed by DENV-2, with DENV-1 and DENV-3 being most closely related to one another.

Most of the sequence data available on dengue viruses in public databases consists of partial genomic sequences of envelope, NS5 and NS3 genes; however, the numbers of complete genome sequences have increased during the last years. Dengue viruses of one serotype can be further separated into several genotypes (or subtypes) based on their grouping in phylogenetic analysis (Figure 6). In phylogenetic analysis, the most widely used genomic region is the envelope gene; however, sequences from various genes have been used in DENV genotype
determination. The phylogenetic analysis also supports the biological separation of dengue viruses based on the differences in their transmission cycles. The dengue virus strains that are originating from transmission cycles between non-human primates and jungle mosquitoes belong to the sylvatic genotype and are separate from the epidemic strains associated with urban human-to-human transmission. For the epidemic DENVs, several different ways and styles of numbering or naming have been proposed depending on the author, resulting in various names for a given genotype.

The main genotypes of the four DENV serotypes are shown in Figure 6. Within serotype 4 viruses (DENV-4), 3 main genotypes are separated including one sylvatic and two epidemic genotypes that were originally described from South East Asia (genotype I) and Indonesia (genotype II) (67,70). The DENV-2 viruses have been separated into 6 genotypes. The sylvatic genotype includes strains from South-East Asia and Africa. The five epidemic genotypes include one with a global distribution (Cosmopolitan genotype [IV]) and one genotype originally described from Central and South America (American genotype [IV]). Additionally, two lineages of Asiatic origin are separated (Asian genotypes I and II) (71) and one genotype from the Americas genetically associated with the Asiatic viruses, is referred to as the American-Asian genotype (III), which has been associated with severe disease (72).

DENV-3 viruses have been separated into 5 lineages found originally from the Indian subcontinent (III), Thailand (II), the Americas (IV), S.E. Asia (V) and one lineage present in South East Asia and the South Pacific (I) (73). No sylvatic strains of DENV-3 have been isolated or sequenced; however, evidence for their existence has been obtained by serological studies (62). The DENV-1 viruses are found to represent 5 separate genotypes, one of them is sylvatic (III). The 4 epidemic genotypes have originated from Thailand (II), other parts of Asia (I), the Americas and Africa (V) and the South Pacific region (IV) (62).
Figure 6. Radial neighbor-joining phylogenetic tree based on envelope genes of 82 dengue viruses depicting the genotypes within the four dengue virus serotypes. DENV-1 genotypes I-V; DENV-3 genotypes I-V and sylvatic genotype; DENV-3 genotypes I-V; DENV-4 genotypes I-II and sylvatic genotype.
1.3.4 Epidemiology

Current pandemic

The disease caused by the four dengue virus serotypes is currently considered the most important mosquito borne viral disease in the world, estimated to cause 50-100 million infections yearly (74,75). The incidence has increased 30-fold in the last 50 years (74). Dengue causes severe public health problems in endemic areas around the tropics and subtropics of the world, and no vaccines or specific treatment currently exist. Prior to 1960s less than 10 countries reported dengue outbreaks. Dengue is now endemic in over 100 countries in all the subtropics and tropics of the world. These include countries in Africa and the Americas, in addition to South-East Asia and the Western Pacific, which are the most seriously affected. It has been estimated that approximately 40% of the whole human population live in risk areas (74-77) (Figure 7).

Prior to the Second World War, dengue was known as a non-fatal febrile disease that caused self-limited epidemics with long intervals. A dramatic change in the epidemiology and disease severity was observed since the Second World War, which was followed by massive epidemics of dengue haemorrhagic fever. This was first observed in Asia where the post war time was characterized by economical boom and urbanization. Prior to the 1980s, severe dengue was a rare disease in the Americas where the observed significant expansion of dengue and increased disease severity was clearly associated with the introduction of multiple serotypes (26).

The global climate change has been blamed for the emergence of dengue (78) and models of dengue emergence have been made based on the climate change estimates (79). It is not clear how climate change will affect dengue transmission even though climatic factors have been linked to DENV epidemiology. Changes caused by the El Niño-phenomenon, such as the amount
of rainfall and temperature, however, have been shown to affect dengue transmission through changes in vector populations and transmission efficiency (80).

The current understanding of the factors affecting the current dengue pandemic situation (62,74) are listed below:

1. Adaptations in both virus and vector to support the urban DENV life cycle
   • Vector specialization to feed on humans and to breed on artificial aqueous environments provided in the urban settings
   • Virus adaptation to the urban vectors and humans, adaptation of DENV serotypes to one another: escape of the cross-neutralization by heterologous antibodies

2. Increased urbanization affecting vector and host densities
   • Human population growth and concentration to cities
   • Changes in the environment: urban environments, especially slums providing vector breeding habitats

3. Travel related geographic spreading of vectors and viruses
   • Traveling of infected humans, transport of materials containing infected mosquito-eggs and larvae
   • Global invasion of Aedes aegypti and Aedes albopictus
1.3.5 Dengue disease

Dengue virus infection can be asymptomatic or a febrile disease that may develop further to severe disease forms involving haemorrhagic symptoms. The incubation period from the infectious mosquito-bite to onset of symptoms can range from 3 to 14 days (81).

Development of humoral immune response to dengue virus

The dengue virus specific antibodies produced during infection are considered to provide protection through various mechanisms including virus neutralization, activation of the complement system and antibody-dependent cell-mediated cytotoxicity. The protective effects have been demonstrated in passive immunization experiments, where administered dengue virus antiserum protected mice from lethal infection (26,82). The neutralizing antibodies
produced during dengue virus infection include virus serotype specific antibodies and cross-reactive antibodies that are reactive against all serotypes. After the acute phase of the disease the specificity of the neutralizing antibodies increases over time. The protective immunity against the homologous serotype is lifelong, whereas the cross-protective immunity against the other serotypes is short lived, lasting only for a few months (62).

Due to the lack of sufficient cross-protective immunity between the different dengue virus serotypes, sequential or secondary dengue virus infections with heterologous serotypes are possible. The secondary infections are associated with elevated risks of severe disease outcomes. In these infections the pre-existing heterologous, non-neutralizing antibodies have been shown to participate to the pathogenesis by enhancing the infection. Tertiary and even quaternary dengue infections have been documented in endemic settings but their association with disease severity is currently not known (83).

Individual variation occurs in antibody responses to dengue virus. However, the primary and secondary infections are distinguishable based on their antibody responses. A primary infection is characterized by appearance of IgM antibodies early during the acute phase, reaching measurable levels on day 3-5 after onset of fever (Figure 8). The IgM peaks for approximately 2 weeks and declines to undetectable levels after 2-3 months. The levels of IgM-class antibodies are higher than those of IgG antibodies, which in primary infections appear after IgM (Figure 8).

During acute febrile and early convalescent phase of primary infections, IgG antibody titers are low. Whereas IgM dominates the humoral immune response in primary infection, the secondary infection is dominated by IgG. In comparison to the primary infections, in secondary infections the kinetics of IgM are more varied, and the response in most patients is delayed, lowered or even absent (84). In contrast, the IgG antibodies reach quickly high titers and are detectable already in the acute phase of secondary infection (26, 85).
Clinical manifestations of dengue virus infection

Based on symptoms alone, it is not easy to distinguish dengue from other tropical diseases, such as Chikungunya virus infection or malaria, and reliable diagnosis requires laboratory tests (85). The dengue disease is categorized according to disease severity by the World Health Organization into dengue fever (DF), dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS) (85). However, the symptoms of dengue disease can vary greatly from one patient to another.
Dengue fever (DF)

Dengue fever is characterized with sudden onset of fever, however the spectrums of the symptoms vary depending on the patient. Different types of aches and pains are common; often the headache is retro-orbital and is accompanied with e.g. rash, myalgia, loss of appetite, nausea, vomiting and abdominal pain. Additionally symptoms may include changes in taste “metallic taste” and flushing of the face.

In addition to fever, the clinical definition of DF includes two or more of the following symptoms: headache, retro-orbital pain, muscle or joint pain, rash, haemorrhagic manifestation or leucopenia (85). The mild haemorrhagic symptoms of skin, such as petechiae may be observed. Additionally, other symptoms include spontaneous bleeding, such as gum bleeding, increased menorrhagic bleeding, gastrointestinal bleeding and hematuria.

Dengue haemorrhagic fever (DHF)

Approximately 1% of DF develops into DHF, which is differentiated from DF by the increase of vascular permeability. This is seen in haematological studies as thrombocytopenia, elevated haematocrit and hypoproteinaemia. The clinical manifestations include high fever (up to >39°C), which can be biphasic, haemorrhages, thrombocytopenia and hemoconcentration, hepatomegaly and signs of circulatory failure. According to WHO, DHF includes fever, thrombocytopenia and elevated haematocrit.

