Emergence of dengue and Zika virus: Travellers as sentinels for global epidemics

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"I may not have gone where I intended to go, but I think I have ended up where I intended to be."

- Douglas Adams

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1. LIST OF ORIGINAL PUBLICATIONS

I. Huhtamo E, Korhonen E, Vapalahti O. Imported dengue virus serotype 1 from Madeira to Finland 2012. Euro Surveillance 2013 Feb 21;18(8)

II. Erra E^, Korhonen E^, Voutilainen L^, Huhtamo E, Vapalahti O, Kantele A. Dengue in travelers: Kinetics of viremia and NS1 antigenemia and their associations with disease outcome. PLoS One. 2013 Jun 3;8 (6)

III. Korhonen EM, Huhtamo E, Virtala AMK, Kantele A, Vapalahti O. Approach to non-invasive sampling in dengue diagnostics: exploring virus and NS1 antigen detection in saliva and urine of travelers with dengue. J Clin Virol. 2014 Sep 1.

IV. Korhonen EM, Huhtamo E, Smura T, Kallio-Kokko H, Raassina M, Vapalahti O. Zika virus infection in a traveller returning from the Maldives, June 2015. Euro Surveillance. 2016 Jan 14;21(2).

V. Driggers RW^, Ho CY^, Korhonen EM^, Kuivanen S^, Jääskeläinen AJ, Smura T, Rosenberg A, Hill DA, DeBiasi RL, Vezina G, Timofeev J, Rodriguez FJ, Levanov L, Razak J, Iyengar P, Hennenfent A, Kennedy R, Lanciotti R, du Plessis A, Vapalahti O. Zika Virus Infection with Prolonged Maternal Viremia and Fetal Brain Abnormalities. N Engl J Med. 2016 Mar 30.

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2. ABBREVIATIONS

ADE	antibody dependent enhancement
AGE	agarose gel electrophoresis
ALT	alanine aminotransferase
AST	aspartate aminotransferase
BSL	biosafety level
CDC	Centers for Disease Control and Prevention
CHATV	Chatanga virus
DHF	dengue hemorrhagic fever
DSS	dengue shock syndrome
CHIKV	Chikungunya virus
CPE	cytopathic effect
DENV1-4	dengue virus 1-4
DNA	deoxyribonucleic acid
EEEV	Eastern Equine Encephalitis virus
ELISA	enzyme-linked immunosorbent assay
FCS	foetal calf serum
GBS	Guillain-Barré syndrome
HANKV	Hanko virus
HIV	human immunodeficiency virus
IFA	immunofluorescence assay
ILOV	Ilomantsi virus
INKV	Inkoo virus
LAMV	Lammi virus

MBV	mosquito-borne virus
MEM	minimum essential medium
NGS	next generation sequencing
NKV	no known vector
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PRNT	plaque reduction neutralizing test
RNA	ribonucleic acid
SINV	Sindbis virus
SIT	sterile insect technology
SPOV	Spondweni virus
TAE	tris-acetate-EDTA buffer
TBEV	tick-borne encephalitis virus
TLR	toll like receptor
USUV	Usutu virus
UTR	untranslated region
UV	ultraviolet
VE6	Vero E6 cell line
VEEV	Venezuelan Equine Encephalitis virus
WEEV	Western Equine Encephalitis virus
WHO	World Health Organization
WNV	West Nile virus
YFV	Yellow fever virus
ZIKV	Zika virus

3. ABSTRACT

Dengue virus (DENV) serotypes 1-4 are mosquito-borne flaviviruses that are increasingly being diagnosed from travellers returning from journeys to subtropical and tropical regions. This is partly correlated with increasing numbers of travellers visiting endemic areas, to an increase in outbreak frequencies and to the continuing geographical spread of dengue viruses. At present, 50-100 cases of dengue infections are diagnosed annually in Finland. A mosquito-borne flavivirus closely related to DENV, Zika virus (ZIKV), was, for many years, considered to be a relatively harmless virus found in Africa and Asia, with low case numbers and mild disease associations. Since 2013, however, Zika virus has emerged and swept across Polynesia, the Americas and the Caribbean, causing massive outbreaks with new severe complications becoming apparent. Amongst these new complications, neurological symptoms have been observed in adults and, alarmingly, congenital infections resulting in severe developmental disorders, including microcephaly.

The global expansion of these mosquito-borne flaviviruses is most likely due to a combination of factors including globalization and environmental changes, including the climate change. Such changes have enabled two important vector mosquito species, *Aedes* (*Stegomyia*) *aegypti* and *Aedes* (*Stegomyia*) *albopictus*, to spread into formerly uncolonised areas. This is particularly visible for *Ae. albopictus*, a strongly invasive species originating from Asia, which is currently expanding its distribution in Southern and Central Europe. Temporary populations have been reported from commercial greenhouses in the Netherlands, highlighting the possibility of expansion into more northerly countries. Areas with competent mosquito-vectors are at risk for endemic transmission of mosquito-borne viruses, which can be introduced by viremic travellers. Europe has already experienced limited autochthonous dengue outbreaks during this Millennium in Croatia and France and a major outbreak in Madeira, Portugal in 2012, demonstrating the need for surveillance and rapid diagnosis of infections in returning travellers.

This thesis aimed to seek the best possible practices in dengue and Zika diagnostics by studying and comparing the available methods of choice,

their combinations and different sample materials collected from Finnish traveller patients at different phases of the illness. Additionally, this thesis includes case studies exploring the molecular epidemiology of DENV and ZIKV and detailed characterization of a congenital ZIKV infection patient case.

The detection kinetics of DENV NS1 antigen and RNA was studied from serum, urine and saliva samples. It was observed that NS1 antigen was detectable from the sera of travellers for notably longer periods than reported previously for populations living in endemic areas. A positive correlation between viremia at the time of sampling and the probability and duration of hospitalization were also noted, which is in line with previous observations in endemic populations. The association of viremia to a more severe disease outcome has also been observed in endemic populations. Importantly, urine and saliva were demonstrated to be potential sample materials for DENV diagnostics. Urine, in particular, provides new opportunities for molecular diagnostics of DENV as the time window for detection of viral RNA is notably later during the infection when compared to serum samples. From a patient case, it was observed that also ZIKV RNA detection was successful from urine whereas the serum sample taken at the same time point remained negative. Nowadays, urine is considered to be a suitable sample material in molecular diagnostics of flaviviruses, enabling detection of viral RNA beyond the early days of the infection. This also has significance for molecular epidemiological studies as the wider usage of urine samples as study material in flavivirus research has enabled obtaining more sequence data.

Travellers play a role in spreading the viruses from one area to another, but also can serve as sentinels for studying their global circulation. By studying traveller patients, the DENV strain responsible for the Madeiran outbreak in 2012 was identified as DENV-1, most likely of South American origin. The characterization of a viral sequence from a Finnish traveller suspected of having DENV infection revealed the circulation of ZIKV in the Maldives for the first time. This case was followed by a few additional traveller cases identified elsewhere. Subsequently, the local authorities conducted surveillance studies and confirmed local transmission of ZIKV by detecting the virus from local mosquitoes.

A ZIKV patient case studied in this thesis was among the first reports to provide concrete evidence for the causality between ZIKV infection in a pregnant woman and malformations of central nervous system in the foetus. In this study the virus was isolated from the brain tissue of the foetus. The mother's viremia was noticed to be prolonged, an observation that later studies have confirmed to be a common phenomenon in pregnant women infected with Zika virus. This case study demonstrated that the mother's viremia was detectable weeks before the abnormalities in brain development were visible in ultrasound examinations. The evidence provided by the studies herein, along with other recently published data, have shown that there is a need to update the diagnostic guidelines.

Although further studies are needed to examine the predictive value and possible use of a mother's prolonged viremia in early detection of foetal infection, it seems like correctly timed screening of ZIKV RNA from a mother's blood sample might enable detection of the infection in the foetus in an early phase.

4. TIIVISTELMÄ

Denguevirukset 1-4 (DENV 1-4) kuuluvat hyttysvälitteisiin flaviviruksiin. Denguevirusten aiheuttamia infektioita diagnosoidaan yhä enenevässä määrin matkailijoilla, jotka vierailevat virukselle endeemisillä subtrooppisilla ja trooppisilla alueilla. Todennäköisiä syitä aiempaa korkeampaan ilmaantuvuuteen ovat kaukomatkailun ja epidemioiden yleistyminen sekä denguevirusten laajeneva maantieteellinen levinneisyys. Viime aikoina Suomessa on diagnosoitu noin 50–100 uutta tapausta vuosittain. Denguevirukselle sukua olevan hyttysvälitteisen zikaviruksen (ZIKV) ajateltiin aiemmin olevan varsin harmiton, Afrikassa ja Aasiassa satunnaisia lieväoireisia infektioita aiheuttanut virus. Vuodesta 2013 lähtien zikavirus alkoi levitä nopeasti Polynesian kautta Etelä- ja Väli-Amerikkaan sekä Karibialle, aiheuttaen näillä alueilla massiivisia epidemioita. Näiden epidemioiden yhteydessä zikavirusinfektioiden kliinisessä kuvassa havaittiin merkittäviä uusia tautiassosiaatioita. Zikavirusinfektio liitettiin vakaviin sikiön kehityshäiriöihin. Lisäksi myös aikuisilla potilailla esiintyi vakavia neurologisia oireita.

Hyttysvälitteisten virusten levinneisyysalueiden laajeneminen on liitetty globalisaatioon sekä elinympäristöjen muutoksiin, kuten kaupungistumiseen ja ympäristön, mukaan lukien ilmaston muutokseen. Nämä muutokset ovat mahdollistaneet kahden tärkeän dengue- ja zikavirusta levittävän hyttyslajin, *Aedes* (*Stegomyia*) *aegyptin* ja *Aedes* (*Stegomyia*) *albopictuksen* leviämisen alueille, joilla näitä hyttysiä ei ole aiemmin esiintynyt. Erityisesti aasialaista alkuperää oleva *Aedes albopictus* leviää aggressiivisesti ja on nykyään endeeminen Etelä-Euroopassa sekä osassa Keski-Eurooppaa. Hollannissa on jo havaintoja tilapäisistä kasvihuonepopulaatioista, mikä korostaa näiden hyttyslajien pohjoisemmaksi leviämisen riskiä. Hyttysvälitteisen viruksen kotoperäinen leviäminen uudella alueella on mahdollista esimerkiksi kotiin palaavan matkailijan mukana. Tämä edellyttää, että alueella esiintyy virusvektoreiksi sopivia hyttyslajeja. Tällä vuosituhannella Eurooppa on jo kohdannut kotoperäisiä denguetapauksia Kroatiassa ja Ranskassa sekä merkittävän epidemian Portugalille kuuluvalla Madeiran saarella vuonna 2012. Tämä

korostaa kotiin palaavien matkailijoiden infektioiden pikaisen diagnostiikan ja toisaalta myös monitoroinnin tarvetta.

Tämän väitöskirjatyön tavoitteina oli kehittää dengue- ja zikavirusten diagnostiikkaa tutkimalla ja vertaamalla sekä käytettävissä olevia diagnostisia menetelmiä ja niiden yhdistelmiä että erilaisia näytemateriaaleja. Työhön kerättiin näytteitä suomalaisilta matkailijoilta taudin eri vaiheissa. Väitöskirjatyössä tutkittiin myös potilastapauksia joiden flavivirusinfektioita tyypitettiin ja karakterisoitiin tiedon tuottamiseksi dengue- ja zikavirusten molekyyliepidemiologiasta.

Väitöskirjatyössä tutkittiin denguevirusten NS1-antigeenin ja RNA-osoituksen kinetiikkaa seerumista, virtsasta ja syljestä. NS1 antigeeni oli osoitettavissa matkailijoiden seeruminäytteissä huomattavasti pidempään kuin aiemmin oli raportoitu endeemisillä alueilla. Sairaalahoidon tarve ja kesto korreloivat positiivisesti näytteenottohetken viremian kanssa. Myös endeemisissä populaatioissa on huomattu yhteys viremian ja vakavampien tautimuotojen välillä. Virtsa ja sylki osoittautuivat potentiaalisiksi näytemateriaaleiksi denguevirusten diagnostiikassa. Erityisesti virtsa tarjoaa näytemateriaalina uusia mahdollisuuksia denguevirusten molekyylidiagnostiikkaan, sillä RNA-osoituksen aikaikkuna virtsasta sijoittui huomattavasti myöhempään sairaudenvaiheeseen kuin seerumista. Myös zikaviruksen osalta havaittiin, että zikaviruksen RNA osoitus virtsanäytteestä potilastapauksessa onnistui samanaikaisen seeruminäytteen jäädessä negatiiviseksi. Virtsan virus-RNA-osoitusta voidaan jo nyt käyttää potilasdiagnostiikkaan ja se soveltuu siihen myös taudin myöhäisemmässä vaiheessa. Tällä on merkitystä paitsi diagnostiikassa, myös molekyyliepidemiologisessa tutkimuksessa.

Matkailijat voivat levittää viruksia uusille alueille, mutta mahdollistavat samalla kattavan näytemateriaalin virusten maailmanlaajuisen kiertokulun tutkimiselle. Matkailijanäytteiden avulla oli mahdollista osoittaa, että Madeiralla vuonna 2012 puhjenneen dengue-epidemian oli aiheuttanut DENV-1 virus, joka oli todennäköisesti peräisin Etelä-Amerikasta. Suomalaisella matkailijalla epäillyn dengueinfektion karakterisointi paljasti zikaviruksen esiintymisen Malediiveilla. Tämän potilastapauksen jälkeen diagnosoitiin myös muita matkailijatapauksia. Lisäksi paikalliset

viranomaiset ryhtyivät toimenpiteisiin, joiden tuloksena zikavirus osoitettiin myös paikallisista hyttysistä.

Tähän väitöskirjatyöhön sisältyvä tutkimus oli ensimmäisten joukossa tuottamassa todistusaineistoa äidin zikavirusinfektion ja sikiön keskushermoston kehityshäiriöiden välillä. Tässä tutkimuksessa zikavirus eristettiin sikiön aivokudoksesta ensimmäistä kertaa. Lisäksi todettiin, että äidin viremia pitkittynyt ja oli havaittavissa useita viikkoja ennen kuin sikiön kehityshäiriöt olivat todettavissa kuvantamisella. Tutkimuksen havainto odottavan äidin pitkittyneestä viremiasta on vahvistunut myöhempien selvitysten myötä. Pitkittyneen viremian havaittiin olevan yleinen ilmiö raskaana olevilla, tartunnan saaneilla naisilla. Tämän väitöskirjatyön yhdessä muiden tutkimusten kanssa tuottama näyttö on jo vaikuttanut tämänhetkisiin diagnostisiin suosituksiin. Vaikka lisätutkimuksia vielä tarvitaan, on mahdollista, että oikein ajoitettu zikavirus-RNA:n seulonta äidin verestä voi mahdollistaa sikiön infektion havaitsemisen aikaisessa vaiheessa.