Dengue shock syndrome (DSS)

Both DHF and DSS include vascular changes, thrombocytopenia and coagulation disorders. In addition to the symptoms described for DHF, DSS include narrow pulse pressure or hypotension. Approximately one third of DHF
patients develop shock. Dengue fever or dengue haemorrhagic fever can develop into a more severe condition usually after 3-7 days of the disease, when the fever disappears. Before the onset of shock, patients have severe abdominal pain. The typical signs of a circulatory failure can be seen as skin becoming cool and pulse becoming rapid and narrow. The patients in shock are in danger, and plasma volume-replacement therapy is needed to prevent death. The death rates of the severe forms of dengue disease, DHF and DSS, can be significantly reduced by correctly timed supportive treatment. However, without treatment the DHF fatality rates can be as high as 20% (74) and reach 44% in DSS (86).

**Treatment**

Currently, no specific medication is available for treatment of dengue. However, some medicines including corticosteroids (87) have been suggested to aid dengue patients with severe symptoms. In self-treatment of DF, resting and prevention of dehydration by drinking enough water is recommended. Paracetamol is preferred as antipyretic. Aspirin (Acetylsalicylic acid) should be avoided due to the anticoagulant properties. The main hospital-care treatment of DHF and DSS patients is the retaining of the intravenous volume by correctly timed intravenous fluid replacement. Frequent monitoring of platelet and hematocrit of DHF/DSS patients is essential for timing the treatment correctly. Several plasma volume replacement solutions have been used, including plasma expanders and electrolyte solutions. In severe cases, blood transfusions have also been used as treatment (76).
1.3.6 Dengue in travelers

Similar to the populations in endemic areas, most of the travelers infected with DENV are asymptomatic. Based on serological surveys, antibodies have been reported in 2.9-8% of travelers returning from endemic areas (76). The timing of the disease onset and the known incubation period from the infectious mosquito-bite to the onset of symptoms can be used in excluding dengue in travelers. The disease is not likely to be dengue if the symptoms start later than 2 weeks after returning from the endemic area.

In many European countries imported dengue is not reported, however, currently, the TropNetEurop (European Network on Imported Infectious Disease Surveillance) framework is collecting the data on dengue in European travelers. A clear pattern of seasonality of dengue in European travelers has been observed, following the “migratory habits” to warmer climates during winter (88). In some previous studies, malaria, gastroenteritis and fever of unknown etiology preceded the number of dengue diagnoses in travelers (89,90). However, a global network of travel and tropical medicine clinics, the GeoSentinel Surveillance Network, reported dengue as a more frequent infection than malaria in travelers returning from tropics outside Africa (91).

The trend in a global scale resembles the situation observed in European studies, the travelers obtaining the infection most frequently in Asia. In contrary to the situation in Asia, where dengue is primarily a disease of young children, in travelers it is mainly a disease of young adults who are active in traveling (88,91-93).
1.3.7 Pathogenesis

Cell tropism

During the early phase of the disease, dengue virus can be detected from peripheral blood for a relatively short period of time; the levels likely to be corresponding to the quantity of the infected tissues. Based on autopsy and in vivo studies of DENV infected humans, the cell tropism of DENV includes cells of the immune system, liver and endothelial cells of blood vessels. After being injected into the human bloodstream by a mosquito, dengue virus has been shown to infect many cell types. The dendritic cells of the epidermis (skin Langerhans cells) (94) and keratinocytes (95) are possible first targets. When the virus reaches the lymph nodes, monocytes and macrophages are also infected, and become the major targets of the infection disseminating the virus through the lymphatic system to cells of mononuclear lineage including monocytes, myeloid dendritic cells and macrophages in liver and spleen (96).

Current knowledge of the pathogenic mechanisms of dengue

Several virus and host specific factors have been associated with disease severity and pathogenesis of dengue disease. The study of dengue pathogenesis currently lacks a good animal model, as similar clinical manifestations mimicking severe dengue in humans are not seen in other animals. The current animal models include murine models (97,98) generated by experimental infection with DENV, which often develop a neurological infection seen only rarely in infected humans (26). The non-human primates experimentally infected with DENV develop viremia, but the replication is not as effective as in humans, resulting in lower levels of viremia. However, if a high dose of virus is used, subcutanic haemorrhagic manifestations develop also in Rhesus macaques (99).
Several hypotheses and involvement of various factors have been proposed to explain the pathogenesis of dengue, however, the necessary or sufficient factors are not known and the knowledge of pathogenesis of dengue in humans is mainly based on the studied fatal cases. The factors associated with severe disease outcomes include host genetic factors, age, immunological status, chronic diseases, and virus strain properties (100). The observed differences in disease patterns in different regions suggest involvement of various factors, as in South-East Asia, DHF is mainly a disease affecting children, while it affects mostly adults in the Americas (101). It has been suggested that different DENV serotypes and lineages may have differences in their biological properties, which can be linked to the pathogenicity (102). However, those are not likely to explain the observed pathogenicity completely as all DENV types are able to cause DHF/DSS (100).

As the viral load in humans corresponds to the disease severity (103,104), the inability of immunological responses to control the replication of DENV and the effects of the infected cells on other cells in the body are likely to play a major role. The high viral load is considered to result in apoptosis and necrosis in various tissues, and trigger the development of imbalance in the profiles of soluble mediators and cytokines resulting in endothelial cell dysfunction and blood coagulation abnormalities observed in DHF and DSS (96).

The involvement of immune enhancement in dengue disease was suspected based on epidemiological studies associating DHF to secondary infections (105). According to the antibody-enhancement (ADE) theory, the cross-reactive antibodies, which can be maternal antibodies in young children or antibodies from an earlier infection with heterologous DENV serotype, bind to the infection-causing virus but are unable to neutralize it. These antibody-virus complexes enhance the infection of monocyte-macrophages through binding to their Fc-receptors. In vitro studies showed that, indeed, the presence of cross-reactive non-neutralizing antibodies increased DENV infection in cells of macrophage lineage (106). According to ADE theory, the enhanced infection leads to the activation of a chain of reactions leading to immunopathology mediated by T-cell activation, interferon-γ, complement and platelet activation, cytokines and
altered functions of endothelial and epithelial cells. The enhanced infection rate is hypothesized to cause higher viremia in patients with secondary infections, which eventually leads to plasma leakage. However, the onset of plasma leakage occurs after the peak viremia, which suggests an immune–mediated mechanism. The enhancement was thought to be caused by cross-reactive antibodies known to exist against E-protein (107), however, in a recent study they were shown to be mainly targeted against preM protein of DENV (108). Whereas the ADE theory emphasizes the importance of the pre-existing antibody in the pathogenesis of dengue, it does not explain DHF in primary infections.

The dengue-virus specific T-cell responses have also been associated with severe disease, and have been suggested to participate in clearing the infection and in immunopathogenesis (62). From patients with secondary infections, the recovered activated T-cells were found to show low affinity to the infective serotype. Instead, they had high affinity to the heterologous serotype, presumably representing the one encountered previously (109).

Molecular mimicry has also been proposed to play a role in dengue pathogenesis by triggering an autoimmune-type of response. In the DENV-4 envelope protein, a 20-amino acid residue area was found to resemble a protein family of blood clotting factors. The patient antibodies against E-protein were further experimentally shown to cross-react with plasminogen (110). Similarly, antibodies against the NS1 protein have been shown to bind also blood clotting factors, integrins and endothelial cells (111).

The mechanisms of the complement system in dengue pathogenesis are poorly characterized, however, they are considered to play an important role. The activation of complement is associated with plasma leakage, which coincides to high levels of C3a and C5a activation products in patient plasma. Additionally, reduced amounts of complement components are reported from patients with DSS. Dengue virus NS1 protein has been shown to interact with complement inhibitory factor clusterin (112) and the soluble NS1 protein was also found to activate complement, enhanced by anti-NS1 protein antibodies (113).
1.3.8 Laboratory diagnostics

The current laboratory diagnosis of dengue is based on detection of markers of DENV infection in patient serum. These include viral components and antibodies that are present in the patient serum at different time points of the infection (Figure 8). This makes the diagnostics of acute dengue infection complicated, and often several test types or paired samples are needed for reliable diagnosis. Furthermore, information of the timing of sampling in regard to the disease onset is needed for choosing the adequate diagnostic method (85). The diagnostic tests used vary from country to country, and in addition to commercial test kits, in-house diagnostic methods are also widely used. In Europe, the European Network for Diagnostics of Imported Viral Diseases (ENIVD) provides quality control samples for evaluation of the diagnostic tests (114,115).

The laboratory criteria for confirmation of dengue virus infection according to WHO (85) include demonstration of at least one of the following in patient sample(s):

1) Live dengue virus by virus isolation
2) Fourfold or greater change in reciprocal IgG or IgM titers to one or more DENV antigens in paired samples
3) DENV antigen
4) DENV genome by RT-PCR
Detection of anti-DENV antibodies

The most widely used traditional dengue diagnostic methods are based on detecting antibodies against dengue virus in patient serum. All members of the genus flavivirus share structural similarities in their envelope proteins, which are seen as serological cross-reactions in serological tests. The cross-reactive antibodies from previous flavivirus infections or vaccinations can cause false positive test results especially in IgG detection. Diagnostics based on serology are not flavivirus specific methods, and thus a positive test result should be considered as “flavivirus antibody positive”. The only serological methods valid for typing flavivirus infections and dengue virus infections according to the infection causing serotype are neutralization tests. These methods are not used in routine diagnostics as they are time consuming and require working with infectious virus.

Reliable serodiagnosis is based on paired samples where a diagnostic rise in antibody titers can be observed between the acute (<6 days after onset) and convalescent (6-30 days after onset) samples. In primary infections, the IgM antibody titers raise to detectable levels in 80% of the patients by day 5 after the onset of fever (116,117). The IgG antibodies become detectable shortly after IgM and by day 7 most of the patients have detectable levels.

The most commonly used serological method for anti-DENV antibodies is capture IgM enzyme immuno-assay (Mac-EIA) based on inactivated virus antigen (85). In addition to EIA-based methods immunofluorescence assays (IFA), lateral flow/immunochromatography-based rapid tests and immunoblot methods (118,119) have also been used in detecting anti-dengue IgM. The methodologies used in detection of IgG are similar to those used in IgM detection, additional methods include dot-blot methods (120) and IFA (121). These methods have largely replaced the previously used hemagglutination inhibition (HI) tests, which were previously used to measure total antibodies (116).
Detection of DENV genome

The detection of DENV genome in patient serum samples enables specific dengue diagnosis during the early stage of the disease when serological methods are not reliable. The viremic phase of the disease coincides with the fever, with its duration varying from 2 to 10 days (100). A positive test result enables diagnosis, however, a negative test result does not exclude the possibility of dengue as individual variation in viremia levels and timing may occur. The timing of the sampling is crucial for RNA detection, however, the storage and sample handling are also important for retaining viral RNA in the samples.

Compared to the other diagnostic methods, DENV genome detection requires several handling steps including sample RNA extraction and the assay itself. Unlike the serological methods, detection of viral RNA is vulnerable for contamination, and requires specific laboratory facilities and equipment. However, unlike serological methods, DENV genome detection enables specific rapid DENV diagnosis from a single early phase-sample and enables the typing of the infection causing serotype, which is important for the epidemiological follow-up.

Different types of reverse-transcription polymerase chain reaction (RT-PCR)-based methodologies have been used in detection of DENV RNA in patient samples. In principle, viral RNA is extracted from serum sample, transcribed to cDNA in a reverse transcription (RT) reaction either in a separate reaction or in a one step format and amplified by polymerase chain reaction (PCR). Depending on the purpose, the amplification targets include highly conserved regions of the DENV genome, such as the NS5 and 3’ UTR, or areas with more variability, such as the C-preM and E-gene regions.
Conventional RT-PCR

The amplification products of conventional RT-PCR are detected visually on agarose gels using ethidium bromide staining. The amplicons are usually several hundreds of base pairs long, and can be utilized in studying the sequence of the amplified PCR product by site-specific restriction enzyme analysis (122), nucleic acid hybridization-based methods (123-125) or by sequencing the PCR product (126). Running several rounds of amplification can enhance the sensitivity of the RT-PCR assay. In “nested” amplification, the second amplicon is located within the first one. Conventional RT-PCR methods have a separate detection step in addition to the amplification itself, making these methods slower in comparison to newer real-time methodologies combining both steps. However, conventional methods have not been totally replaced by the real-time applications, as they are robust, less expensive and can be carried out using basic thermal cyclers and reagents (127-130).

Real-time RT-PCR

Two principal methodologies of real-time PCR have been widely used in virus nucleic acid detection. These include sequence specific detection of amplified PCR products using fluorogenically labeled probes, and methods that are based on accumulation of a fluorescent dye bound to the double-stranded DNA amplicon in a sequence unspecific manner (131). In both ways, the produced fluorescence is proportional to the amplified target sequence and can be quantified (132). The real-time PCR amplicons are usually shorter than those used in conventional PCR. Additionally, the PCR instruments and reagents used allow very rapid PCR steps, detecting the results simultaneously thereby shortening the time required for the assay. Several types of instruments have been developed by different manufacturers, differing in the technologies used to perform the thermal cycling, detecting the fluorescence and analyzing the obtained data.
Most of these enable real-time follow up of the accumulation of the fluorescence in samples while the protocol is running.

**TaqMan chemistry**

The sequence-specific real-time PCR methods are based on probes binding specifically to the target sequence, which is amplified in PCR. These include various probe types and detection principles. One of the most widely used probe-based systems in DENV RNA detection utilizes the 5’ nuclease activity of Taq DNA polymerase and dual-labeled probes (also called TaqMan® chemistry). TaqMan probes have a fluorescent dye at the 5’ end, and a quencher dye at the 3’ end. When the probe is intact, the quencher dye prevents the fluorescent reporter dye from emitting fluorescence through fluorescence resonance energy transfer, which is dependent on the distance between the reporter and quencher molecules. The probe is designed downstream of PCR primer site in the target sequence, and during the primer extension step of the PCR amplification, the probe is cleaved by Taq DNA polymerase (133). This results in separation of the quencher and reporter from another, and enables the reporter to emit fluorescence that can be measured (Figure 9). This does not occur unless the probe-specific sequence is amplified, making the method highly specific. In the presence of a probe-matching amplification product, the reporter molecules are cleaved from the probes in each cycle of PCR creating an increase in fluorescence intensity that corresponds to the amount of amplicon produced. Several TaqMan-based RT-PCR methods have been used to detect and type DENV RNA (134-139).
1. Review of the literature

Figure 9. Principle of TaqMan® chemistry.
1. When the probe is intact, the quencher molecule (Q) prevents emission of fluorescence of the reporter dye (R). In the absence of probe specific target sequence no fluorescent signal is emitted.
2. Probe and primers bind to their specific target sequence. 3-5. Taq DNA polymerase cleaves the probe during PCR amplification and reporter and quencher become separated. Reporter dye emits fluorescence that can be detected and measured by a real-time PCR instrument.
SYBR Green based methods

Unlike the sequence-specific methods that utilize probes, the SYBR Green-based methods measure fluorescence produced by SYBR Green I dye that binds to any double-stranded DNA. In an assay with sequence specific primers, the increase in fluorescence is proportional to the amount of the product produced. The specificity is thus dependent on the primers used, but allows the detection of variable sequences, which can be differentiated based on melting curve analysis of the amplification products. This analysis shows the specific temperatures, in which the fluorescent dye dissociates from the dsDNA-PCR product. The melting temperature is dependent on the amplified product sequence, as the proportions of C/G and A/T result in different melting temperatures. This has been used in real-time detection of DENV genome, giving a melting temperature profile that can be separated from those of other flaviviruses (140-142). However, as all double stranded DNA will produce fluorescent signals, including primer-dimers and unspecific amplification products, the interpretation of the results of SYBR Green based real-time PCR assays can be challenging, and depend on a clearly recognizable, unique melting temperature of the target sequence.

Other RNA detection methods

In addition to the above-mentioned methods, RT-PCR based DNA microarray detection (143) and amplification by nucleic acid sequence-based amplification (NASBA) have also been used in detection of DENV RNA (144).
Detection of viral antigens

The dengue virus NS1 antigen detection methods have been shown to be suitable for early diagnosis as the antigen is detectable in patient serum prior to the appearance of antibodies (145-147). Commercially available assays for detection of DENV antigens in patient serum have been available for a fairly short period of time (148). These are now available in EIA and rapid immunochromatography-test formats, however, the EIA format has been reported to be most sensitive (149). The current data on the NS1 antigen testing has showed that the test may have different sensitivities for primary and secondary infections, as pre-existing anti-NS1 antibodies may interfere with the test in secondary infections, competing with the monoclonal antibody used in the antigen-capture assay. Furthermore, it has been suspected that the monoclonal antibodies used in the tests may prefer some DENV types to others. However, more studies on the performance is needed for this test, but the preliminary results seem promising, as NS1 antigen detection is a DENV specific method suitable for early diagnosis. Furthermore, unlike RT-PCR based methods, in a rapid test format the NS1 antigen test does not require specific laboratory facilities to carry out the test (150), and is very easy to use (151). However, further improvements and more extensive evaluation of the rapid test format have been suggested necessary, including an internal positive control (152). In addition to NS1 detection, commercial kits are also available for E-protein antigen detection, but these have been shown to be less sensitive in comparison to NS1 detection (149).
Virus isolation

Virus isolation is not commonly used in routine diagnostics, but it constitutes definitive proof of DENV infection. As in virus genome detection, the sampling time and optimal sample storage are crucial (153,154). Currently, the most widely used method of DENV culture from patient sera is to use cultured mosquito cells (Aedes albopictus C6/36), but several other mosquito-cell lines and mammalian cell lines commonly used in virus cultures such as monkey kidney cells are also suitable. The most sensitive although impractical method reported is inoculation of live mosquitoes (100). The ability of dengue virus to cause cytopathic effects (CPE) on infected cells varies and is likely to be dependent on virus strain properties. From virus isolation cells DENV can be detected and serotype determined using dengue virus specific RT-PCR methods or monoclonal antibodies in an immunofluorescence format.