5. LITERATURE REVIEW

5.1 Mosquito-borne viruses

Mosquitoes act as vectors for a large variety of human and veterinary pathogens, including viruses. The mosquito-borne viruses (MBV) responsible for causing human diseases mainly belong to the virus families *Flaviviridae, Togaviridae* and *Bunyaviridae,* and occur worldwide. The most important MBVs, globally, include dengue 1-4 (DENV1-4), Zika (ZIKV), West Nile (WNV), Chikungunya (CHIKV) Yellow fever (YFV) and Japanese encephalitis (JEV) viruses [1,2].

The genomes of these viruses are composed of RNA, which permits a faster rate of evolution than DNA, and therefore DNA viruses, due to the lower fidelity of RNA polymerase than DNA polymerase. Since mutations in a virus genome may lead to changes in features of the virus, for example its pathogenesis or mode of transmission, this may in turn lead to serious outbreaks of a virus or the expansion of a virus' distribution. In spite of this, the necessity for MBVs to survive in two very different hosts (arthropods and vertebrates) is proposed to result in a restricted mutation rate compared to other human pathogenic RNA viruses [3,4].

5.1.1 Mosquito-borne flaviviruses

The family *Flaviviridae* consists of three genera: *Flavivirus*, *Pestivirus* and *Hepacivirus*. This thesis focuses on mosquito-borne members of genus *Flavivirus* (in this thesis term *Flavivirus* refers to genus), which includes mainly arthropod-borne viruses (arboviruses) that mainly infect, and are transmitted by, mosquitoes or ticks (Figure 1.).

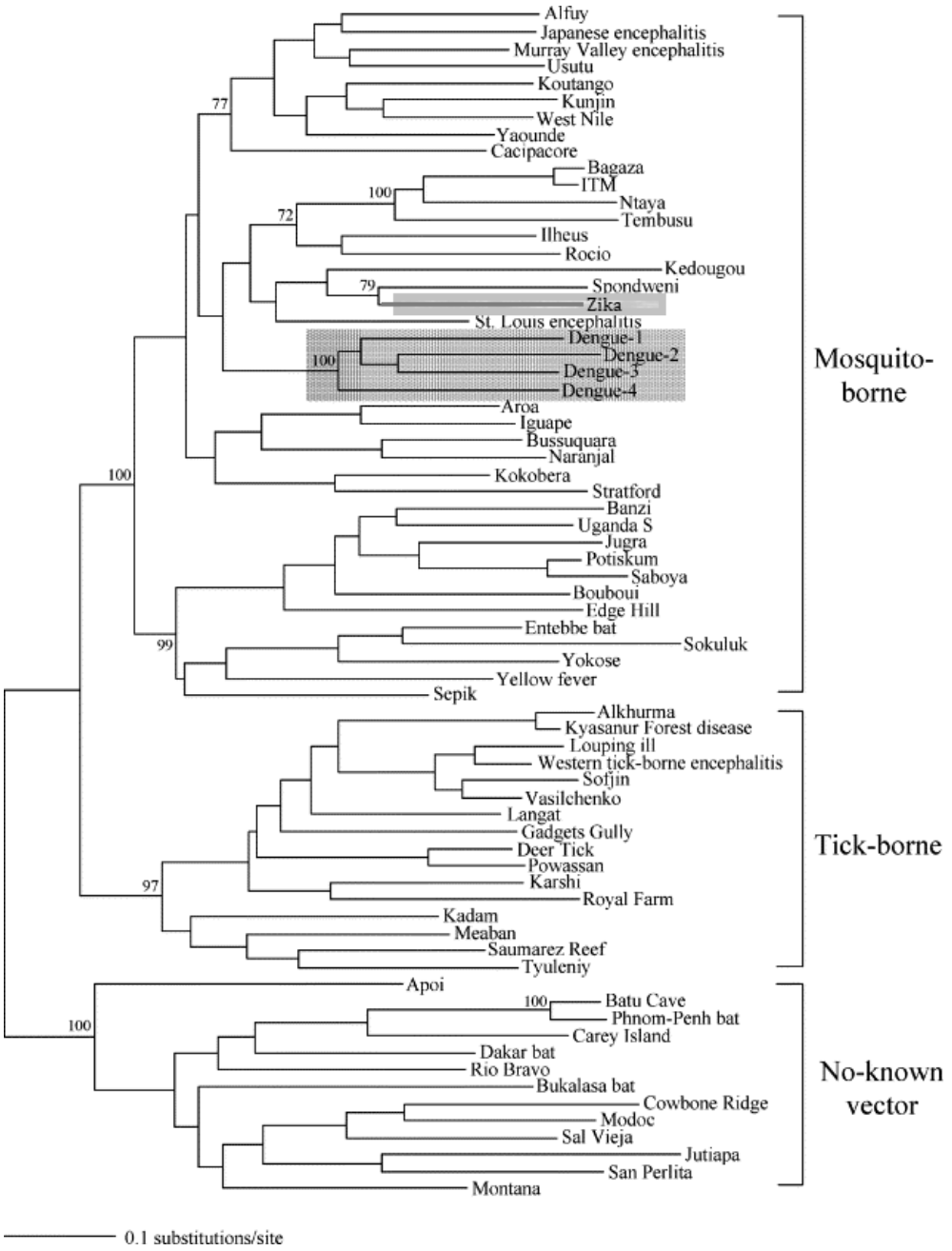


Figure 1. Maximum likelihood (ML) phylogeny based on *NS-5* gene sequences from 70 viruses of the genus *Flavivirus*. Dengue and Zika viruses are highlighted. Modified from Infection, Genetics and Evolution, Vol 3, Holmes EC & Twiddy SS, The origin, emergence and evolutionary genetics of dengue virus, Pages 19-28 No. 1, Copyright (2017), with permission from Elsevier.

Of the 71 species of *Flavivirus* currently reported, 38 species are confirmed to be mosquito-borne, 19 are tick-borne and 16 have no known vector (Figure 1.). Tick-borne viruses compose only one serocomplex and are all closely related, whereas mosquito-borne flaviviruses comprise seven groups (Figure 1.)[5]. Many mosquito-borne and tick-borne flaviviruses are globally important human pathogens, such as YFV, JEV, ZIKV, dengue, and tick-borne encephalitis (TBEV), whereas some have no known association to human disease. A summary of human pathogenic mosquito-borne flaviviruses is shown in Table 1. [1,6,7].

Table 1. Human pathogenic mosquito-borne flaviviruses, the year of isolation, distribution area, host if known, vector and disease association.

Virus	Isolation year	Geographical distribution	Host	Main vector	Disease
Bagaza	1966	Afica	unknown	*Cx.* spp	fever
Banzi	1956	Afica	unknown	*Cx.* spp	fever
Bussuquara	1956	Brazil, Colombia, Panama	unknown	*Cx.* spp	fever
DENV-1	1944	Essentially worldwide	humans	*Ae. aegypti*	fever, rash, shock syndrome
DENV-2	1944	Essentially worldwide	humans	*Ae. aegypti*	fever, rash, shock syndrome
DENV-3	1957	Essentially worldwide	humans	*Ae. aegypti*	fever, rash, shock syndrome
DENV-4	1957	Essentially worldwide	humans	*Ae. aegypti*	fever, rash, shock syndrome
Illheus	1944	South and Central America	birds	*Cx.* spp	fever
Japanese encephalitis	1935	Asia	birds	*Cx. tritaeniorhynchus*	encephalitis
Murray Valley encephalitis	1951	Australia, New Guinea	birds	*Cx. annulirostris*	encephalitis
Ntaya	1943	Africa	unknown	mosquitoes	fever
Rocio	1975	Brazil	birds	mosquitoes	encephalitis
St. Louis encephalitis	1933	The Americas	birds	*Cx.* spp	encephalitis
Sepik	1966	New Guinea	unknown	mosquitoes	fever
Spondweni	1955	Africa	unknown	*Ae. circumluteolus*	fever
Usutu	1959	Africa, South and Central Europe	birds	mosquitoes	fever, rash
Wesselbron	1955	Africa, Asia	unknown	*Ae. spp*	fever
West Nile	1937	Worldwide	birds	mosquitoes	fever, rash, encephalitis
Yellow fever	1927	Africa, South America	non-human primates	*Ae. aegypti*	pantropic
Zika	1947	Africa, Asia, the Americas	non-human primates	*Ae. aegypti*	fever, rash, GBS, abnormalities in fetal brain development

The type species within the genus *Flavivirus* is yellow fever virus (YFV), which originated in Africa about 1500 years ago and spread 300-400 years ago by slave trade to South and Central America but not to Asia. YFV spread rapidly in South America and has now been established to circulate in sylvatic cycle between mosquitoes and non-human primates [8]. The main

urban vector of YFV is *Ae. aegypti*, a very important vector species for several flaviviruses, but other mosquito vectors include species in genus *Aedes* and there are sporadic reports of YFV virus from other mosquito genera and even *Amblyomma* ticks [9,10]. Symptoms of YFV infection include self-limiting febrile disease but the severe infection that develops to approximately 15% of patients, is characterized with hemorrhages, jaundice and abdominal pain. Severe form is fatal in around 50% of cases [10-13].

Mosquito-borne flaviviruses are phylogenetically divided into seven groups (Figure 1.): Aroa virus group, Japanese encephalitis group, Ntaya virus group , Kokobera virus group, dengue virus group, Spondweni virus group with Yellow fever virus group [5].

5.1.2 Mosquito-borne flaviviruses in Europe

WNV is endemic in parts of Southern, Central and Eastern Europe [14-20]. Usutu virus (USUV) has also been reported from South and Central Europe, from mosquitoes and non-human vertebrates in addition to human cases [20-30]. Both WNV and USUV are MBVs that originated in Africa and are transmitted by *Culex* mosquitoes, and thus have different life cycles to dengue and Zika due to their preference for different vertebrate hosts (birds). Of these, WNV has more importance as a human pathogen and is associated with encephalitis, whereas USUV is considered more as an infection of birds[24,31,32]. Several flaviviruses with no known disease associations have been identified from mosquitoes in Europe. Finnish viruses without disease associations include Lammi (LAMV) and Ilomantsi (ILOV) viruses, genetically associated with human pathogenic flaviviruses [33,34] and Hanko (HANKV) virus grouping with insect-specific flaviviruses [34-36]. Even though LAMV and ILOV have not been shown to replicate in certain vertebrate cell lines (including cell lines from monkeys, hamster, canine and human) or in newborn mice that usually are susceptible for MBV infections to date, it remains unclear whether they are able to infect some vertebrate species *in vivo* [33,34].

Dengue viruses have caused several autochthonous, self-limited, outbreaks in Europe. In Greece, the most serious outbreak occurred in the 1920's when over a million cases occurred, and resulted in around 1500 deaths. Prior to this, Greece had also had several, smaller, outbreaks in the late 1800s and early 1900s [37,38]. After decades without any autochthonous cases, outbreaks with just few cases in France (in Nice) and Croatia took place in the 2000s [39-41]. Shortly after, in 2012, a large outbreak with 1080 confirmed cases took place on the Portuguese island of Madeira, though no severe complications or fatalities were reported. [42-44]. Between 2013 and 2015 there were sporadic outbreaks with a few cases in France. For most of the outbreaks outlined above, DENV-1 was the serotype responsible for the human cases, although the sequence data from the French case caused by DENV-2 is lacking. The vector mosquito was *Ae. aegypti* in at least the Greek and Madeiran outbreaks [37,43,45] and *Ae. albopictus* in France [39]. These European outbreaks most likely result from the expansion of the dengue vectors distribution, and therefore the virus distribution into Europe from Asia, increase of travelling to dengue endemic areas and an overall increase in air traffic and therefore human mobility.

5.1.3 Other mosquito-borne viruses

Other mosquito-borne viruses with medical importance include alphaviruses, orthobunyaviruses and members of *Reoviridae*. These viruses are partly present in the same areas as DENV and ZIKV, and symptoms often overlap, making these viruses important for differential diagnostics.

The genus *Alphavirus* in family *Togaviridae* includes many medically important mosquito-borne viruses, which have single-stranded positive sense RNA genomes [46]. The most important human pathogen of this genus, globally, is arguably CHIKV, which is transmitted by several species of *Aedes* mosquitoes. Symptoms of infection reflect those of DENV and ZIKV but severe and often chronic arthalgias are more descriptive of CHIKV infection than DENV and ZIKV. In contrast to these flaviviruses, most of the CHIKV infections are symptomatic [47]. Though the first reports of CHIKV epidemics were made from Tanzania in 1953, it wasn't until after 2004 that it became a widespread problem when a new epidemic strain emerged. This new strain first spread into Asia and Africa, then in 2013 was reported in

the Western hemisphere, later causing outbreaks in Europe [48,49]. Human cases of DENV, ZIKV and CHIKV are transmitted by the same two mosquito species, *Ae. Aegypti* and *Ae. albopictus*, therefore their geographical distributions overlap. [50,51]. In Finland, Sindbis virus (SINV) is transmitted by *Culex* mosquitoes. Sindbis causes a disease in humans with fever, rash and prolonged arthalgias [52]. Despite viruses of which clinical picture is characterized by arthritis, there are alphaviruses causing encephalitis namely western- (WEEV), eastern- (EEEV), Venezuelan (VEEV) equine encephalitis viruses are transmitted by several mosquito species. These viruses are often referred to as New World viruses as their distribution area is the Americas [53].

Most virus species in *Bunyaviridae* infect animals, being either arthropod- or rodent-borne viruses and have segmented, negative sense RNA genomes [54,55]. The genus *Orthobunyavirus* includes many mosquito-borne viruses, including La Crosse, Oropouche and Ngari viruses. The clinical picture of human pathogenic orthobunyaviruses varies and the outcomes include, in addition to self-limiting febrile illness, encephalitis and haemorrhagic fever. Orthobunyaviruses are also important veterinary pathogens, which notably include Schmallenberg virus, which recently spread to Europe [56,57]. In Finland, seroprevalence against Inkoo (INKV) and Chatanga (CHATV) viruses is high. These mosquito-borne orthobunyaviruses are known usually to cause mild self-limiting disease but both are occasionally associated with neurological complications [58].

5.2 DENV and ZIKV: phylogeny and epidemiology

DENV and ZIKV share antigenic similarity despite belonging to different groups (dengue virus group and Spondweni virus groups, respectively). The amino acid sequence of the E gene, the main target of neutralizing antibodies, shares 54-59% similarity between the dengue virus group and ZIKV [59].