1.3.9 Prevention

Vaccine and drug development

In future, antiviral compounds against DENV are likely to become commercially available. Compounds inhibiting DENV replication, enzymatic activity, receptor binding and fusion are currently studied. The aim of these studies is to identify potential drugs that could prevent DF from developing to DHF or DSS (155). Additionally, therapeutic antibodies are currently studied for their potential in the treatment of dengue (156).

The lack of an animal model and the poor understanding of the pathogenesis of dengue have made the development of an effective and safe dengue vaccine difficult. An optimal vaccine would raise an equally effective neutralizing antibody response to the four serotypes simultaneously, as otherwise
the vaccine could predispose to severe disease. The current types of dengue vaccines in development include chimeric, live-attenuated, inactivated, replication incompetent and subunit vaccines. Many of these have not provided sufficient protection or have caused side-effects (157). One of the leading candidates is a chimeric vaccine based on a yellow-fever live-attenuated vaccine backbone, where the preM and E genes have been replaced by those of DENV1-4. This vaccine is currently in phase 2 clinical trials, and has given promising results on human volunteers (158).

Vector control

Vector control is currently the main dengue prevention method. The adult mosquitoes do not require water and the mosquito eggs can tolerate desiccation for months. However, aquatic habitats are required for the larvae and pupal stages. These are readily available in urban environments, including domestic freshwater containers and everything that collects rainwater from trash cans to flower pots (26). Restriction of aqueous environments outdoors and prevention of mosquitoes both indoors and outdoors by nets and insecticides are the main ways of vector control in the endemic areas of the world. Ae. aegypti is a day active mosquito, and travelers to endemic areas should wear protective clothing and apply insect repellents on skin and clothing also during daytime and while traveling in cities (76).
2. Aims of the study

Dengue is not a new disease, however, the recent widening of endemic areas and increase of severe disease forms have made dengue one of the most important mosquito-borne viral diseases of mankind. The infection caused by the four dengue virus serotypes is currently a major public health problem in the endemic areas of the subtropics and tropics of the world. The increased incidence and prevalence of dengue in the endemic areas also affects the travelers visiting these areas. The increased importance of dengue as a disease of travelers was also observed in Finland. Within the last ten years, the numbers of positive diagnoses have risen steadily, now reaching over 30 diagnoses yearly including one confirmed fatality. Severe dengue disease and fatalities among travelers are rare, and relatively little information is available of these cases making their description and investigation important. Due to the risk for severe disease outcomes, the recognition of dengue infection in travelers is important. The diagnostics of dengue virus infection is complicated by the serological cross-reactions with antibodies from other flaviviral infections. Diagnostic methods that provide a reliable and specific diagnosis of dengue virus infection in the early stage of the disease are needed.

The global distribution and composition of dengue virus sero- and genotypes circulating in different geographical regions is constantly changing. The characterization and phylogenetic analysis of dengue virus strains from travelers provide up-to-date information on the global epidemiology including areas lacking active surveillance. In the countries endemic for dengue viruses, the follow-up of the circulating dengue virus strains provides information of the local epidemiological situation and evolution of these viruses. The epidemiology of dengue virus type 2 was earlier studied in Venezuela in the late 1990s when evidence of serotype 2 diversification and recombination were observed. A collaboration study with Venezuelan researchers aimed at updating the current situation in Venezuela.
The specific aims of the study were to

- Carry out a virological investigation of the first fatal dengue case in Finland for confirmation of the etiology of the infection and aiming at identification of the infective virus serotype

- Develop a DENV RNA-detection method to be able to diagnose dengue from early time-point patient samples and to compare this method to other available methods including NS1 antigen detection, virus isolation and serological methods for choosing the optimal methodologies for diagnosing dengue in travelers

- Isolate and characterize DENV strains from Finnish travelers for identification of the infective DENV sero-and genotype and for studying their genetic relationships to previously characterized DENV strains

- Analyze a collection of 23 DENV-2 isolates from an endemic country, Venezuela, for a follow-up of DENV-2 epidemiology and evolution by determining and analyzing envelope gene sequences
3. Materials and methods

3.1 Study materials

3.1.1 Patient samples

Dengue patient serum samples were obtained from HUSLAB department of virology, the viral zoonoses diagnostic unit that currently performs dengue diagnostics as the only laboratory in Finland. The sera were stored at -20°C, aliquots for virus isolation trials and RT-PCR were stored at -70°C until use. Information on the travel history, onset and variety of symptoms of the patients was collected by a questionnaire from the clinicians who treated the patients in hospitals around Finland.

As information on the onset of symptoms was not available for all the patients, the patient sera were selected for virus isolations and early time-point diagnostic tests by choosing the first available serum from a diagnosed patient using a criterion of IgG titer (IFA) 320 or less. Using these criteria, a panel of 99 samples was selected from patient samples collected in 1999-2008.

Autopsy samples including the paraffin embedded tissue samples (brain, lung, kidney) and brain tissue sample used in publication I were obtained from Tampere University Hospital.

3.1.2 Viruses

The reference dengue virus strains used in neutralization assays and as positive controls in IFA and RT-PCR included DENV-1 (Hawaii), DENV-2 (New Guinea C), DENV-3 (H87) and DENV-4 (H241) from the collections of Haartman Institute. The
Venezuelan DENV-2 strains were obtained from Regional Laboratory for Diagnosis and Research of Dengue and other virus diseases (Lardidev) Aragua State, Venezuela, where the 23 DENV-2 strains were isolated from patient sera collected in 1999-2005 in *Aedes albopictus C6/36* cells.

### 3.1.3 Cell lines

Cultured cell lines used in this study were originally obtained from ATCC collections including Vero E6 (ATCC: CRL-1586) green monkey kidney cell line grown in cell culture bottles in MEM supplemented with fetal calf serum, glutamine and antibiotics (penicillin, streptomycin) at +37°C at 5% CO2 and C6/36 (ATCC:CRL-1660) *Aedes albopictus* mosquito-cell line, grown in Gibco Leibovitz L-15 media (Invitrogen, Carlsbad, California, USA) at room temperature or at +27 °C with closed lids.

### 3.1.4 Monoclonal antibodies

The monoclonal antibodies (MAbs) against dengue virus E-protein were obtained as mouse hybridoma cell lines 15F3 (DENV-1), 3H5 (DENV-2), 5D4 (DENV-3), 1H10 (DENV-4) and 813 (flavivirus cross-reactive MAb), (159) were kindly provided by professor Ernest Gould from the University of Oxford, UK. The cell lines were cultured in RPMI-media. The supernatants from confluent cells were clarified by centrifugation and used as primary antibodies in immunofluorescence assays. The flavivirus cross-reactive MAb HB-112 was provided by Sirkka Vene from Swedish Institute for Infectious Disease Control, Stockholm, Sweden.
3.2 Methods

All virus culture and isolation experiments were carried out in biosafety-level 3 (BSL-3) laboratories in Haartman Institute, University of Helsinki.

3.2.1 Virus isolation in cell culture

Virus isolations from patient serum samples were performed in 25 cm² culture bottles using Vero E6 and C6/36 cells. The cells were first rinsed with PBS with added antibiotics to remove the culture media, and infected with 50 μl of the serum sample for 1 hour. After 1 hour, fresh media was added. The Vero E6 cells were grown at +37 °C in a CO₂ incubator and C6/36 cells at room temperature. The infected cells were observed for cytopathic effects and studied further for viral antigens in IFA and the culture media samples for the presence of viral RNA by RT-PCR.

3.2.2 RNA extraction and RT-PCR

RNA from virus isolation culture supernatants and human serum samples were extracted using QiaAmp viral RNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. RNA from tissue samples was extracted using TRIPURE isolation reagent (Roche Applied Science, Mannheim, Germany).
Conventional RT-PCR

The dengue virus typing semi-nested RT-PCR was performed using the previously described primers targeting the C-preM region in DENV genome (127). In brief, a 511 bp region of all DENV types was amplified by RT-PCR (primers D1 and D2), which was used as a template in the second round with D1 primer and DENV type specific reverse primers (TS1-4) producing DENV type specific amplification products (DENV-1: 482 bp; DENV-4: 392 bp; DENV-3: 290 bp; DENV-2: 119 bp). In addition to this protocol, another semi-nested RT-PCR was also used, amplifying a 220 bp conserved region of NS5 gene of most flaviviruses (160). The DENV-2 envelope gene-sequences were amplified using previously described primers (58).

The RT-reactions were performed using Expand Reverse Transcriptase (Roche Applied Science, Mannheim, Germany) either by gene specific reverse primers or random hexamers. The PCR steps were carried out using Recombinant Taq DNA polymerase (Fermentas, Vilnius, Lithuania) and Taq Extender PCR additive (Stratagene, La Jolla, California, USA) according to the manufacturer’s instructions.

TaqMan real-time one step RT-PCR

For setting up the TaqMan assay, a few modifications of the primers were first tested for their performance in PCR amplification using SYBR Green I. These tests were done on the positive control RNAs (DENV-1-4) that were first reverse transcribed to cDNA using Expand Reverse Transcriptase (Roche Applied Science, Mannheim, Germany). The PCR was done using Light Cycler Fast Start DNA Master SYBR Green I Master kit (Roche Applied Science, Mannheim, Germany) on Light Cycler real-time PCR instrument using a program of 95 °C 1 min following amplification 40 x a 10s cycle of 95 °C, 60 °C s and 72 ° C. Based on the amplification results and melting curve analysis, the best primers (forward 5’-GGACTAGAGGTAGAGGAGACCC, reverse 5’-GAGACAGCAGGTCTCTGGTC) were
chosen for further testing on TaqMan real-time RT-PCR using a 6-FAM-AGCATATTGACGGTGGGA-MGB-BHQ1 probe. The one-step test format was chosen for avoiding contaminations and for the ease of performance. The test was optimized on Qiagen QuantiTect One-step Probe RT-PCR kit (Qiagen, Hilden, Germany) in a 50μl reaction volume using 900 nmol of each primer and 250 nmol of the probe, including 5 μl of template RNA using a program of 50 °C 30 min followed by 45x cycle of 95 °C 15 s, 60°C 1 min in Applied Biosystems optical 96-well plates using ABI PRISM 7700 Sequence Detection System-instrument. The probe was purchased from Applied Biosystems and primers from Oligomer.

3.2.3 Sequencing and sequence analysis

The RT-PCR products were enzymatically purified for sequencing using ExoSAP-IT (USB Corporation, Ohio, USA) and when necessary using gel extraction by Qiagen gel extraction kit (Qiagen, Hilden, Germany). The sequencing reactions were done at the sequencing core facility of Haartman Institute (Sequencing unit, Haartmaninkatu 3, PL21, 00014, University of Helsinki, Helsinki, Finland). The obtained sequences were trimmed and combined to consensus sequences using the program 4Peaks (available at http://mekentosj.com/science/4peaks/), and programs of the Staden package (available at http://mekentosj.com/science/4peaks/ and http://staden.sourceforge.net/).

The multiple sequence alignments were done using ClustalW2 or MUSCLE programs available online (http://www.ebi.ac.uk/ clustalw and http://www.ebi.ac.uk/Tools/muscle/index.html). The phylogenetic analysis of nucleotide sequences was performed using MEGA 4 software (161).
3. Materials and methods

3.2.4 IFA

The immunofluorescence assay (IFA) was used in this study for two purposes, for detection of antibodies against a known DENV antigen and for screening for viral antigens using a known antibody. The primary antibody in diagnostic procedures was a patient IgG or IgM detected by a fluorescent (FITC-labeled) secondary antibody, anti-human IgG or IgM (Jackson Immunoresearch, West Grove, Pennsylvania, USA). In detection of viral antigens from virus isolation cells, known DENV-antibody positive human sera or MAb was used. The MAbs were detected by FITC-labeled anti-mouse conjugate (DAKO Cytomation, Glostrup, Denmark).

IFA slides for diagnostic IFA tests were made by infecting Vero E6 cells for 3-7 days depending on the virus, detaching the cells by trypsin-EDTA and growing the cells overnight on pre-sterilized 10-well slides at +37°C in a moist environment. The next day, the slides were gently rinsed in PBS to remove the unattached cells. Finally, the slides were air-dried and fixed in chilled acetone for 7 minutes and air dried again. The slides were stored at -70°C prior to use.

The IFA slides from virus isolation trials were made similarly, with the exception that the infected cells (Vero E6 or C6/36) were dried directly on slides.

3.2.5 EIA

Commercially available enzyme immunoassays were used for detection of anti-dengue virus IgM using a capture-EIA kit (FOCUS Technologies, Cypress, California, USA) and for detecting dengue virus NS1 antigen from patient sera, an antigen capture-EIA, BioRad dengue NS1 AG-ELISA (Bio-Rad, Marnes-la-Coquette, France) was used according to the manufacturer’s instructions.
3.2.6 Immunohistochemistry

Immunohistochemistry (IHC) was performed by Ventana Discovery immunohistochemistry Slide Stainer (Ventana Medical Systems, Tucson, Arizona, USA) using paraffin-embedded thin sections of the tissues. Thin sections of paraffin-embedded DENV-1-4 infected Vero-E6 cells were used as positive controls in the IHC stainings. Slides were incubated with primary antibody (pan-flavi cross-reactive MAbs and DENV-1-4 specific MAbs) and anti-mouse conjugate and detected using Ventana DAB biotin-avidin detection kit.

3.2.7 Neutralization test

Plaque reduction neutralization (PRNT) tests were carried out in Vero E6 cells using reference virus strains adapted to this cell line, with a known titer in plaque forming units (PFU) in 6-well plates. A virus dilution producing easily readable amount of separate plaques in a well (approximately 50 pfu) was chosen as the working amount of the virus. The neutralization capability of a serum sample was tested in a dilution series using a standard amount of virus. The patient serum samples were first heat-inactivated by incubating them 30 min at 60 °C prior to incubation with virus at +37°C for 1 hour. Freshly confluent monolayers of Vero E6 cells on 6-well plates were infected with the serum-virus mixture for 1 hour, and then the inoculum was removed. Cells were overlaid with a 1:1 mixture of 2xMEM and 2% agarose. The plaques were allowed to develop for 3-7 days depending on the virus and then the cells were fixed in 10% formaldehyde solution. The agarose was removed and the plates were stained using crystal violet solution for visualization of the remaining monolayer and the plaques. The result from patient samples in pfu’s was compared to those of the controls treated similarly without the serum. A dilution of the patient serum, which caused an 80% reduction in the plaque formation of a given virus, was considered as the PRNT$_{80}$ titer of a serum.
4. Results and discussion

4.1 Diagnosis of dengue virus infection in travelers

The number of diagnosed dengue cases in Finnish travelers has tripled in a short period of time (Figure 10), now reaching over 30 diagnosed cases each year (Huhtamo et al., unpublished). This may be partly due to increased recognition of the disease, but is also affected by the increase of people traveling to endemic areas (162), and the extent of dengue transmission in these areas. An analysis of the statistics of the Finnish travel destinations show that Thailand is the most popular single long-distance travel destination. Since 2006, approximately 100 000 trips were made yearly from Finland to Thailand (163).

![Number of trips to endemic areas and number of positive dengue diagnoses](image)

Figure 10. Number of trips by Finnish travelers aged 15-74 to areas endemic and potentially endemic to dengue virus including travel destinations in Central-and South America, Asia, Oceania and Africa (obtained from Statistics Finland, http://pxweb2.stat.fi/Database/StatFin/livi/smat/smat_fi.asp) and number of positive dengue diagnosis in Finland 2000-2009 based on serological testing (HUSLAB) (Huhtamo et al., unpublished).
4.1.1 Virological examination of a fatal dengue case (I)

In travelers, dengue is usually a primary infection with relatively mild symptoms. The sudden death of a previously healthy young Finnish woman who was recovering from a severe dengue virus infection raised a lot of questions and triggered a thorough examination of the case. The patient fell ill during her journey in South East Asia in May 2002. Based on her travel history, the infection most likely originated from Malaysia, where she traveled during the weeks prior to onset of the disease.

The disease started as a typical DF including high fever, headache and rash. From this stage the symptoms gradually became more severe, and the patient was hospitalized with nausea, vomiting and cough, in addition to leukocytosis, thrombocytopenia and reduced blood pressure. The fever subsided on day 4 after the onset, she developed edema on the face and limbs and laboratory tests revealed abnormal blood clotting. One week after the onset of fever, the patient’s condition developed to DHF. She became unconscious, had renal failure and developed haemorrhagic symptoms including nose bleeding and petechiae on the skin. Additionally, she had pulmonary edema and pleural effusions.

The symptoms were prolonged and on the fourth week the patient was transferred to Finland. The patient still had pleural and abdominal effusions, renal failure, liver damage with icterus and bleeding from intravenous lines. The renal failure was treated by hemodialysis, and the prolonged sepsis syndrome by methylprednisolone. The patient was recovering, however she had a sudden brain hemorrhage after a routinely performed dialysis. The patient died 3 days later, and the autopsy revealed diffuse hemorrhages in brain, meninges, endocardium, pancreas and ovaries.