Dengue viruses are classified into four serotypes (1–4) that are genetically related but antigenically distinct from each other, sharing 60–70% sequence identity [3,60]. They were first isolated from Japan and Hawaii during the Second World War in 1943 and 1945, despite the fact that the disease had been known for centuries [61]. The Hawaiian virus was designated as DENV-1 and subsequently it was noted that Japanese virus also represented DENV-1. DENV-2 was first isolated from New Guinea [61] while DENV-3 and DENV-4 were later isolated following an outbreak of Dengue in the Philippines in

1956 [62]. All further dengue viruses subsequently fitted into one of these four serotypes until 2013 when a possible fifth serotype was reported. This 'new' serotype was reported to have been isolated from a sample taken from a farmer from Malaysia, Sarawak in 2007 but at the time of writing this thesis no sequence data has been provided to support this claim [63,64]. All serotypes are further classified into genotypes, which share about 92–96% sequence similarity at the nucleotide level, based on their phylogenetic relationships. Genotypes are generally associated with different geographical distributions and they vary in their abilities to cause severe disease [3,65,66].

DENV-1 is classified into five genotypes based on the E gene, or on complete genome sequences [65,67-70]. In addition to genotypes representing epidemic strains, the classification includes the sylvatic genotype, which is represented by two strains originating from Malaysia. The sylvatic origin of these strains has, however, been questioned and it has been suggested that they are possibly spillbacks from humans to monkeys [71,72]. Genotype II comprises old strains (1950-1960s) from Thailand. These strains have not been reported since the 1960s but they may still circulate with low frequency. Asian genotype I consist of strains from across Asia and has caused numerous outbreaks in the area in the 2000s. Genotype IV, or the South Pacific genotype, comprises strains from the West Pacific and Australia. Genotype V represents all strains from the Americas, in addition to African and some Asian strains, and is designated as the American/African genotype [65,67-70].

Based on E gene sequences DENV-2 is classified into five genotypes; two Asian, a Cosmopolitan, an American and an American/ Asian genotype, of which one represents sylvatic strains from West Africa and South East Asia, and four represent epidemic strains. The Asian genotype is divided into two: Asian genotype I, comprising strains from Malaysia and Thailand; and Asian genotype II with strains from Vietnam, China, Taiwan, Sri Lanka and the Philippines. The Cosmopolitan genotype consists of strains with a wide geographical distribution from Pacific and Indian Ocean islands, to the subcontinent of India, Australia, Africa and the Middle East. The American genotype includes, in addition to strains from the Americas until the 1980s, older strains from Pacific islands, the Indian subcontinent and Caribbean area (collected in the 1950s and 1960s). The American/Asian genotype spread from Asia to the Americas in the 1980s and comprises the American strains from the last few decades in addition to strains from Thailand and Vietnam. Introduction of American/Asian genotype to Americas and the replacement of the American genotype by it were followed by DHF

outbreaks in Americas. American/Asian genotype has been linked to more severe disease. [65,73-75].

Classification of DENV-3 into four genotypes is based both on complete genome sequences and on sequences of the prM/E region [76,77]. Genotype I consist of Asian strains (from Indonesia, Malaysia and the Philippines) and strains from islands of the South Pacific. Genotype II comprises Asian strains similar to genotype I but which originate from Thailand, Vietnam and Bangladesh. Genotype III includes strains from Asia (Sri Lanka, India), Africa and Polynesia (Samoa). Genotype IV consists of the American strains and strains from Tahiti [76,77].

DENV-4 is classified into four genotypes based on the E gene, and complete genome sequences [78,79]. Of these, one represents sylvatic strains from Malaysia (genotype IV) and three represent epidemic strains. Genotype I represents Asian strains (Thailand, Sri Lanka, Japan and Philippines) whereas genotype II consists of American and Caribbean strains in addition to Asian (Indonesia and Malaysia) strains. Genotype III includes recent strains from Thailand [79].

DENV is distributed worldwide across the tropics and subtropics, in mainly urban and semi-urban environments. While many areas have varying numbers of serotypes present at any one time, some areas, e.g. South East Asia and Brazil, are hyperendemic for dengue [80,81]. A region is considered hyperendemic when all four serotypes circulate simultaneously.

It has been estimated that 390 million people become infected with dengue annually, and of these approximately 96 million cases are symptomatic. Of all clinical DENV infections approximately 70% occur in Asia [82], but the DENV endemic areas and also the global incidence of DENV have rapidly increased in recent years. The two primary factors behind the expansion of the distribution of dengue are considered to be the introduction of the vector mosquitoes into previously uncolonised areas, which is considered to be the only limiting factor of establishment of dengue, and the importation of viruses via viremic travellers [43,83-86]. Urbanization has also had a great impact on the increase and expansion of dengue. The artificially dense human populations in urban areas create a multitude of microhabitats, which can act as mosquito breeding sites. Vector mosquito species take full advantage of these sites, and dense mosquito populations can thrive. When

dense populations of both humans and mosquitoes inhabit the same environment, the possibility for disease transmission is substantially increased [87]. The two primary vectors of DENV and ZIKV, *Ae. aegypti* and *Ae. albopictus*, are both able to inhabit these urban environments, and therefore can cause high disease burdens in some areas. Dengue is currently considered to be the most important mosquito-borne viral disease, globally, from economic and public health perspectives [82,88]. In 2013 Shepard *et al* estimated the annual global burden of dengue to be 8.89 billion US$. This figure takes into account hospitalised and ambulatory non-fatal cases, non-fatal non-medical cases and fatal cases, of which the hospitalised non-fatal cases accounted for 46% of the total cost. They also observed that the costs of dengue exceed costs incurred by other infectious diseases with comparable data [88,89].

Zika virus was first discovered from Ziika forest, Uganda, where it was isolated from a sentinel monkey in 1947 and from *Ae. africanus* mosquitoes (a forest canopy species) collected from the same area in the early 1960s [90,91]. The first human cases of ZIKV were recognised in Nigeria in the 1950s by observing neutralizing antibodies against ZIKV, and one case by viral isolation during an outbreak of jaundice that was mistakenly thought to be caused by YFV [92,93]. Only sporadic human cases (n=13) were recorded from Africa and South-East Asia from the 1950s until 2007 (Figure 3), when a major outbreak from Yap Island, Micronesia, was reported. In this outbreak, a third of Yap residents were infected with ZIKV. Considering the incidence of the cases prior to the outbreak, the number of infections was unprecedented [94]. Zika then spread to French Polynesia, where it caused outbreak with approximately 32 000 cases in 2013–2014 [95,96]. In 2015 the first reports of ZIKV in the Americas were made from Brazil, after which it rapidly swept across South and Central America and the Caribbean area [97]. To date, ZIKV is distributed across sub-Saharan Africa, South-East Asia, and the Americas (Figure 2) [98].

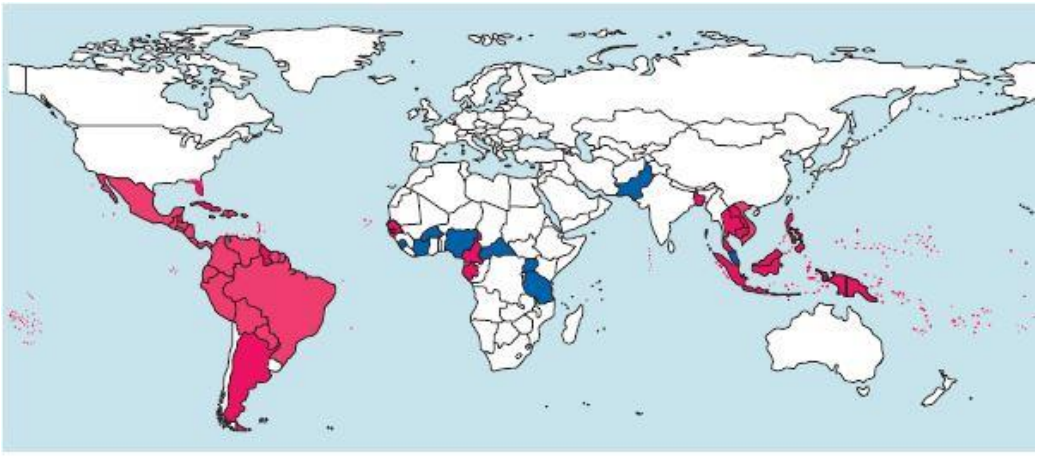


Figure 2. The distribution of ZIKV. Areas coloured blue represent areas with ZIKV cases prior to 2007 and red areas are where Zika emerged after 2007. From Huhtamo E, Jääskeläinen AJ, Sane J, Nohynek H, Vapalahti O.: Zikavirus. Duodecim 2016;132:1521–9. Reprinted with permission from Aikakauskirja Duodecim.

Phylogenetically, ZIKV shows most homology with Spondweni virus (SPOV). Phylogenetic analyses show that there are two lineages of ZIKV; the African lineage and the epidemic Asian lineage. The African lineage has further been classified into West and East African (which includes the prototype MR766 strain from Uganda) lineages [99,100]. Combining the epidemiological data with the phylogeny of ZIKV it has been estimated that the Asian lineage underwent genetic change, which resulted in the epidemic strain with higher virulence [100-102]. The complications associated with ZIKV infection (e.g. microcephaly and Guillain-Barre syndrome) were not observed until the French Polynesian outbreak. Prior to the 2007 Yap Island outbreak, fewer than 20 human cases had been reported in several decades [101].

5.3 Coding strategy, replication and structure of flaviviruses

Flaviviruses are enveloped, positive sense single-stranded RNA viruses, and their genome functions as messenger RNA in a host cell. The virus particles are spherical, with a diameter around 50nm. Genome size varies between

species, but is approximately 11 kb; DENV serotypes vary between 10.6 to 11 kb [103] and ZIKV is 10.7 kb [104].

Flaviviruses utilise receptor-mediated endocytosis in a clathrin-dependent manner to enter host cells. E protein of the virus interacts with the receptor of the host cell's plasma membrane. Dengue virus uses several different receptors for the internalisation, which include glycosaminoglycans, the mannose receptor (MR) of macrophages, the adhesion molecule of dendritic cells (DC-SIGN) and stress-induced proteins [105-107]. Once in a cell, the virion fuses with the endosomal membrane, triggered by acidification of the late endosome, which enables the release of RNA into the cytosol (Figure 3.) [105,108]. After initial translation of the genome, the replication complex is formed in the perinuclear endoplasmic reticulum (ER) followed by RNA replication and protein translation (Figure 3.). Flaviviruses' genomes code for ten proteins; three structural and seven non-structural proteins (Figure 4.). These are translated in the cytoplasm as one large polypeptide and then cleaved post-translationally into single proteins by proteases of both the virus and the host [109,110]. Newly synthesised positive-sense RNA is packed into the capsid protein and assembled into an enveloped virion, formed by prM/E heterodimers. The maturation of the virions occurs in the Golgi apparatus by cleavage of prM protein by furin protease. Partially or fully mature virions are finally secreted out of the cell and go on to infect new host cells to repeat the cycle (Figure 3.) [111].

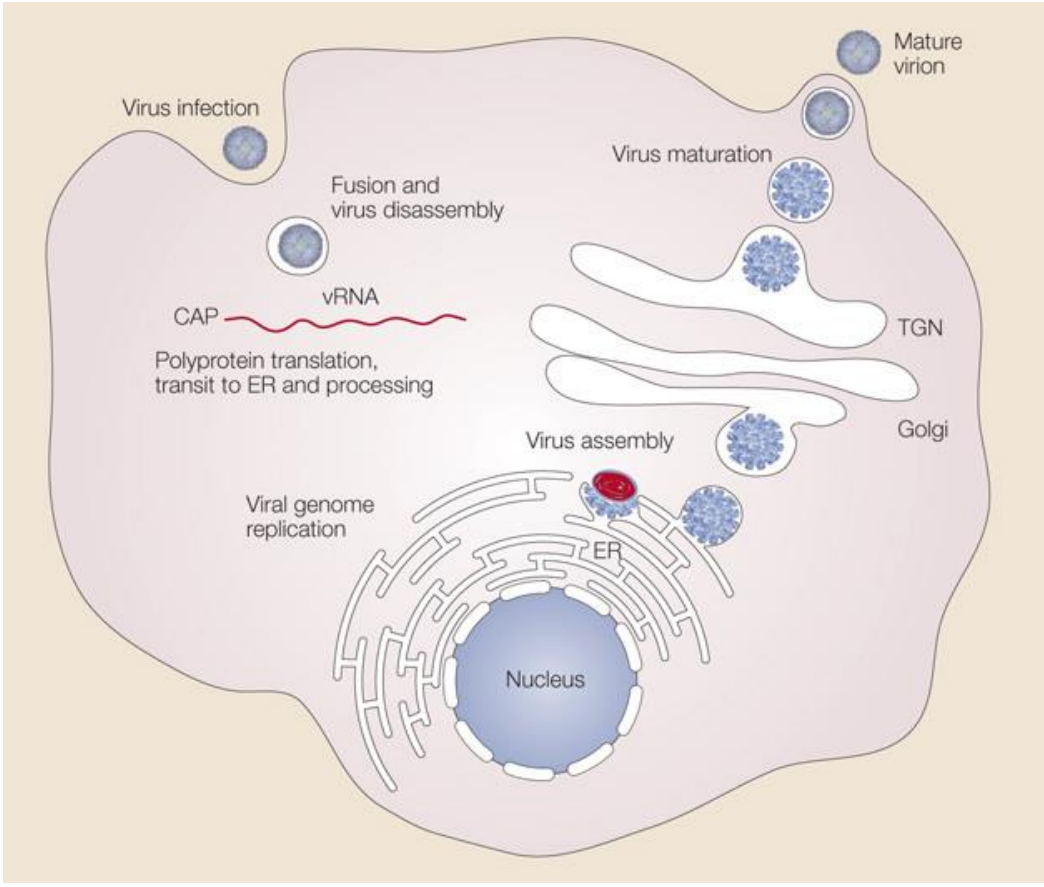


Figure 3. Replication cycle of flaviviruses in mammalian cells. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Microbiology, Mukhopadhyay, S et al, A structural perspective of the flavivirus life cycle, copyright (2005).

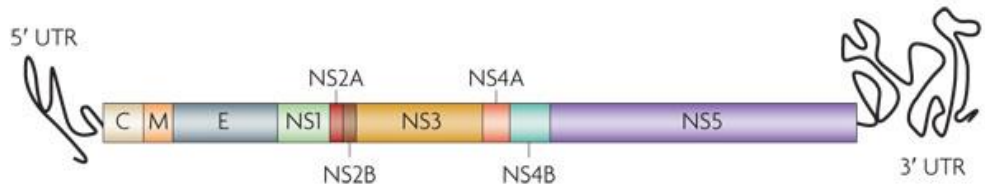


Figure 4. Flavivirus genome organization. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Microbiology, Guzman MG et al, Dengue: A continuing global threat, copyright (2010).

The three structural proteins, capsid (C), membrane (M) and envelope (E) are components of a flavivirus virion. The capsid protein forms the icosahedral capsid (Figure 5.), while the M and E proteins are displayed on the surface of the virion. The M protein is first synthesised as prM, which is then cleaved by furin into to an M protein and a pr peptide as the virion is secreted from the cell (Figure 5.). The E protein interacts with cellular receptors and is considered to be the main target of neutralizing antibodies [112,113].

The seven non-structural (NS) proteins, designated as NS1, NS2A and NS2B, NS3, NS4A and NS4B and Ns5, are present in the infected cells [103]. The role of these NS proteins is at present only partially understood but they are known to mediate virus replication, interplay with host cell and have associations to pathogenic properties of the virus [114]. The open reading frame is flanked with 5' and 3' untranslated regions (UTR). The promoter region of viral RNA lies within the 5' UTR region and these regions include secondary structures that are necessary for replication, translation and pathogenesis in vertebrate cells [103,115,116].

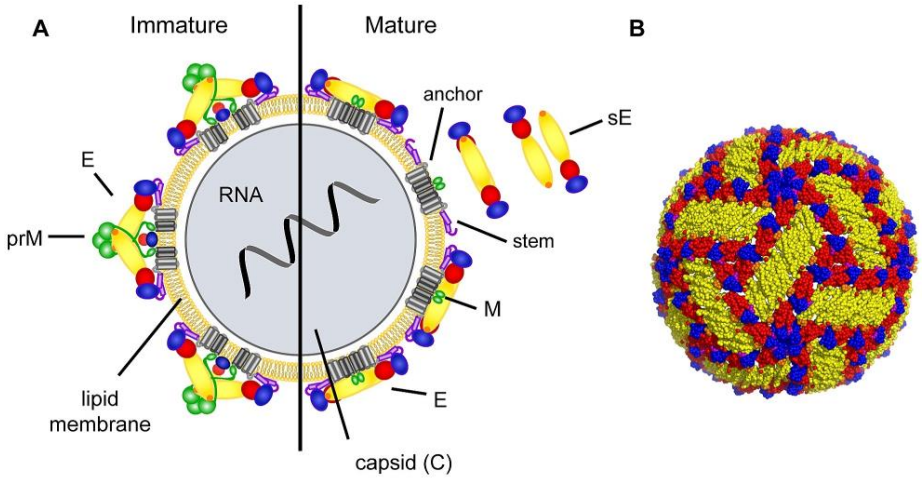


Figure 5. Structure of immature and mature flavivirus particle (Yellow fever). A. A cross-section of the virion showing both an immature (left) and mature (right) virion. Mature particles are formed after cleavage of prM. B. Arrangement of E dimers at the surface of mature dengue 2 virion. Adopted and modified from Vratskikh O et al:. Dissection of antibody specificities induced by yellow fever vaccination. PLoS Pathog. 2013;9(6). Reprinted with permission from PLoS Pathogens.

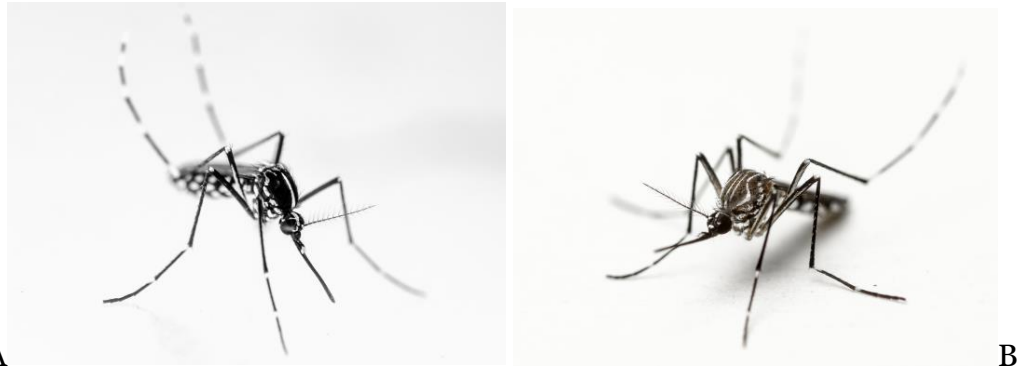
The NS1 protein has many functions in flavivirus infections. These functions have been studied with different flaviviruses and they might not be the same in all flaviviruses. Both the secreted and membrane-bound forms have been shown to elicit an immune response, while the intracellular NS1 plays a crucial role in viral replication. NS1 is represented in three forms; monomeric, dimeric and hexameric. Secreted (hexameric) and membrane-bound (dimeric) forms of the NS1 antigen can interact with the complement components and activate the complement system and thus elicit complement-dependent cytotoxicity in endothelial cells. This might also play a role in antigen dependent enhancement of the DENV infection. [117-121]. Additionally, the NS1 protein has complement inhibiting activity [121]. The NS1 protein has been demonstrated to activate Toll-like receptor 4 (TLR4), which leads to cytokine production and leakage of endothelial cell monolayer, in DENV infections. It has also been demonstrated that there is an association between high NS1 levels in a host's circulation and the enhancement of mosquito infectivity of ZIKV. The cytokines induced by NS1 might significantly contribute to the vascular leakage in severe dengue [122,123]. The extended form of NS1, NS1', is reported in some flaviviruses, namely JEV serogroup and WNV. This extension is produced as a result of -1 ribosomal frameshifting and has been associated with neuroinvasiveness of the virus [120,124-126].

The relatively smaller non-structural proteins NS2A, NS2B, NS4A and NS4B are all hydrophobic and are thus identified as membrane-associated proteins [103]. They have not been shown to possess enzymatic activity and are therefore considered to function via protein-protein or protein-lipid interactions [114]. NS2A plays a role in the processing of NS1 [127], while NS2B is part of a protease complex with NS3 [128].

Among flaviviruses, NS3 and, in particular, NS5 are highly conserved proteins. NS3 has helicase and protease activity, while NS5 plays a role as an RNA-dependent RNA polymerase and is also involved in capping viral RNA [114,129]. NS5 has additionally been shown to play a role in evading the host's immune response by downregulating the interferon response in DENV infections [130].

5.4 Transmission of dengue and Zika viruses

As dengue and Zika viruses are mosquito-borne, their lifecycles include replication in both mosquito and mammalian hosts. They share similar transmission cycles that include the urban cycle between humans and *Aedes*[1] mosquitoes (Diptera: *Culicidae*), mainly *Ae. aegypti* and *Ae. albopictus*, (Figure 6.) which differs from e.g. JE serogroup MB viruses that require a wild vertebrate (birds) amplifying host for maintaining the life cycle, and a sylvatic cycle in the jungle between mosquitoes and non-human primates (Figure 7). Many mosquitoes from the large and cosmopolitan genera *Aedes* and *Culex* can act as vectors for flaviviruses. Viruses transmitted by *Aedes* mosquitoes frequently have transmission cycles between non-human primates and cause hemorrhagic fevers in humans, like DENVs and YFV. In contrast, viruses transmitted by *Culex* mosquitoes typically are transmitted between birds and cause encephalitis-like WNV and JEV when human infection occurs [1].



A B

Figure 6. The primary vector species of DENV and ZIKV. A. *Ae.* (*Stg.*) *albopictus*, and B. *Ae.* (*Stg.*) *aegypti*. Photographs courtesy of Anders Lindström, SVA.

The sylvatic dengue cycle, between mosquitoes and primates in the jungle (Figure 7.), is well studied. Sylvatic cycles of DENV-2 have been shown to occur in West Africa, Senegal, [131-133] South East Asia, in peninsular Malaysia,

[1] This thesis follows the mosquito classification of Wilkerson, et al (2015) rather than the earlier classification of Reinert et al (2009).

whereas sylvatic cycles of DENV-1 and DENV-4 have been recorded only from South East Asia [66,74,134]. Sylvatic strains of DENV-3 have not been isolated to date, but serological data indicate that sylvatic DENV-3 also circulates between primates in the Malaysian jungle of South East Asia [66]. Sylvatic cycles of DENV in the Americas has not as yet been proven but serological data from members of an indigenous Indian tribe in Bolivia living in a restricted area with no presence of *Ae. aegypti*, may suggest that sylvatic transmission of dengue is possible [72,135]. The mosquitoes transmitting the virus between non-human primates in sylvatic dengue cycles are arboreal species, in Africa *Ae.* (*Stg.*) *luteocephalus* and *Ae.* (*Dic.*) *furcifer* and in South East Asia *Ae.* (*Dow.*) *niveus* [3] and the main reservoirs in Africa include the Patas monkey (*Erythrocebus patas*), African green monkey (*Chlorocebus sabaeus*) and Guinea baboon (*Papio papio*), whereas in Asia the primary hosts of sylvatic cycle seem to be cynomolgus macaques (*Macaca fascicularis*), Southern pig-tailed macaques (*Macaca nemestrina*) and silvered leaf monkeys (*Presbytis cristata*) [3].

The sylvatic cycle of ZIKV has been documented in West Africa with isolations from non-human primates and the sylvatic *Aedes* mosquitoes collected from the forest canopy and floor [136,137]. Serosurveys have also recovered antibodies against ZIKV in African non-human primates [138]. To date there is no evidence of an Asian ZIKV sylvatic life cycle, but it cannot be excluded as the surveillance of arboviruses in Asia is not comprehensive [137].

The main vector in urban life cycle of DENV and ZIKV is *Ae. aegypti*. Dengue viruses are also transmitted by *Ae. albopictus*, which has only recently been shown to be susceptible for ZIKV replication. ZIKV has been found in the saliva of *Ae. albopictus* experimentally infected with the virus [139] and ZIKV RNA was detected in similar proportions as DENV RNA from *Ae. albopictus* during the 2007 DENV outbreak in Gabon [140]. Even though *Ae. aegypti* and *Ae. albopictus* are established vectors of Zika virus, Chouin-Carneiro et al. demonstrated that the vector competence of both species for the Asian genotype of the virus was lower than expected. Among the mosquitoes tested, a low proportion were able to transmit Zika virus yet the transmission rates of the mosquito populations from different geographical regions varied [141]. ZIKV RNA has also been detected from other *Aedes* species and some of these have been found susceptible for

ZIKV; the role of these species in ZIKV transmission remains to be studied [94,142]. Yet there is cumulating evidence for the vector-competence of *Cx. quinqefasciatus* for ZIKV, other *Cx.* species have not been demonstrated to be able to transmit ZIKV [94,143,144].

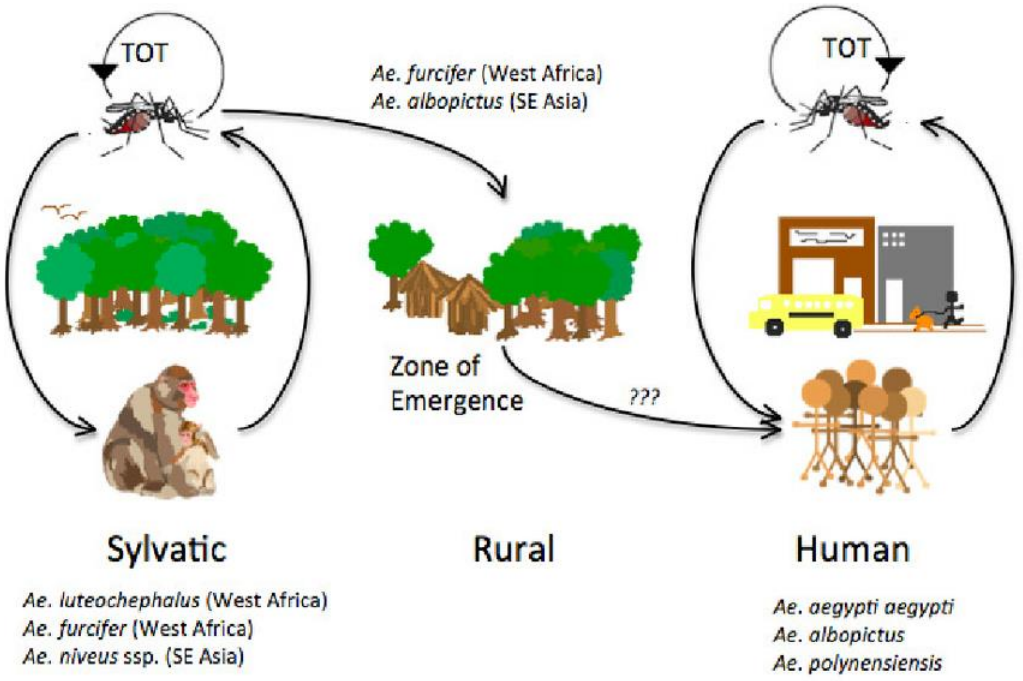


Figure 7. Sylvatic and urban transmission cycles of DENV. From Chen R & Vasilakis N: *Dengue--quo tu et quo vadis?*. Viruses 2011 Sep;3(9):1562-608. Reprinted with permission from MDPI.

In addition to vector-borne transmission, Zika virus is efficiently transmitted both prenatally and sexually. This feature is remarkable within genus *Flavivirus* as the main transmission routes of these viruses are via arthropod vectors. Even though cases of vertical transmission of DENV from mother to neonate have been reported, these reports have been incidental [145-148]. If a mother is infected with ZIKV then the foetus is predisposed to contract the infection. The risk of prenatal transmission and the complications of infection for the foetus seem to be greatest during the first trimester, though both transmission and foetal damage can occur during any trimester [149-151]. In addition to prenatal transmission, Besnard et

al (2014) reported perinatal transmission of ZIKV prior to current outbreak [152]. Prenatal and sexual transmission make ZIKV extraordinary in the genus *Flavivirus* as none of the other members are known to be transmitted via these routes with same efficiency and significance. Another remarkable feature is the prolonged excretion of ZIKV to semen in infected males up to six months post-infection suggesting persistent infection [153-156].

When the virus is prenatally transmitted from mother to foetus a persistent infection may also occur in the foetus [157,158]. Although transmission via breastfeeding has not yet been reported, ZIKV has nonetheless been detected from breast milk [159,160]. The experiments with rhesus macaques showed low infectivity of ZIKV of oral secretions, yet there is a risk of mucosal route of transmission [161]. Transmission has also been demonstrated via blood transfusions [162-164], and via monkey bites or laboratory infections [165-167]. Reports for other flaviviruses describe other, rare, routes of transmission, such as blood transfusions, needle-stick injuries and organ transplants [168-173]. WNV is known to be transmitted via blood transfusions and blood donors have been screened for WNV in United States since 2003 [171,174] TBEV, a tick-borne flavivirus, is known to be transmitted via unpasteurised goat milk and to have caused occasional milk-borne outbreaks [175-177]. In addition, transmission via blood transfusion has been reported for TBEV [178].