The diagnostic tests of the early stage samples were performed at hospitals in Asia and the late stage samples were studied in Finland. During the acute phase of the disease, IgM but no IgG was detected characterizing the disease as a primary dengue infection. As no evidence of virus was detected from
the examination of the late stage serum or the autopsy samples studied by virus isolation, RT-PCR or immunohistochemistry, the patients prolonged symptoms of renal failure, abnormal blood clotting and hemorrhages likely were caused by indirect effects of the infection. Corticosteroids have been used in treating sepsis syndrome (164) and were also administered to the patient described here with observed improvement in the patient’s condition.

The acute phase samples taken in Asian hospitals were not available for further studies, such as virus isolation or RT-PCR. The antibody responses of the patient were found to be cross-reactive, and non-typable by the plaque reduction neutralization (PRNT) tests. These, however, provided evidence that the infection was caused by DENV, as the PRNT titers were higher to DENV than to JEV. The IgM-IFA titers were found to be higher against DENV-1 and DENV-2 (1:160) than against the rest of the dengue virus types (1:40-1:60) suggesting that these DENV types may have been involved (165). The reported concurrent circulation of DENV-1 and 2 in Malaysia 2002 (166) would fit the antibody findings of the patient, suggesting the possibility of a multiple serotype infection, which are known to occur in areas where DENV types co-circulate (167,168). The significance of the IgM-IFA result alone, however, remains unclear as those were not supported by the PRNT.

While the severe dengue disease is often associated with secondary infections, explained by the ADE-theory, relatively little is known about the risk factors affecting the severe disease outcomes of primary infections, which also include DHF and fatalities (169,170). Several host specific factors have been suggested to affect the disease outcome including race, sex and nutritional status. The infecting virus type and its pathogenicity have also been suggested to influence disease outcome. In a study of DHF of primary infections in Thailand involving mainly young patients, an association of severe disease forms to DENV serotypes 1 and 3 was observed (171). Various symptoms considered unusual in dengue have been reported from travelers such as skin tenderness, pancreatitis, meningitis (92) subarachnoidal haemorrhage (172) and encephalitis (173). Additionally, symptoms mimicking typhoid fever (174), complications during pregnancy (175) and fatalities (176) have been recently reported. In classification
of dengue disease severity in travelers, the WHO classifications have been questioned as the disease in travelers can be severe and yet not fulfill the current DHF classification (93, 177). In conclusion, our investigation confirmed the serological diagnosis of dengue virus infection in the patient by neutralization tests but was not able to identify the infective DENV serotype or serotypes involved. The results suggested that our patient experienced a prolonged primary dengue virus infection that was the underlying cause of the fatal brain hemorrhage.
4. Results and discussion

4.1.2 Development of a new real-time RT-PCR method for detection of DENV RNA (II)

Assay design

The requirements for a diagnostic real-time RT-PCR test included specificity for DENV RNA and a sufficient sensitivity to detect DENV RNA from patient serum. From the diagnostic laboratory’s point of view, reliable qualitative results in a simple and affordable format preceded the needs of quantification and typing of dengue viruses in patient samples. To meet these criteria, we aimed to design an assay based on a single TaqMan probe simultaneously detecting the four dengue virus types. The suitable genomic regions that included short highly conserved sequences were searched visually from DENV complete genome alignments of the four DENV serotypes. Based on the alignments, the identified conserved regions were further examined by using them as query sequences in searches against GenBank using the BlastN algorithm (available at http://blast.ncbi.nlm.nih.gov/Blast). Based on the results, the 3’UTR region was selected as a target for the PCR amplification. The primers and probe were designed using Primer Express Software Version 3.0 (Applied Biosystems) using the settings for minor groove binder (MGB) –probe. The use of a probe with attached MGB molecule, a conjugated hairpin-ligand increasing the melting temperature (Tm) of the probe, enabled us to use a short probe sequence that suited our target sequence.
**Determination of assay sensitivity and specificity**

For the assay sensitivity estimation, the numbers of viral genomes in the positive control virus supernatants were estimated based on the measured RNA concentration using a spectrophotometer. The RNA was extracted without carrier RNA that would influence the spectrophotometrical measurement and the measured RNA quantity was considered to contain only viral RNA. The obtained values were used in the calculation of number of viral genome copies in the RNA extract, which was further tested in real-time RT-PCR. A 10-fold dilution series of the RNA samples was run in duplicates with the assay. This sensitivity estimation resulted in detection of less than 1000 genome copies for all DENV types per reaction.

The specificity of the test for DENV RNA was evaluated by testing RNA extracts of other flaviviruses including YFV, JEV, TBEV, USUV and WNV. Testing demonstrated that none of these templates gave positive results. Additionally, patient serum nucleic acids (n=32) from randomly selected unrelated patient material was tested negative. These results demonstrated that the designed primers and probe were specific for DENV RNA. The test was further evaluated using two external control panels from the European Network for Imported Viral Diseases (ENIVD) (114). The first control panel was provided for the assay testing and included the sample identity information. The samples included 12 lyophilized serum samples that were resuspended into 100 μl of PCR grade H₂O for RNA extraction. The samples included seven samples of DENV-1-4 RNA, TBEV and YFV RNA and one negative sample. The results of the DENV-RNA containing samples were found to be positive and the non-dengue and negative samples negative.

In 2009 we participated in the ENIVD External Quality Assurance round for DENV RNA detection. The 12 samples provided were tested without any sample information and the results were sent to ENIVD. The results of the control samples were provided after the results were collected from 31 European laboratories anonymously, including information of the method used. The results of each participant were provided confidentially, including the identity of each
sample tested. The samples included 9 DENV samples (DENV-1-4), 2 unrelated flaviviral RNA containing samples and one negative sample. Only two laboratories got the correct results in detecting the presence or absence of DENV RNA, our laboratory being one of them (ENIVD, unpublished). The sample information provided by ENIVD showed that the lowest amount of viral RNA in the controls was 250 genomes/ml, corresponding to 25 genome copies in the 100 µl sample which was used in the RNA extraction, eluted into 50 µl, and a total of 5µl was used in a 50 µl reaction volume in our assay. In our assay, this sample repeatedly gave similar results, one of the duplicates turning out positive on late cycles, and the other resulting negative. The sample was considered positive, the result, however, suggested that this level of viral RNA (approximately 2,5 genomes/reaction) in the sample was at the detection limit of the assay. The original sensitivity estimations based on calculations of spectrophotometrically measured total RNA concentrations of the virus supernatants gave results that indicated lower assay sensitivity than what was concluded based on the external controls provided by ENIVD. One reason for this could be the possible traces of cellular RNA in the RNA extracts that could have given falsely high RNA concentrations in relation to the actual viral RNA concentration.

The sensitivities of different real-time PCR based methods are reported as detection levels estimated based on different methods that cannot be directly compared. These include in vitro transcribed RNA controls, numbers of pfu's and calculated numbers of genomes in purified viral RNA. Based on RNA transcripts, the detection sensitivities reported have been in the range of hundreds or thousands genome-copies/reaction (137) and based on pfu's 0,5- 0,002 pfu per reaction (136). The levels reported in patient serum have been reported as high as 10^9 RNA copies/ml (178) and to vary from 0,002-1400000 pfu/ml (135,136). In regard to these values, the estimated level of our assay sensitivity, under 10 genomic copies per reaction, can be considered highly sensitive.
Evaluation of the assay using patient samples

The developed assay was further evaluated using a panel of serum samples from serologically diagnosed dengue patients. Only the samples that gave clear amplification plots (Figure 11) were considered positive. Out of 98 selected early samples, 59/98 gave a positive real-time RT-PCR result. Of these, 43 were confirmed by virus isolation and 12 by a conventional RT-PCR amplification and subsequent sequencing. The results demonstrated that all 4 DENV serotypes were detected with the assay, including different genotypes. In 3 of the real-time RT-PCR positive samples, the result could not be confirmed by virus isolation or other RT-PCR methods, likely due to low amount of RNA or loss of infectivity during the storage. The results from the test evaluations demonstrate the developed DENV RNA detection method is both sensitive and specific for detection of DENV RNA and therefore suitable for diagnostic use.

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**Figure 11.** Amplification plots of three DENV-RNA positive patient samples (in duplicates) and the negative controls in TaqMan real-time one-step RT-PCR.
4. Results and discussion

4.1.3 Comparison of the early diagnostic methods (II)

The diagnosis of dengue infection during the early phase of the disease is complicated, and often paired samples and several methods are required. These methods include IgM detection, which is vulnerable to cross-reactions, and dengue virus specific tests detecting dengue virus RNA and antigens. During the first days of the disease, the IgM test often gives negative results, whereas in most patients viral RNA or NS1 antigen can be detected (179).