5.5 Clinical manifestations and pathogenesis of DENV and ZIKV infections

The clinical manifestations of acute DENV and ZIKV infections, including fever, rash, myalgias, resemble one another as well as CHIKV infection. For DENV and ZIKV infections the clinical picture varies from asymptomatic infection to serious and even life-threatening complications. Despite many similarities in the clinical pictures, the severe manifestations of DENV and ZIKV infections are different. In ZIKV infection, the severe complications are associated with neurological symptoms (brain abnormalities in foetuses and Guillain-Barre syndrome in adults), whereas the hallmark of severe dengue infection is characterized with plasma leakage due to increased vascular permeability leading possibly to hypovolemic shock.

Dengue infection is often asymptomatic or exhibits symptoms resembling a mild, flu-like disease. The classical form of dengue infection, dengue fever, originally termed breakbone fever, is characterized by fever, rash, headache and severe muscle pain (Table 2) [179,180]. The incubation period of DENV infection varies from 3–10 days and the viremic phase often sets in before symptoms occur [181]. Severe complications of dengue infection include plasma leakage, hemorrhages and organ impairment (Table 2). This condition is classified as severe dengue by the WHO (former classified in dengue hemorrhagic fever, DHF, and dengue shock syndrome, DSS) and it may be fatal. The critical point is defeverscence 3–7 days after onset of initial symptoms. Warning signs to severe dengue include severe abdominal pain, persistent vomiting, hematemesis, rapid breathing, bleeding gums, fatigue and restlessness. It has been estimated that 1–2% of human infections develop into the severe forms with fatality rate of 5%. Fatal cases are often caused by shock due to plasma leakage and subsequent hypovolemia (Table 2.). Maintenance of the patient's body fluid volume is thus essential in treatment of severe dengue [180,182].

Table 2. Clinical manifestations of different forms of DENV infections. The classification according to WHO [180].

Form of disease	Clinical manifestations
Mild dengue	unspecific, flu-like symptoms
Dengue fever	high fever, headache, ocular pain, muscle and joint pains, nausea, vomiting, swollen glands, rash
Dengue with warning signs	Abdominal pain, persistent vomiting, mucosal bleeding, fluid accumulation, lethargy, restlessness
Severe dengue	plasma leakage, fluid accumulation, respiratory distress, severe bleeding, organ impairment

The mechanisms underlying severe dengue are complicated and still the subject of research. Many immunopathogenetic mechanisms have been identified underlying behind severe dengue symptoms. Non-neutralizing, infection-enhancing antibodies promote the intake of the viruses by phagocytic cells like dendritic cells and macrophages through Fc-receptors leading to enhanced infection of these cells. Plasma leakage is thought to be mediated by the TLR-4 activated cytokine storm and NS1 triggered complement system. Chymases, mediators produced by mast cells, have also been associated with increase in vascular permeability during severe dengue infection [59,183].

Infection by one serotype of DENV gives a lifelong immunity against subsequent infections by that serotype but only short-term (2–3 months) immunity against the other serotypes [184]. Previous infection with another serotype of DENV is actually considered to predispose the patient to a more severe infection via antigen dependent enhancement (ADE) [185-188]. In this phenomenon, existing but non-neutralizing antibodies invigorate the secondary infection by enhancing the uptake of DENV to cells with Fc receptors (monocytes, macrophages), which display the major replication site of DENV *in vivo* [188,189]. It has been suggested based on functional studies that immature DENV particles are non-infectious, yet antibodies against prM (the uncleaved M protein, present in immature virus particles) are shown to enhance the infection. However, it was shown that in the presence of prM antibodies, primarily in secondary infections, immature virus particles became as infectious as mature virion. Subsequently, study by Richter *et al* showed the ability of immature DENV particles to infect immature human dendritic cells via DC-SIGN molecule in primary infections. [189-191]. Children born to mothers with prior exposure to DENV, who are themselves later exposed to the virus, are at risk while they still carry maternal antibodies during their first few months [192]. Even though the severe forms of dengue are often associated with secondary infections, they can occur also in primary infections. In Finland one case of lethal primary dengue infection has been reported [193].

Pathogenesis of DENV infections is not yet fully understood, yet it is known that properties of both the virus and the host have an effect in the outcome of the infection. Some of the sero- and especially genotypes of the viruses are more often associated with the severe forms of the infection than others [194-198]. Host-dependent factors associated with higher risk for severe dengue include being young, female, having a high body-mass index and the host's genetics [199-201]. As described above, secondary infection is known to be a predisposing factor for severe forms of dengue. Yet, no associations of tertiary infections and severe dengue have been observed and it has been suggested that antibodies produced due to secondary infection would provide neutralizing protection even to serotypes other than the initial infecting strain [112]. The scene between endemic countries and travellers from non-endemic areas differs greatly: in endemic areas primary cases are mostly seen in children while adults have secondary infections; whereas

within travellers the patients are usually adults who develop primary infection.

Zika virus infection, until recently, was considered to resemble a mild version of DENV. The symptoms are often very similar in both infections but with notable differences: plasma leakage in severe cases is not characteristic for ZIKV infection, whereas conjunctivitis is typical for Zika rather than dengue. Up to 80% of ZIKV infections are asymptomatic and usually the possible symptoms are quite mild and the disease self-limiting with symptoms like fever, rash, conjunctivitis and arthalgias. In 2015, during the epidemic in South America, the number of cases of foetal microcephaly increased by 20-fold. This quickly raised questions of a possible association between maternal ZIKV infection and foetal microcephaly, with rapid investigations confirming the association [149,150,202,203]. Later, it was discovered that the microcephaly cases are just part of the myriad of foetal developmental disorders caused by Zika, jointly called Zika congenital syndrome. Complications of foetal ZIKV infection include spontaneous abortion, stillbirth, hydranencephaly and other neurological, ophthalmologic, and auditory manifestations in addition to microcephaly [150,151,157,204-211]. Abnormalities in the placenta, umbilical cord and amniotic fluid have also been reported in pregnant women with ZIKV infections [149,151]. Subsequent studies have observed microcephaly and other neural abnormalities in the epidemics of French Polynesia between 2013–2014 [212,213].

In addition to foetal abnormalities, ZIKV also causes neurological symptoms in adults. An increase in the number of cases of Guillain-Barré syndrome (GBS) occurred during the outbreak in French Polynesia in 2013 [214,215] and 2015 in South America [216-218]. Guillain-Barré syndrome is a disorder in which the immune system targets parts of the peripheral nervous system and symptoms include weakness or tingling sensations in the legs progressing towards the upper body. In the severe form of the syndrome the patient becomes almost totally paralyzed and the condition can be life threatening. In most cases, a spontaneous recovery occurs, but some patients may develop chronic manifestations. Data based on French Polynesia outbreak suggest a risk of GBS at a rate of 2.4 per 10 000 ZIKV infections [219]. In GBS cases due to ZIKV infection, symptoms have been observed to manifest already during or immediately after the acute

infection which is atypical for GBS related to infections in general and prolonged viruria has been associated with this complication [217].

Other severe neurological manifestations associated with ZIKV infections are rare and mostly associated with other, chronic, health disorders. Cases with meningoencephalitis, encephalopathy and myelitis have been reported sporadically [220,221]. Sporadic lethal ZIKV cases have been reported amongst adults but these are associated with co-morbidities [221,222].

DENV and ZIKV can cause co-infections [223]. This may be especially significant since existing non-neutralizing antibodies against DENV have been shown to enhance ZIKV infection by promoting ADE and a correlation between ZIKV related GBS and previous DENV infection has been observed [187,217,224]. This suggests that prior dengue infections might predispose to more severe outcome in subsequential ZIKV infection, yet the most recent studies show controversial results [225,226]. This still is a phenomenon to be considered when developing vaccines against these diseases. The true significance of this phenomenon is currently unknown, but given the subject further research is expected during the next few years.

5.6 Laboratory diagnostics of DENV and ZIKV infections

As the symptoms of infections caused by ZIKV or DENV resemble each other and these viruses co-circulate in several geographical regions, these infections cannot be distinguished on the basis of the clinical picture of the disease. Clinical manifestations of other febrile vector-borne diseases, particularly CHIKV or malaria, may likewise, resemble the clinical course of DENV and ZIKV infections and a laboratory confirmation is required for diagnosis. The diagnostic criteria of WHO for DENV is either detection of the virus by virus isolation or viral RNA or seroconversion of IgM or IgG antibodies in paired sera samples (or fourfold rise in IgG titer) [227].

Table 3. Summary of methods in diagnostics of DENV and ZIKV infections from serum. *in traveller population longer detection of NS1 Ag has been reported [228], ^cross-reactions with other flaviviruses and previous flavivirus vaccines

	Dengue			Zika		
Method	Benefits	Disadvantages	Timing (after onset of symptoms)	Benefits	Disadvantages	Timing (after onset of symptoms)
Virus isolation	Golden standard, confirmation of the infecting virus	Slow, demands BSL-3 facilities and a lot of hands-on work	Days 1-7	Same as with dengue	Same as with dengue	Days 1-14 (?)
Viral RNA detection	Virus specific, fast	Expensive	Days 1-7	Same as with dengue	Same as with dengue	Days 1-14, with pregnant women prolonged period
NS1 Ag detection	Specific, easy, fast	Variation of sensitivity in secondary infections and possibly between serotypes	Days 1-8 (15) dependent on the population*	Not available	Not available	Unknown
IgM antibody detection	Low costs, easy, fast	Cross-reactions, not positive during the first few days	After 3-4 days, up to 3 months	Rather specific, easy, inexpensive	Cross-reactions	After 7-8 days, up to 3 months
IgG antibody detection	Low costs, easy, fast	Not detectable in acute phase, cross-reactions^	After 6-7 days	Same as with dengue	Same as with dengue	After a week

Virus isolation

Viral isolation is no longer in routinely used for diagnostics since the method is slow and requires special facilities, demanding manual techniques and skilled personnel (Table 3.). In research, by contrast, viral isolation is still widely used as it is considered as a golden standard for confirmation and typing of the infecting virus.

The traditional approach to virus isolation of arboviruses has been culturing in mouse brain, a method which is still in use [229]. Generally, flaviviruses are grown in several vertebrate or mosquito cell lines. Vero cells (from green monkey kidney epithelium), LLC-MK2 (rhesus monkey kidney) and BHK-21 (baby hamster kidney) of mammalian origin cells and mosquito C6/36 *Aedes albopictus* cell lines are frequently used in isolation trials of MBVs [227,230,231].

Antibody detection

Measurement of the IgM and IgG class antibody response against DENV and ZIKV infections is widely used in diagnostics. Methods for the antibody

detection include enzyme-linked immunosorbent assay (ELISA) and immunofluorescence assay (IFA) based methods and immunochromatographic rapid tests. Specific IgM antibodies are detectable in primary DENV infections in 4-5 days (Figure 8.) and remain in the circulation at detectable levels for up to 3 months (Figure 8.); specific IgG antibodies are found in about seven days after onset of illness yet may remain detectable even for decades (Figure 8.). In secondary infections IgM class antibodies are not always measurable at all while IgG antibodies are produced within couple of days after onset of illness in levels higher than those seen after primary infection [112,227]. The kinetics of antibodies against ZIKV or markers associating with ZIKV infection is not thoroughly understood to date yet comparisons of DENV infection markers and their kinetics have revealed some differences.. RNA detection from serum suggests that the viremic phase of ZIKV infection is longer, up to two weeks after onset of illness compared to week in DENV (Figure 8.) infections. In ZIKV infections IgM detection is recommended since day four after onset of symptoms, yet there is gathering evidence that IgM is reliably detectable approximately 7-8 days after onset of illness [232-235]. Lustig *et al* reported ZIKV IgM detection from day two and IgG detection from day five after onset of symptoms in traveller population [236]. Commercial tests to detect antibodies against ZIKV have been developed [237,238]; antibody tests for detection of IgM against ZIKV are available from several manufacturers (Diasorin Incorporated, Inbios International, EuroImmun).

The differential diagnostics between different flaviviruses, such as DENVs and Zika, is complicated by significant cross-reactivity between these related viruses and, thus, methods other than antibody detection are often required. Also prior vaccinations against other flaviviruses (YFV, JEV, TBEV) may result in false positive results in IgG serology. Commercial anti-DENV IgM tests have proved to give false positive results from patients with ZIKV infections [239-242]. Tests to detect ZIKV IgM have been shown to have high specificity, yet shortcomings in sensitivity of some tests have been observed [243].

The identification of the aetiological agent is difficult and often even impossible on the basis of the antibody tests, neutralization assays excluded. Neutralization assays are based on the neutralizing antibodies in the patient serum interacting with the virus. The interaction inhibits

attachment of the virus to cells. These assays provide a specific tool for convalescent phase of the infection. Yet neutralization assays require facilities and skilled personnel as they include lots of steps, are slow, demand a lot of hands on work, cell culture work and BSL facilities. and are though not in routine diagnostic use [244,245]. The plaque reduction neutralizing test (PRNT) has traditionally been considered as a golden standard in the differential diagnostics of flaviviruses. In addition to neutralization assays detection of the nucleic acids and the virus isolation are the most reliable methods for differential diagnostics.

NS1 antigen detection

In dengue infections the NS1 protein is secreted into serum and is therefore often detectable during the early days of the disease (Figure 8.). The benefits of the NS1 antigen detection include detectability in early phase of the disease, the specificity, simplicity of testing and the stability of NS1 protein compared to RNA. NS1 antigen tests are either ELISA or immunochromatographic rapid tests which do not require special facilities and are easy to conduct. Moreover, it has been reported that especially in primary DENV cases the NS1 ag is detectable notably longer than viral RNA [246]. However, there are some reports of NS1 detection being less sensitive in secondary infections [247,248]. Pre-existing antibodies in secondary infections could interfere with the antigen detection assay by forming antigen-antibody complexes and they may also influence in more rapid clearance of the NS1 antigenemia in secondary infections [248]. In addition to ELISA and immunochromatographic methods, rapid tests for detection of DENV NS1 Ag have been developed and methods based on immunosensors and detection of optomagnetic nanoclusters and fluorescent nanoparticles utilizing immunospot assay are under development [249-251].

Even though NS1 antigen test has been considered as a DENV specific method, some DENV NS1 Ag rapid tests have given false positive results in ZIKV infections [252]. To date, the proof-of-concept of utilization of ZIKV NS1 antigen tests in diagnostics remains to be shown and there are not yet commercial tests available for the detection of ZIKV NS1 Ag. In dengue

infections, NS1 antigen has been shown to be secreted to urine and saliva in addition to serum [253-255] which enables non-invasive sampling. In ZIKV infections the usability and secretion to different sample materials of NS1 antigen has to be investigated further.

Detection of viral nucleic acids

There are many assays developed, both conventional RT-PCRs and also real-time RT-PCRs to detect dengue and ZIKV RNA and also the variation in target regions is wide. RNA detection requires considerably more from the infrastructure and personnel of the laboratory when compared with serological methods. The detection of DENV RNA is recommended from samples taken within the first week of illness, whereas in ZIKV infections RNA detection from serum and urine is recommended for the first 14 days after onset of symptoms (Figure 8.) [99,239,256-258] It has though been observed that the level of viremia is greater in primary than secondary DENV infections [248,259,260]. Whole blood has also been reported as a sample material that contains detectable levels of ZIKV RNA for long periods, up to two months, on the contrary to DENV infections, in which serum is considered preferable sample material over whole blood [261-263]. The viral RNA levels of saliva samples of ZIKV patients have been reported to be higher compared to serum yet the timing of the presence of RNA in both samples does not seem to differ [264]. However Paz-Bailey et al found detection of ZIKV RNA in saliva rather infrequent when compared to serum [233]. Within pregnant women with ZIKV infection the duration of viremia has been shown to be prolonged. The viral level in the mother's serum (but not in urine) stays stable up to 14 weeks after onset of symptoms; the viral load has been reported to be higher in the amniotic fluid than in mother's serum. Neutralizing antibodies in the maternal serum have also been detected [157,265,266].

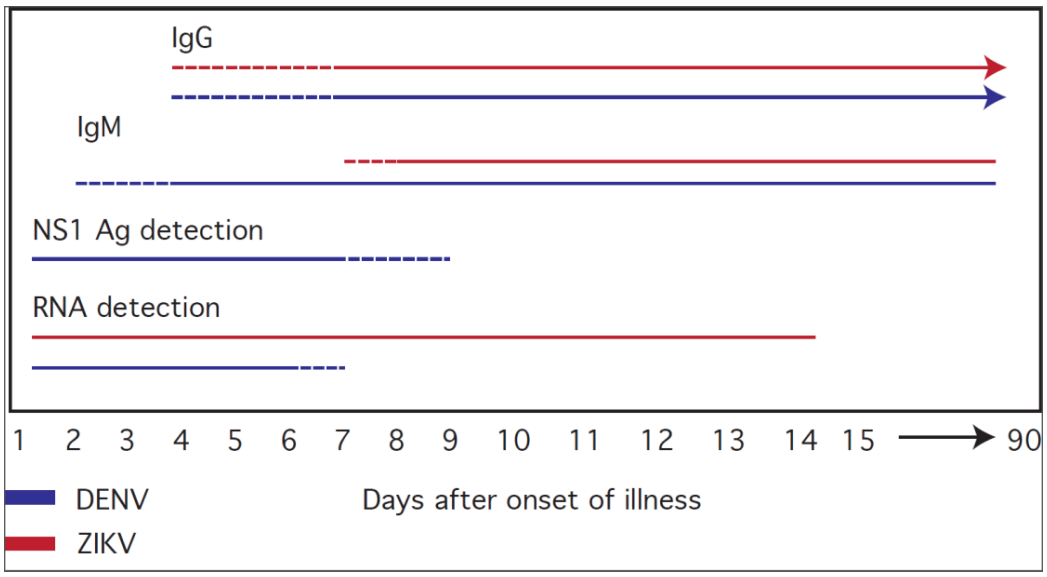


Figure 8. Timing of different diagnostic methods in primary DENV and ZIKV infections. Currently the usability and kinetics of NS1 Ag in ZIKV infections is still unclear.

There is no single assay that would reliably detect infection with DENV or ZIKV throughout the clinical course of the disease but a combination of tests targeted to the early phase and convalescent phase are needed. In the acute phase of the disease, approximately days 1-4 after onset of symptoms, methods detecting the virus or its components (RNA or in case of DENV NS1 antigen) are the preferred choices. In convalescent phase, approximately from day 5 on, methods detecting antibodies (with IgG from day 7 on) are recommended to supplement the direct detection of the virus. A combination of NS1 antigen testing with detection of IgM class antibody tests is widely used in DENV diagnostics [267]. This combination enables detection of the infection in the early phase but also in later phase, when viremia has passed. In ZIKV diagnostics detection of viral nucleic acids is combined with antibody (especially IgM) detection. Yet, in dengue endemic regions the role of ZIKV IgM testing is hampered by cross-reacting antibodies against DENV.

5.7 Prevention of DENV and ZIKV infections

There is no specific treatment for DENV or ZIKV infection so the actions are taken towards prevention. There are two main strategies; vector control and vaccine development. The eradication of the vector mosquitoes has been attempted but has not proven a successful strategy in the long term. In many countries of South America, for instance Brazil, Colombia and Bolivia, *Ae. aegypti* was eradicated with quite success in the large campaign during 1947-1970 [268]. This campaign was extremely efficient and only one DENV-2 genotype was circulating in the countries with eradication campaign until 1970s [269]. The success of the campaign greatly relied upon the utilization of DDT but as it turned out that DDT is carcinogenic, its use was eventually banned in 1970s [268,270]. As the program waned, *Ae. aegypti* was rapidly re-introduced and spread even to regions it had not been present previously. To date, it has been highlighted that massive spraying of the insecticides does not have notable impact in dengue transmission [271]. One way is to remove all the possible breeding sites, namely stagnant water around houses and elsewhere. Also the utilization of larvicides helps in controlling the mosquito breeding. This is an efficient approach which people themselves can carry out and it has been stated that the community participation in vector control is crucial for its success [271].

Wolbachia, a bacterial symbiont of insects, has been shown to produce resistance against RNA viruses in *Drosophila melanogaster* [272] and also to induce cytoplasmic incompatibility in insects [273], allowing this bacterium to spread rapidly amongst the population. These findings arose interest of utilization of *Wolbachia* in MBV control. When a strain of *Wolbachia*, not effecting on the lifespan of the mosquito, was introduced to *Ae. aegypti* population, it was soon observed that mosquitoes became resistant for DENV as *Wolbachia* prevented the dissemination of the virus to salivary glands of the mosquitoes and thus reducing the vector competence of these mosquitoes [274,275]. In addition to Wolbachia, other technologies in biological prevention have been developed. Genetic modification to reduce vector-competence by making the mosquitoes more resistant for DENV infection [276] and reducing the mosquito population by killing mosquitoes in larval stage [277] have been shown to be promising efficient ways in mosquito

control. These technologies are still under studies and ways improve the methods are explored. In Sterile Insect Technique (SIT), the radiated male mosquitoes are released. Their offspring is not viable thus reducing the populations [278,279]. Considering the increasing resistance of mosquitoes to insecticides, *Wolbachia* SIT technique and genetic modification of vector species provide a promising approach to MBV control and decreasing the environmental impact of mosquito control as reducing the utilization of insecticides and larvicides. Vector control has been calculated to be profitable and cost-effective when compared to disease treatment of MBVs [280].

Clinical trials are currently ongoing for candidate vaccines against DENV and ZIKV. Problem with DENV and probably also ZIKV vaccine development is circumventing ADE. With DENV this means that the vaccine should raise equal immune response against all the four serotypes of DENV. Sanofi Pasteur has 2015 licensed a tetravalent live recombinant dengue vaccine, Dengvaxia (CYD-TDV), which is now available several endemic countries. The vaccine is registered for use in individuals living in endemic settings (age 9-45) and it seems to function well in that population, among people with previous exposure to DENV [281,282]. However, there has been discussion whether vaccine is recommended for person not primed with DENV, as the antibodies elicited may predispose the vaccinee to a more severe outcome if they get a natural infection [283]. There are also five other vaccines under clinical trials, in different phases, at the moment of writing this thesis. These vaccines are based on live-attenuated or purified inactivated viruses and DNA or subunit technologies. In addition there are virus-vectored and VLP-based vaccines in preclinical trials [281,284]. There is not yet licensed vaccine against ZIKV but several vaccine candidates are in different phases of trials, some already in clinical trials. As with other flaviviruses live-attenuated and inactivated purified vaccines are approaches with ZIKV vaccine but also new technologies like mRNA vaccines are under development [285,286].

5.8 Finnish travellers

As for the trips to (sub)tropical regions, the numbers have been constantly increasing over the past years. In 2016 Finns made 6,1 million overnight

holiday journeys and 1,8 million work-related trips abroad; in approximately 350 000 trips the destination was in the (sub)tropics. According to Association of Finnish Travel Agents (AFTA) the most popular destinations of Finnish travellers during the last years have been destinations within Europe. The trips to Middle East and Northern Africa have been in increase in recent years. Thailand is the most popular destination for long-distance journeys even though within recent years trips to Central America and Caribbean area have become increasingly popular. AFTAs statistics are based on package tours but they can be interpreted as indicative of the travel habitats of Finnish travellers [287,288].

While DENV and ZIKV are not endemic in Finland, cases are seen regularly among travellers returning from visits to endemic areas in the (sub)tropical regions. Indeed, along with the increasing numbers of travels to these regions [287] and expanding endemic regions, the numbers of cases have been estimated to increase every year. Likewise, while no cases of ZIKV were recorded in 2006, from 2007 to 2016 79 countries or territories have reported ZIKV transmission and 31 countries cases of microcephaly or other malformations of central nervous system [289]. Of note, the incubation period for DENV infections is short (3-10 days) which means that it is not rare that the symptoms start already at the destination and the patient is treated at a local hospital [181,290] and the case is never recorded in Finland. Furthermore, in diagnostics of febrile travellers, DENV infection can be immediately excluded from the differential diagnostics if the symptoms start longer than two weeks after return, whereas this is not as clear in case of ZIKV infection.

Travellers have a role in the epidemiology of mosquito-borne viruses as they may transport the viruses from endemic to non-endemic areas (and by doing so increasing a risk of establishing virus circulation in new areas if a suitable vectors are present). Indeed, outbreaks and non-traveller cases have been described e.g. following travel-related transmission of CHIKV [291,292].

Travellers contracting mosquito-borne diseases may also provide a tool for studying epidemiology and molecular epidemiology of MBVs and act as sentinels by providing a sign of an outbreak in an early phase. Sometimes the (traveller) infections originate in areas with limited facilities for diagnostics. As the viremic travellers return to their countries of residence,

laboratory confirmation of the virus and possibly even serotyping or sequencing can be performed. This offers a view to global circulation of MBVs.

6. AIMS OF THE STUDY

1. Study the kinetics and secretion of virological markers of dengue infection and their associations with disease outcome and other laboratory parameters (Study II)
2. To explore the utilization of non-invasive samples in laboratory diagnostics of dengue virus infection (Study III)
3. To characterize congenital ZIKV infection to seek evidence for causality between ZIKV infection and foetal brain abnormalities (Study V)
4. To study the global epidemiology of DENV and ZIKV using Finnish travellers as sentinels (Studies I and IV)

7. MATERIALS AND METHODS

7.1 Study samples

The studies are mainly based on serum samples of suspected dengue patients sent to HUSLAB, the laboratory service of the Hospital District of Helsinki and Uusimaa (HUCH), Department of Virology and biobank with archived samples from HUSLAB. The biobank includes serum aliquots, stored in -70°C, from all Finnish patients suspected with dengue. Summary of the samples of all studies of this thesis is shown on the Table 4.

Table 4 Study samples. Numbers of samples and sample types in studies of the thesis. In Study V the tissue samples comprise of placenta, foetal membrane/umbilical cord, rain, spleen, lung, liver, muscle and thymus and other samples include plasma (n=1), amniotic fluid, PBMC and semen samples (n=2/each sample type).

	Sample type						
Study	**Serum/n**	**Urine/n**	**Saliva/n**	**Tissue**	**Others (n)**	**Total/n**	**Patients**
I	5					5	5
II	139					139	93
III	39	50	48			137	14
IV	5	1				6	5
V	6	3	2	8	7	26	2
Total	194	54	50	8	7	313	119

Blood/serum samples

Serum samples for studies I-IV were samples sent to HUSLAB collected from patients suspected to have dengue infection / explored for dengue as one of the alternative causes for fever after travel to (sub) tropics. Samples for RNA extraction and isolation were stored in -70°C as 100µl aliquots and samples for NS1 antigen detection and antibody screening were stored at -20°C.

Plasma and PBMC samples in study V, obtained from Sibley Memoriam Hospital, United States, were stored in -70°C and -135°C.

Urine and saliva samples

Urine and saliva samples were collected on daily basis during the hospitalization from the patients taking part in the study in study III. Samples were aliquoted and stored in -70°C. Urine samples were also obtained from the patients (studies IV and V) and from the husband of the patient urine and semen samples were obtained in study V. All these samples were stored in -70°C.

Tissue samples

In study V foetal and maternal tissue samples were obtained from Division of Pathology and Center for Genetic Medicine Research of Sibley Memoriam hospital after the abortion of the pregnancy. Brain, spleen, muscle, lung, liver, placenta, membrane/cord and thymus samples were studied. Samples were stored in -70° and homogenized with mortars and pestles with sterile sand and suspended in Dulbecco's BSA supplemented with antibiotics prior to use.

7.2 Patient data

Necessary patient data was collected for each study from patients themselves, the doctors taking care of the patients' or from patient records within the data of HUSLAB and HUCH.

The data collected consisted of travel information (destination, duration and purpose of the trip), days of onset of illness, symptoms, laboratory values, possible co-infections and flavivirus vaccines, duration of hospitalization and previous flavivirus infections.

7.3 Research permits

A research permit for collecting the necessary data about the patients whose samples were sent to HUSLAB was applied and gained for HUSLAB (TYH2014251). This permit included authenticity to obtain data that was classified as confidential. This permit covered all the studies of this thesis.

The permit for data collecting of patients outside HUSLAB area was obtained from the Ministry of Social Affairs and Health (STM/2523/2009).

The study protocol of work III (HUS DNRO 388/13/03/01/09) was approved by the Ethics Committee of the Department of Medicine, HUCH. Written informed consent was obtained from all patients. The protocol included collection of daily non-invasive samples (urine and saliva) that were not necessary for their routine care.

7.4 Immunofluorescence assay (IFA)

Immunofluorescence assay (IFA) was used in all studies for IgG and/ or IgM antibody detection. It was also used in studies III and V for detection of viral antigens from the cell culture isolates. IFA is in routine diagnostic use for DENV and ZIKV IgG in HUSLAB.