The NS1 detection methods have become commercially available relatively recently, and comparative information of DENV NS1 and RNA detection methods remains scarce (180,181). As the performances of different ‘in-house’ DENV RNA detection methods vary, we wanted to compare a commercial NS1 antigen detection method (BIORAD Platelia Dengue NS1 AG EIA) to our own ‘in-house’ TaqMan real-time one-step RT-PCR. The comparison of the available methods was carried out using a panel of 99 selected serologically diagnosed patient serum samples. The aim was to study whether the performances of RNA and NS1 detection methods differed from one another in regard to IgM-EIA. Additionally, the samples were tested by virus isolation and for IgG-antibodies in IFA.

When considering the results simply based on whether the diagnosis was obtained from a single sample, regardless of the information of the sampling time, the IgM-EIA test appeared to be the best method. A positive IgM result was obtained in approximately 80% of the samples followed by NS1 detection (approximately 67%) and RNA detection (approximately 60%). This result is likely to be affected by the time-distribution of the study material, which included mostly samples taken after day 4 following onset of symptoms, which favors the IgM detection. Our study material included altogether 19 samples that were found to be IgM negative. Based on the information of the timing of the sampling after onset of symptoms, these were taken within approximately one week after the onset, with a few exceptions (Table 2). All of these samples were positive in real-
4. Results and discussion

time RT-PCR, and 16 were positive in the NS1 antigen test, demonstrating that RNA detection performed slightly better than NS1 detection in our test material.

The RNA and NS1 detection methods have been shown to be suitable for early diagnosis of dengue infection during the time when the IgM responses are not yet detectable. However, secondary infections have been shown to be problematic for both IgM and NS1 antigen detection, as IgM responses can be very low and pre-existing anti-NS1 antibodies may affect NS1 detection sensitivity. In two of the NS1 negative samples (no. 2 and no.17 in table 2), a secondary infection could have explained the obtained test results. However, secondary infection could also be suspected in sample 13, which had IgG but no IgM on day 3 after onset, but the real-time RT-PCR and NS1 tests were positive.

Our results were in agreement with earlier reports in showing that in general the time span when NS1 can be detected in patient serum is longer than that of DENV RNA. The time for NS1 antigenemia in patient serum also coincided with IgM for a longer period of time than the viral RNA. This would suggest that NS1 detection would be the method of choice over RNA detection, as it is also a very easy method to perform, and currently also available as a rapid test format where the result can be read in 15 minutes without special laboratory equipment. However, the few NS1 negative results in samples that were found positive for DENV RNA raise questions of the sensitivity and performance of the NS1 detection assay, and of the kinetics of NS1 in patient serum. The NS1 antigen detection rates in primary and secondary infections have been variable in different studies, however, also the detection sensitivities of NS1 proteins from different virus sero- and genotypes should be evaluated. The thorough study of individual variation and kinetics of DENV NS1 and RNA in patients requires larger sample panels, and preferably serial samples from each patient. Our study material was small, and selected based on a prior serological diagnosis that may have influenced the obtained results. The study material included 25 samples that were negative in DENV specific tests, and thus the original serological test results could be cross-reactions. Furthermore, the reliability of the information available to us regarding the onset of the disease in some patients, where the diagnostic test results suggested earlier phase of the disease than reported, could be questioned.
It can be concluded that combinations of methods are needed for obtaining the most reliable results. In our sample material the diagnostic rates were higher when IgM results were combined to RNA or NS1 detection, yielding 99% sensitivity for combined RNA and IgM, and nearly 96% sensitivity for the IgM and NS1 combination.

The question of which one of the two DENV specific tests, NS1 or RNA detection, would be a better method to be combined with IgM detection remains to be thoroughly studied with larger sample panels. Additional studies aimed at providing information of the kinetics of viral RNA and NS1 in patient samples would be important and might provide insights to the course of DENV infection in human body. NS1 is a viral non-structural protein that is secreted out of infected cells, thus it is not surprising that NS1 can be detected during viremia. However, it is interesting that it can sometimes also be detected post viremia as defined by virus isolation and RT-PCR. The reasons for this are not known, however, as the viremia and elevated NS1 levels have been associated with increased disease severity, NS1 kinetics may be linked to the course of infection and pathogenesis.
4. Results and discussion

Table 2. Test results of the IgM negative patient samples.

<table>
<thead>
<tr>
<th>IgG titer</th>
<th>Virus isolation</th>
<th>Real-time RT-PCR</th>
<th>NS1 Ag</th>
<th>Days after onset</th>
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<tbody>
<tr>
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<td>+</td>
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<td>4</td>
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<td>13 80</td>
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<td>3</td>
</tr>
<tr>
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<td>+</td>
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<td>+</td>
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<td>15 &lt;10</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>2</td>
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</tr>
<tr>
<td>19 &lt;10</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>5</td>
</tr>
</tbody>
</table>
4. Results and discussion

4.2. Molecular epidemiology of DENV strains

4.2.1 A global collection of DENV from Finnish travelers (III)

Prior this study, no information existed on the sero- or genotypes of dengue viruses infecting Finnish travelers. A virus isolation approach was taken with the aim of isolating and characterizing these viruses. The virus isolation was carried out in parallel in cultured mosquito (C6/36) and mammalian cells (Vero E6), both of which also could support the growth of flaviviruses other than DENV, that could be the possible cause for the observed seropositivity of the patients.

A total of 40 serum samples were selected from patient samples collected in 1999-2005 for the isolation trial, using a criterion of an IgG titer less or equal to 320, and the first available sample from a given patient. The virus isolation trial cells were observed for cytopathic effects, and harvested when necessary. In absence of a CPE, the cells were passaged on day 7 and harvested on day 24. The cells from days 7 and 21 post infection were studied for the presence of viral antigen in immunofluorescence assay (IFA) using DENV type specific MAbs (159). From the IFA positive culture supernatants, RNA was extracted and amplified in DENV typing RT-PCR (127). Based on the typing RT-PCR and MAb IFA, a total of 11 DENV were isolated including 4 DENV-1, 2 DENV-2, 3 DENV-3 and 2 DENV-4 (Table 3). The virus isolation positive samples were obtained from patients aged 22-56, including 6 females and 5 males.

The virus strains differed in their growth pattern in the two cell lines, 5 of the isolates only grew in mosquito cells, and based on the low amount of positive cells seen in IFA, the serum samples either contained a low amount of virus, or the virus strains grew poorly in vitro. 6 of the isolates grew in both cell lines. Based on the proportion of positive cells observed in IFA, only one strain grew equally well in both cell lines, and the rest of the dual tropic viruses appeared to grow better in mammalian than in mosquito cells, as defined by the amount of positive cells
observed in IFA. However, even if some strains seemed to prefer the mammalian cells, none of the strains failed to grow in C6/36 mosquito cells. Our overall isolation results confirm that C6/36 mosquito cells are more sensitive than mammalian cells in isolation of DENV from patient samples (85). The culture properties of some strains preferring mammalian cells is interesting, and merits further investigation as the culture properties could be related to the adaptation to primate hosts and pathogenicity for humans.

The RT-PCR product amplified in dengue virus typing RT-PCR (127) from the C-preM region (454 bp) was sequenced and used for the phylogenetic analysis. The results showed the strains to represent all four serotypes and various genotypes (Figure 13), mostly fitting to the available travel information of the patients (Figure 12). Four DENV-1 viruses were isolated from travelers returning from Asiatic origins, representing two different genotypes, the Asian genotype I and the American-African genotype V (also referred to as genotype III) (182). The two DENV-2 isolates were obtained from travelers returning from Ghana and Sri Lanka, represented by Cosmopolitan and Asian genotypes. The three DENV-3 viruses originated from Cuba, Brazil and Sri Lanka, all representing genotype III. The two DENV-4 isolates from Sri Lanka and Indonesia represented genotypes I and II respectively. Up-to-date information on dengue epidemiology in Africa is currently incomplete, however, recent reports show increasing recognition of DENV in several African countries (183-185). Information obtained from DENV infected travelers can provide up-to-date information on epidemics (186) and novel virus types (187). The first isolation of DENV-2 from Ghana demonstrates the potential of travelers to act as sentinels for DENV in areas that do not have national surveillance systems.
### Table 3. Dengue virus isolates from Finnish travelers.