The IFA slides for showing the presence of viral antigen in virus isolation cell cultures or antibody determination from patient samples were prepared with infected cells. For antibody titer determination Vero E6 cells infected with DENV-3 (studies I-V) and ZIKV MR766 (study V) were used as antigen. The slides were prepared by infecting VE6 cells with DENV or ZIKV and cells were incubated in 37◦C for 7 or 3 days for DENV and ZIKV respectively. With virus isolations the slides were prepared when subculturing or harvesting. The infected cells were detached and in case of slide preparation for antibody determination mixed with clean cells in ratio of 3:1. Cells were washed four times with and suspended in PBS. The suspension was dropped on the microwell slides and let to dry overnight. Cells were fixed with ice-cold acetone for 7 minutes and stored in -70°C, prior to use.

Serum samples were diluted 1/10-1/1280 with PBS and added to IFA slides containing the virus infected, acetone fixed cells. The samples were incubated on the slides for 30 minutes in 37°C and washed three times with PBS. A secondary antibody against human IgG (Jackson ImmunoResearch) conjugated with fluorescent label (FITC) was added and after 30 minutes' incubation in 37°C, excess conjugate was washed with three times 5 minutes PBS and once for 5 minutes in Milli-Q water. For IFA, human serum (knowing to contain antibodies against DENV or ZIKV) or monoclonal antibodies (against DENV) were used as positive controls. A

serum known not to contain antibodies against flaviviruses was used as a negative control and PBS to control background staining from the conjugate.

The presence of antibodies in the studied human sera or presence of viral antigens in viral culture samples was observed visually with help of Olympus BX51 Fluorescence microscope.

7.5 Enzyme-linked immunosorbent assay (ELISA) and immunochromatographic rapid test

A commercial test (Dengue Virus IgM Capture DxSelect ELISA, Focus Diagnostics, USA) was used to detect antibodies against DENV IgM. The test was used according to manufacturer's instructions.

NS1 antigen was detected from serum, urine and saliva samples (studies I - IV) with Bio-Rad Platelia NS1 Antigen EIA according to manufacturer's instructions. This test is a one-step sandwich format microplate enzyme immunoassay. Though the test is only validated for serum, urine and saliva samples were treated with the same protocol.

The lateral flow immunochromatographic Dengue NS1 Ag Strip (Bio-Rad, USA) was used in study III for serum and urine samples. Both sample materials were processed according to manufacturer's instructions indicated for serum.

7.6 Virus isolation

Virus isolation trials were conducted in studies III and V. All the viral isolation trials were conducted in a biosafety level 3 laboratory (BSL-3).

The tissue homogenates, serum, urine and saliva (study III) samples were inoculated to 80% confluent monolayers Vero E6 (green monkey kidney epithelium), and C6/36 (*Aedes albopictus*) cells in 25cm2 culture bottles. Prior to infection cells were washed once with Phosphate buffered saline

(PBS) and after that 50µl of serum or saliva and 200µl of urine was inoculated to cells. After inoculation, cells were incubated for one hour with gentle rocking either at room temperature (C6/36) or 37°C (VE6). After incubation cells were washed once with PBS and culture medium, L-15 for C6/36 and MEM for Vero E6, supplemented with antibiotics (penicillin and streptomycin), Fungizone and 2% Foetal calf serum (FCS) was added.

The cells were checked every other day for cytopathic effect (CPE) and if noticed, cells were harvested. If no CPE was observed, cells were subcultured to T75 culture flasks after seven days incubation period. After 24 days, all cells were harvested regardless of CPE. When harvesting an IFA slide was made to test the cells for the presence of viral antigens.

7.7 Real-time RT-PCRs

Real-time RT-PCRs were used in all studies to detect the presence of flavivirus RNA in serum, plasma, PBMC, urine, saliva, semen and tissue samples. The assays were performed with Stratagene MX3005P real-time qPCR instrument.

Dengue real-time RT-PCR used in the studies I-III is an in-house MGB probe method developed earlier [258].

ZIKV RT-qPCR protocol targeting the NS5 gene used in the study V was adopted from a previously described method [293] and the MGB probe was modified from Faye et al [157,256].

In studies IV and V nested PCR detecting a wide variety of flaviviruses was used. The method is adopted from Moureau et al [294,295]. The method targets the NS5 gene and is two-step PCR the first round being a SybrGreen real-time RT-PCR and the second round a conventional PCR.

A second seminested traditional PCR was conducted for all the samples, regardless of the positivity or negativity in the real-time RT-PCR. 2 µl of the first round PCR product was used for the nested round amplification.

The first and second rounds of the PCRs were pipetted in different laboratories to avoid and water controls included to control potential contamination risks.

The results were observed with agarose gel electrophoresis (AGE) using 1,5% Seakem agarose in 1x TAE buffer. 10µl of PCR product was used in the electrophoresis. The results were visualized under UV light from ethidium bromide (EtBr) stained gels.

7.8 Other RT-PCR protocols

Reverse transcriptase reaction

RNAs were converted to cDNA for subsequent PCR protocols utilizing random hexamers and Expand RT reverse transcriptase enzyme (Roche).

DENV serotyping nested RT-PCR

In studies II-III the original serotyping of the DENV strains were performed utilizing a protocol and primers of nested serotyping RT-PCR [296] but using Expand RT Reverse transcriptase (Roche) for RT reaction and TaqDNA polymerase (Thermo Scientific). The first round produces an amplicon of 511bp of all serotypes but the second-round amplicons length is dependent on the serotype of DENV (482bp DENV-1, 119bp DENV-2, 290bp DENV-3 and 392 DENV-4). The results of all conventional PCRs were confirmed with AGE, using 1% Seakem agarose gel stained with either ethidium bromide or GelRed (Biotium, United States). For visualization UV light visualization was utilized.

E gene PCRs

In studies I-III the E gene PCRs for phylogenetic analyses was utilized. The nested method was adopted from Gaunt and Gould [297]. 5µl of the cDNA and 500nM of each primer in total reaction volume of 50µl was utilized in the first round PCR and 2µl of the first round PCR product with the equal concentration of primers in the second. The PCR protocol for the both rounds was 95°C for 10 minutes followed by five cycles of 94°C for 1,5 minutes, 45°C for 1,5 minutes and 72°C for 1,5 minutes. After that followed 45 amplification cycles: 94°C for 50 seconds, 55°C for 60 seconds and 72°C for two minutes. The final elongation step was conducted in 72°C for 10 minutes.

7.9 Sequencing

In all studies of the thesis sequencing of the genes or whole genomes of the viruses was applied to ensure the species, serotype or genotype and for the phylogenetic analyses.

Sanger sequencing

Samples for Sanger sequencing were prepared for the sequencing either by straight purifying the PCR product with ExoSAP-IT™ PCR Product Cleanup Reagent (ThermoFisher Scientific). The PCR products were T-A cloned, if necessary, to pGEM-T vector (Promega) according to manufacturer's instructions and T7 and SP6 colony PCR. The purified PCR products were mixed with sequencing primers, which in all these cases were also the PCR primers, and delivered into a commercial sequencing laboratory.

Next Generation sequencing (NGS)

In study V next generation sequencing was used to obtain the complete coding sequence of the virus strain (FB-GWUH), isolated in SK-N-SH cells. RNA was extracted using QIAamp viral RNA Minikit (Qiagen) from the cell culture supernatant. In library preparation Illumina TruSeq Stranded Total RNA LT with Ribo-Zero Gold sample preparation kit (Illumina) was applied and library quantitation step was performed employing NEBNext Library Quant Kit for Illumina (New England Biolabs) according to manufacturer's instructions. For sequencing Illumina Miseq system was utilized. The sequences were assembled utilizing Bowtie2 using KU509998; Haiti/1225/2014 strain as a reference sequence.

7.10 Phylogenetic analyses

Phylogenetic analyses of the viral sequences were conducted in studies I, IV and V.

In study I phylogenetic analyses was based on the E gene sequence aligned with sequences from GenBank showing high homology in BLAST (Basic Local Alignment Search Tool) and also sequences of representatives of different genotypes of DENV-1. Sequences were aligned with MUSCLE and the neighbor-joining phylogenetic tree was constructed utilizing the maximum composite likelihood method implemented in Mega software.

In study IV, the obtained 160bp sequence of NS5 gene was aligned with all the overlapping ZIKV sequences deposited in GenBank and in study V the complete genome or coding region sequences in GenBank were used and aligned with the obtained genome from the foetal brain by ClustalW algorithm in Mega software, version 6.06 [298]. In the construction of the phylogenetic tree Bayesian analyses (Beast version 1.8.0) was utilized in study IV using Tamura-Nei (TN93+G) model of substitution, strict molecular clock and constant population size demographic model. In study V GTR+G substitution model, uncorrelated log-normal distributed relaxed

molecular clock and Bayesian skyline demographic model were used in phylogenetic analyses [299].

7.11 Statistical analyses

In studies II and III statistical analyses was used in the interpretation of the results.

In study II all statistical analyses were performed with R software (R Development Core Team 2011). The kinetics of infection markers in serum were studied using generalized additive mixed models (GAMM) [300] and associations between virological markers (serum DENV RNA and NS1 antigen) and clinical parameters were studied by regression models in patients (n=89/93) from who we could obtain all the data needed.

In study III SPSS (IBM Corp. Released 2012. IBM SPSS statistics for Windows. Version 21.0. Armonk:NY, IBM Corp.) was utilized for the statistical analyses of the results. In some cases, the small number of the observations limited the analyse methods that were suitable for the analyses of the material.

8 RESULTS

General patient data

All together this thesis is based on samples of 119 patients with laboratory confirmed DENV or ZIKV infection. The age range was 6 – 74 and 53% (n=62) were men and 47% (n=55) women (gender of the three children in study IV is not known). Most of the patients originated in Finland but 14 were immigrants from Africa or Asia. The patients in all studies were travellers returning from journeys to tropics or subtropics (Figure 9.). The continent, most frequently visited by the study patients was Asia (Figure 9.)

with four most popular countries of destination: Thailand (n=37), Indonesia (n=12), India (n=11) and Sri Lanka (n=9) (Figure 9.).

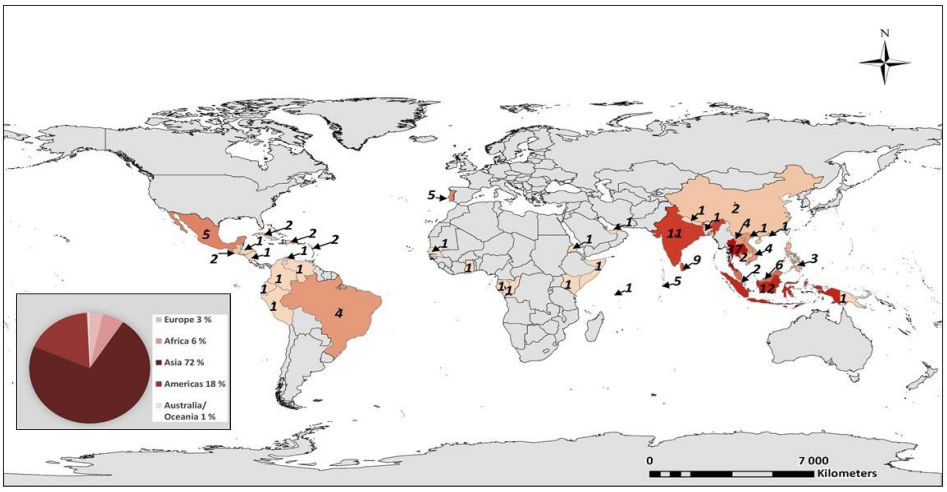


Figure 9. The travel destinations of the patients in studies I-V. The countries visited are indicated with coloring;. The color gets more intense with more travellers visiting. Some of the travellers visited more than one country.

Kinetics and secretion of markers of DENV infection (study II)

In study II, the kinetics of dengue virus infection markers and the potential associations of various routine laboratory parameters with disease severity were studied from 139 serum samples of 93 patients with a diagnosis of acute dengue infection. The diagnosis was based on IgG seroconversion between first and last sample and positive IgM result at least from one sample. The diagnosis was confirmed by virus isolation, viral RNA detection or NS1 Ag detection in 44, 67 and 70 cases, respectively. Based on travel history and serological results the infections were considered to be mainly primary. In three cases, secondary infection was suspected based on antibody responses (moderately high IgG titer in first sample while IgM was negative). Although most of the patients were hospitalized with a mean duration of hospitalization of 4.5 days, none of them had complications,

such as hemorrhages. The travel destinations included Asia, Africa, Caribbean, South and Central America. Altogether 72% of the infections were obtained from Asia and 50% specifically from Southeast Asia. In 56 cases (60%), the infecting serotype could be determined either by virus isolation or direct RT-PCR and subsequent sequencing. All dengue virus serotypes were represented within the study patients: most common were DENV-1 and DENV-3 (24/56 and 22/56 respectively). In 17/91 cases (19%) co-infections with other non DENV pathogenic microbes were either diagnosed or suspected. In most cases these agents were bacteria causing gastroenteritis.

The comparison of the results obtained from dengue NS1 antigen and viral RNA detection tests from 139 sera suggested clear differences in the detection kinetics of these markers. The NS1 Ag was detectable for notably longer period of time than the viral RNA in serum samples. Whereas viral RNA was mainly detected on days 2-9 after onset of illness, the NS1 Ag was found to be most probably detected from day 2 up to day 15. Individual positive samples were still found positive for NS1 Ag up to day 21.

When all the diagnostic assay (IgM, IgG, NS1 Ag, RNA detection) results were considered, it was clear that none of them alone reached a sufficient level of sensitivity for the diagnosis of acute dengue virus infection as none of them reached 90% of sensitivity when examining all samples in total (Table 5.). With the first available serum sample from each patient (ranging from day 1 to 25 since onset of fever), or the samples altogether, IgM showed the best diagnostic sensitivity (Table 5.). Examining by days of acute phase, during days 1-4 after onset of illness, RT-PCR showed greatest diagnostic sensitivity, whereas during days 5-8 after onset of illness IgM showed best diagnostic sensitivity (Table 5.)

Table 5. Results of individual diagnostic methods in study II for a selected set of confirmed dengue patients and their samples. In all categories the method that gave highest positivity rate is highlighted.

	Sample			
Method	First available n/n (%)	All in total n/n (%)	Days 1-4 n/n (%)	Days 5-8 n/n (%)
RT-PCR	65/88 (74%)	70/131 (53%)	21/24 (88%)	39/64 (61%)
NS1 Ag	68/88 (77%)	98/136 (72%)	18/23 (78%)	52/63 (83%)
IgM	68/87 (78%)	105/124 (85%)	11/22 (50%)	55/59 (93%)
IgG	61/89 (69%)	101/132 (77%)	10/22 (45%)	47/63 (75%)

Best positivity rate

When combining the assays, the acute phase diagnosis was significantly enhanced by using IgM detection in parallel with either NS1 Ag or viral RNA detection. The viral RNA or NS1 Ag with IgM detection showed the best sensitivity during the first three weeks after onset of illness: 99% and 97% of the sera, respectively, were found positive with these combinations (the proportion of positive samples with combinations of IgG and viral RNA or NS1 Ag detection being 98% and 97%, respectively). Combining antibody detection (IgG and IgM) gave positive results in 90% of the samples whereas with the combination of NS1 Ag and viral RNA detection 75% of the samples were found positive.