<table>
<thead>
<tr>
<th>Patient sex and age</th>
<th>Isolate no.</th>
<th>Serotype</th>
<th>Origin country</th>
<th>Year</th>
<th>Cell culture virus isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Vero E6</td>
</tr>
<tr>
<td>F/23</td>
<td>3</td>
<td>DENV-1</td>
<td>Thailand</td>
<td>2002</td>
<td>7</td>
</tr>
<tr>
<td>F/43</td>
<td>4</td>
<td>DENV-1</td>
<td>Malaysia/ Thailand</td>
<td>2002</td>
<td>7, 24</td>
</tr>
<tr>
<td>F/56</td>
<td>8</td>
<td>DENV-1</td>
<td>Thailand</td>
<td>2005</td>
<td>7, 24</td>
</tr>
<tr>
<td>M/31</td>
<td>11</td>
<td>DENV-1</td>
<td>India</td>
<td>2005</td>
<td>5</td>
</tr>
<tr>
<td>M/54</td>
<td>6</td>
<td>DENV-2</td>
<td>Sri Lanka</td>
<td>2003</td>
<td>7, 24</td>
</tr>
<tr>
<td>F/22</td>
<td>9</td>
<td>DENV-2</td>
<td>Ghana</td>
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<td>7</td>
</tr>
<tr>
<td>M/55</td>
<td>2</td>
<td>DENV-3</td>
<td>Cuba</td>
<td>2002</td>
<td>7</td>
</tr>
<tr>
<td>M/26</td>
<td>5</td>
<td>DENV-3</td>
<td>Brazil</td>
<td>2003</td>
<td>7, 24</td>
</tr>
<tr>
<td>F/39</td>
<td>7</td>
<td>DENV-3</td>
<td>Sri Lanka</td>
<td>2004</td>
<td>4</td>
</tr>
<tr>
<td>F/42</td>
<td>1</td>
<td>DENV-4</td>
<td>Sri Lanka</td>
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<td>M/37</td>
<td>10</td>
<td>DENV-4</td>
<td>Indonesia</td>
<td>2005</td>
<td>7, 24</td>
</tr>
</tbody>
</table>

### Figure 12. Origins of DENV isolates from Finnish travelers in 2000-2005. The areas endemic for dengue viruses in 2005 are shaded in grey (modified from CDC, 2005).
Figure 13. Neighbor-joining phylogenetic tree based on C-preM region (454 bp) nucleotide sequences of 70 dengue virus strains. 1000 bootstrap replications were conducted, the support values above 50% are indicated. Isolates from Finnish travelers are marked in grey.
4. Results and discussion

4.2.2 A collection of DENV-2 strains from an endemic country, Venezuela (IV)

Of all the countries in Latin America, Venezuela reports the highest number of DHF cases (188). The major epidemics with DHF have occurred in Venezuela since the 1980s, and have been associated with the introduction of Asiatic-origin DENV-2. Similar to other countries in the region, in Venezuela the Asiatic DENV-2 virtually replaced the serotype that circulated in the area previously, designated as the American genotype DENV-2 (189,190), which was not associated with severe disease (191). Since the introduction of the lineage of Asiatic origin, referred to as American-Asian genotype (192), it has been shown to diversify into different lineages in Venezuela. Additionally, recombinant virus between the Asiatic and American-Asian genotypes has been detected from Venezuela (58).

A collaboration with Venezuelan researchers provided a collection of DENV-2 isolates for this study from Aragua State, Venezuela (Figure 14) which aimed at updating the epidemiological situation of this serotype in Venezuela. Venezuela is currently a hyperendemic region for the four DENV types (193). At the time of the previous phylogenetic study of DENV-2 in Venezuela, including isolates from 1997-2000, DENV-2 was one of the predominant serotypes detected in epidemics and associated with DHF (58). During the last decade, a change in DENV serotype dominance has been observed in Venezuela, and DENV-3 and DENV-1 have surpassed DENV-2 as the most prevalent genotypes. However, DENV-2 is still constantly detected in patients. In this study, the envelope (E) gene sequences were determined for 23 novel DENV-2 strains isolated in 1999-2005 in Aragua State (Figure 15). The sequence analysis included all the available DENV-2 strains from Venezuela including recent sequences until 2008. Additionally, a global collection including representatives of all 6 known genotypes of DENV-2 were included in the analysis.
The results of the sequence analysis suggest that DENV-2 evolution has continued in Venezuela, and all the isolates represented the Asian-American genotype. In comparison to previously published sequences and their analysis, genetic diversification was detectable, as the Venezuelan isolates were now separated into 3 separate lineages (Figure 15). The envelope protein sequence comparisons revealed several amino-acid changes, some of them found exclusively in Venezuelan isolates. The identified virus lineages could not be linked with disease severity, as all lineages included isolates from both DF and DHF cases. All the DENV-2 isolates studied here represented the same genotype, which was already known to circulate in Venezuela. Our results based on isolates from Aragua state suggests, that unlike in several other countries in the South American region, no recent introduction of foreign DENV-2 lineages have occurred in Venezuela. Based on the previous massive DENV-2 epidemics in Venezuela, it could be speculated that the majority of the adult population is likely to be immune to this serotype, leaving only the younger generations vulnerable to DENV-2 infection. The population immunity could restrict the virus transmission and lead to serotype replacement. The majority of the virus isolates studied here originated from young patients aged less than 20 years, however, isolates were also obtained from patents representing older age groups (Table 4). The epidemiological factors enabling the sustained maintenance of several lineages of DENV-2 as minor serotype in the co-circulation of the four serotypes in Venezuela are not currently known. Those could include mechanisms independent from human population immunity, such as the maintenance of the virus in mosquito populations.
Table 4. Venezuelan DENV-2 strains.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Strain name</th>
<th>Month/Year</th>
<th>Grading</th>
<th>Sex</th>
<th>Age</th>
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<tbody>
<tr>
<td>L2d00</td>
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<td>DF</td>
<td>M</td>
<td>8</td>
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<tr>
<td>L5d01</td>
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<td>06/2001</td>
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<td>F</td>
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<td>F</td>
<td>19</td>
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<td>LARD8139</td>
<td>01/2001</td>
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<td>F</td>
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</tr>
<tr>
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<td>LARD26853</td>
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<td>DF</td>
<td>F</td>
<td>15</td>
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<td>L9d01</td>
<td>LARD8336</td>
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<td>LARD26062</td>
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<td>LARD28105</td>
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<td>DF</td>
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Figure 14. Map of the geographical location of Aragua State, Venezuela.
Figure 15. Neighbor-joining phylogenetic tree based on envelope gene sequences of 60 DENV-2. 1000 bootstrap replications were conducted, the support values above 50% are indicated. The 23 Venezuelan DENV-2 isolates sequenced in this study are marked in grey.
Concluding remarks and future prospects

The last decades have shown that even if dengue is not a new disease, in a relatively short time, it has become a major vector-borne viral disease of mankind. Currently, no effective and safe vaccines are available against dengue. The geographical spreading of dengue viruses requires constant monitoring in the risk areas that have suitable mosquito vectors (Ae. aegypti, Ae. albopictus) readily available, including parts of Europe.

The effects of dengue virus infection reach far beyond the endemic areas, as dengue is also increasingly affecting travelers visiting these areas. The popularity of South-East Asian tourist attractions among Finnish travelers have clearly influenced the observed rise in diagnosed cases. However, this study has shown the origins and causative agents of dengue infection in Finnish travelers to include various endemic areas and all DENV serotypes. Within this study, a novel DENV RNA detection method was developed for routine diagnostic use and compared to other early diagnostic tests. With the traveler samples, it was evident that the best diagnostic rates were obtained by combining IgM detection to viral NS1 antigen or RNA detection.

Fortunately, the majority of the infections of travelers are mild and severe disease and fatalities are extremely rare. However, it seems likely that the repeated traveling to endemic areas will increase the occurrence of secondary infections, which may cause more severe disease outcomes in travelers in the future. Currently, no specific drug against dengue is available, however, it is important to diagnose dengue in travelers, as the diagnosis is a prerequisite for the monitoring and hospitalization of the patient in the case that the disease turns worse. Therefore, in the future, rapid and reliable diagnosis for dengue will increasingly be in demand. Additionally, when informed about the potential risks of secondary infections, the patient may take the mosquito prevention measures seriously the next time when traveling in the endemic areas.

The greatest effects of dengue to public health are seen in the areas that are endemic for these viruses. Factors such as the population immunity status and
vector competence are likely to affect DENV serotype prevalence and their co-circulation, however, their effects are not well known. The study of dengue virus strains from the endemic areas, both from travelers or local patients, is therefore important and provides information that could prove useful in the future prevention of the disease. The mapping of the endemic areas and the knowledge of the diversity of the circulating dengue viruses are needed for planning vector control measures and for developing vaccines and drugs. A dengue vaccine is likely to become available in the future, however, it may turn out too expensive for the people who live in the endemic areas. Vector control is an effective measure against dengue, and requires more knowledge than money. Travel agencies have advertised their efforts in taking care of environmental issues caused by tourism in the travel destinations. This could be well extended to vector control efforts in dengue endemic areas, as these measures would prevent dengue and other mosquito-transmitted diseases in both travelers and residents in the area.

The recent developments on dengue diagnosis, such as the NS1 antigen detection in a rapid test format have made the assay itself easy, but still a serum sample is needed as a test material requiring special equipment. The majority of described diagnostic methods for dengue use patient serum as the sample material, although evidence of detectable amounts of viral components and antibodies has been shown from other sample types, such as saliva. Easily collectable, non-invasive sample materials merit further studies on their potential as diagnostic sample material with novel methodologies, such as NS1 antigen and RNA detection.
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Helsinki, September 2010,

Eili Huhtamo
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Original publications