The detection rate of NS1 Ag seemed to vary between serotypes during the first 12 days of illness but not after that time point; respective differences in the detection rates of the different serotypes was not observed in the detection of viral RNA. Our results showed that NS1 Ag was detectable from all the patients with DENV-1 infection while from patients with DENV-3 infection only 69% proved positive for NS1 Ag during the early phase of the infection.

Associations of markers of DENV infection with disease outcome and other laboratory parameters (study II)

We found no correlations between the detected viremia and the outcomes of the disease when individual symptoms were considered. However, the presence of viremia was found to correlate with the probability of hospitalization and the level of viremia, as determined from the relative RNA levels derived from the real-time RT-PCR Ct (cycle treshold) values. In contrast, neither the positivity of the NS1 antigen test nor the level of the antigenemia correlated with either hospitalization or length of hospitalization. NS1 antigenemia, derived from the optical density value, was found to be associated with fatigue and gastrointestinal symptoms (abdominal pain and diarrhea).

Leukopenia, thrombocytopenia and elevated liver transaminase (ALT, AST) levels are characteristic for dengue fever and elevated hematocrit and hemoglobin levels may indicate plasma leakage in dengue patients. The performed analysis suggested associations between clinical laboratory parameters and a positive test result in either NS1 ag or viral RNA The overall NS1 antigen positivity at the time of hospitalization was associated with increased probability of leukopenia and thrombocytopenia and elevated ALT level whereas the level of antigenemia was found to correlate positively with maximum hemoglobin level and levels of hematocrit and liver transaminases (ALT, AST).

Secretion and kinetics of viral RNA and NS1 Ag to urine and saliva (studies III and IV)

The study population consisted of 14 travellers (Study III) each of whom was interpreted to have a primary dengue infection with no severe complications. The inclusion criteria were suspected dengue infection and positive dengue NS1 Ag rapid test result from serum sample. All the patients, with one exception, were hospitalized and samples of urine, saliva and from some patients also serum, were collected daily during the

hospitalization. The most common symptoms included fever, rash and headache. The origins of the infections were in Southeast or South Asia and Africa. All DENV serotypes were represented (DENV1-4).

The methodologies developed by the manufacturers for serum samples were applied as such in this study on urine and saliva samples and were found to be applicable in general for the detection of DENV NS1Ag and RNA from urine and saliva, although some problems were encountered with saliva as discussed below. (Figure 10.). Virus isolation was not successful from any urine or saliva samples tested, regardless of the success in virus isolation from a corresponding serum sample. A cell culture isolate was obtained from 4/8 of the tested sera and subsequent sequencing showed these to represent serotypes DENV-2 (n=3) and DENV-4 (n=1).

NS1 Ag detection rates and kinetics

The NS1 Ag was detected from all available sample materials collected between days 1-13 after the onset of symptoms. The results obtained from the different sample materials however showed substantial variation in NS1 Ag detection rates within this timespan. NS1 Ag detection from serum showed the greatest detection rate overall as all but one of the serum samples were tested positive (Figure 10). Overall detection rate from serum was 97.3% while it was 54.2% and 55.5% for urine and saliva, respectively. Several urine and saliva samples showed borderline results, which were considered as negative (Figure 10).

RNA detection positivity rates and kinetics.

The kinetics of the RNA detection from saliva did not differ notably when compared to that observed from serum. Of these two sample materials, serum seemed superior with a 100% sensitivity of detection during days 1-6 as compared to 71.4% from saliva; the overall sensitivity (days 1-13) was

70% and 60% for serum and saliva, respectively. However, the kinetics of RNA detection from urine was found to differ from that recorded from saliva and serum samples as viral RNA was detectable in a later phase from urine than from serum and saliva. The overall detection rate of RNA from urine was shown to be 64%. However, the detection rate of RNA detection from urine was superior to other sample materials during the days 7-13: 74% of the samples were positive for viral RNA from urine but only 31% and 50% from serum and saliva, respectively. The RNA detection from a urine sample proved positive for each of the study patients 14/14 from at least one of the tested time points; whereas only 10/14 patients were found positive for viral RNA in any of the tested saliva samples (Figure 10).

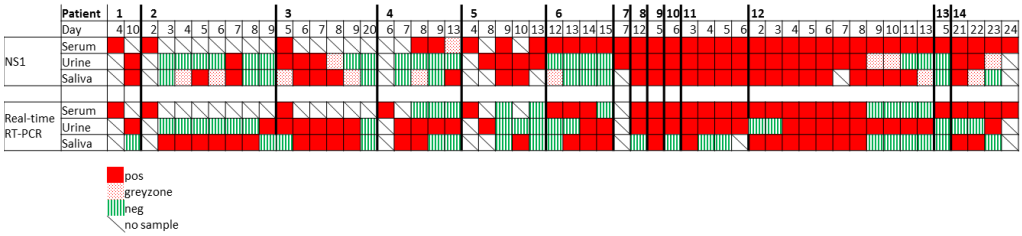


Figure 10. Results of viral RNA and NS1 Ag detection in 14 patients. Samples were taken of serum, urine and saliva on the given days following the onset of symptoms. Greyzone indicates a repeated borderline result.

Statistical analyses showed that urine NS1 Ag levels correlated positively with both urine total protein levels and NS1 Ag levels in saliva. Neither RNA nor NS1 Ag levels of urine correlated with urine osmolality. NS1 Ag levels of serum and saliva correlated with each other but not with urine NS1 Ag levels. Of the basic laboratory parameters, urine NS1 Ag levels were found to correlate with decreased platelet levels.

In study IV flavivirus RNA was detected from a patient urine sample whereas the sera remained negative. (Figure 11.) (serum taken 7 days and urine 8 days after onset of illness). Subsequent sequencing and phylogenetic analyses revealed that the infecting virus was ZIKV belonging to epidemic Asian lineage. Patient's serum sample was positive for DENV IgG (IFA) and low positive for IgM (Figure 11.) while no DENV NS1 antigen was detectable using a rapid test.

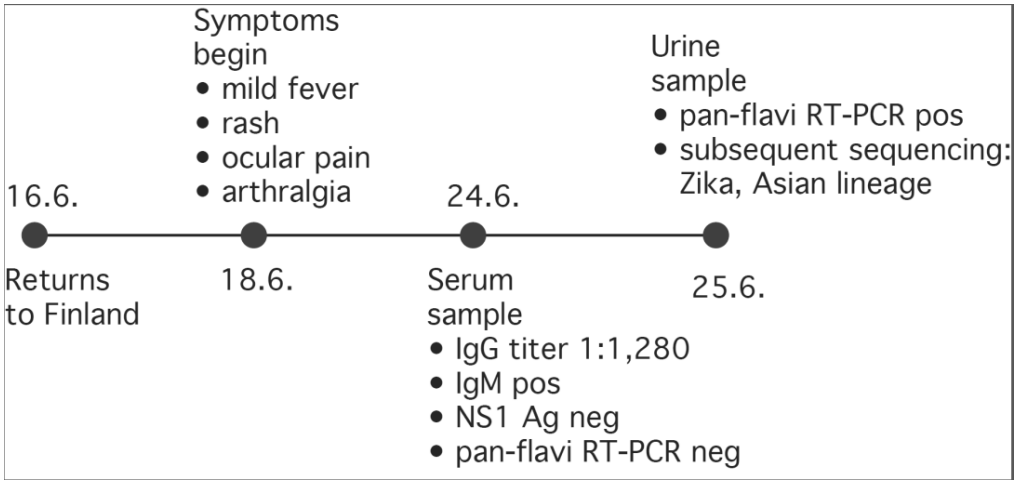


Figure 11. Timeline and laboratory results of the traveller case of ZIKV infection from Maldives in 2015. Serum sample was tested for DENV IgG, IgM and NS1 Ag.

ZIKV congenital infection (study V)

In study V, the patient was a pregnant woman who had been travelling with her husband in Central America, and been heavily bitten by mosquitoes, in November 2015. When she returned from the holiday, she got symptoms (fever, rash) consistent with ZIKV infection. First suspicions of the association of ZIKV infection of the mother and microcephaly of the foetus had just been published in the lay press during this time. Ultrasonographic examinations showed no abnormalities at gestation weeks 13, 16 and 17. Yet, a blood sample was taken from the mother four weeks after onset of symptoms and this was found positive in pan-flavi RT-PCR and subsequent sequencing revealed the infecting virus to be ZIKV (Figure 12.).

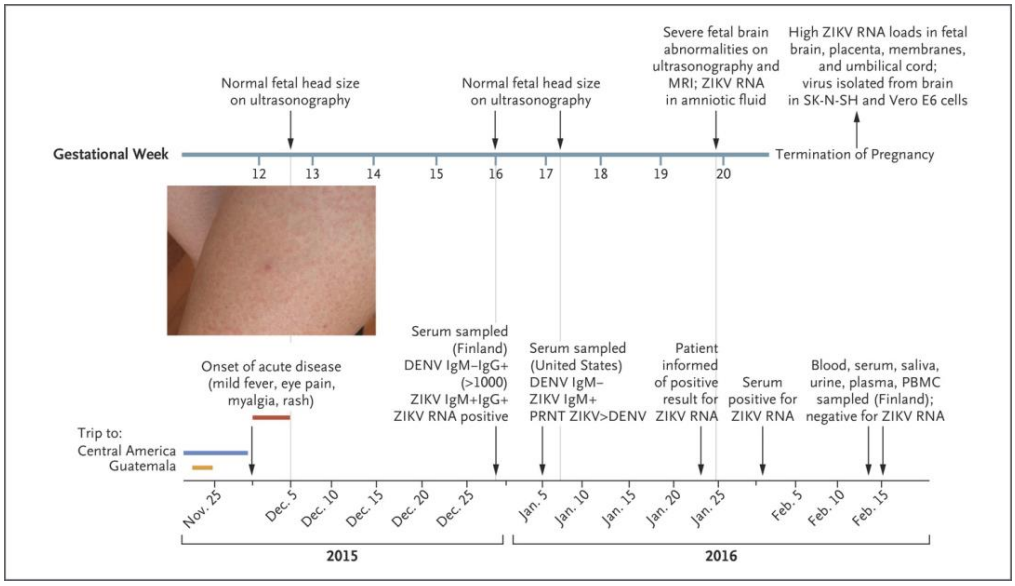


Figure 12. The timeline and the laboratory results of the ZIKV congenital infection. From New England Journal of Medicine, Driggers R et al, Zika Virus Infection with Prolonged Maternal Viremia and Fetal Brain Abnormalities, 2016 Jun 2;374(22):2142-51. Copyright © (2017) Massachusetts Medical Society. Reprinted with permission.

ZIKV RNA from mother's serum sample was detected prior to any notable abnormalities on ultrasonography examination. The patient obtained the infection during gestation week 11. ZIKV RNA was detected in serum sample taken at gestation week 16 (and 21), whereas ultrasonography examination of the foetus at gestation weeks 13, 16 and 17 did show no evidence of calcifications or microcephaly (Figure 12.). Ultrasonography at gestation week 17 showed abnormalities in the foetal brains and head circumference measurements decreased between gestation weeks 16-20 from the 47th percentile to the 24th percentile. Explicit brain abnormalities were detected at gestation weeks 19 on ultrasonography and 20 on magnetic resonance imaging (MRI). The pregnancy was terminated on gestation week 21.

ZIKV RNA was detected from mother's serum up to 10 weeks post infection, until the abortion was carried out. Serum samples taken after the abortion were negative for ZIKV RNA. Samples taken on the day of the abortion from membrane/cord, placenta, amniotic fluid and spleen, liver, lungs and

muscle of the foetus were positive for ZIKV RNA whereas thymus was negative. The samples from the spouse of the mother (and the father of the foetus) - serum, urine and semen - were all negative for ZIKV RNA but the serum sample showed IgG and IgM class antibodies against ZIKV virus with titers of 1:320 and 1:20 five weeks after the infection. From mother's urine, peripheral blood mononuclear cells (PBMC) and saliva ZIKV RNA could not be detected. Serum samples from the mother showed IgM and IgG class antibodies against ZIKV with titers of 1:80 and 1:640 five weeks after the infection (Figure 12.).

Isolation of ZIKV was successful from the brain tissue of the foetus in SK-N-SH and VeroE6 cells but not in C6/36 mosquito cells. From the other studied tissues (membrane/cord, placenta and spleen) or mother's serum the isolation was not successful. The replication of the virus was observed as decreasing CT values in a ZIKV real-time RT-PCR test of the culture supernatants sampled and studied daily. The replication of the virus was observed since day one after inoculation in SK-N-SH cells and after three days in VeroE6 cells. The presence of viral antigens in the cells was shown using IFA and the presence of the virus was confirmed by observing virions compatible with description of a flavivirus in electron microscopy (EM). Additionally, a complete coding sequence was obtained from the culture supernatant by NGS.

Molecular epidemiological studies from travellers (studies I, IV and V)

In study I we reported five Finnish travellers returning from Madeira in the end of 2012 diagnosed with DENV infection. All five seroconverted for IgG against DENV and showed class IgM antibodies against DENV. Three out of five were also positive for DENV NS1 antigen. From two patients DENV RNA was detected and found to represent DENV-1. Phylogenetic analysis showed the Madeiran origin strain to associate with American/African genotype strains known to circulate in South America.

In study IV a Finnish traveller was diagnosed with flavivirus infection turning out to be ZIKV infection originating from the Maldives. At the time, no previous reports of the existence of ZIKV within the area of Western Indian Ocean had been published. The sequence analysis suggested the Maldives strain to represent the epidemic Asian lineage which had caused the outbreaks of Yap Island, French Polynesia and later in the Americas and the Caribbean region.

The complete genome sequence of ZIKV strain isolated from the foetal brain tissue in study V (supernatant of the SK-N-SH cell culture, day 5 was sequenced using NGS methods. This virus was shown to be of Asian lineage in phylogenetic analyses and to share highest homologies with Guatemalan strains originating from cases with mild disease. This virus, designated as FB-GWUH-2016, was identified to have eight amino acid substitutions compared to other American ZIKV sequences available in the GenBank database. Five amino acid substitutions were found to be unique to FB-GWUH-2016 strain and three where shared with the Guatemalan strains but not with other American strains.

9 DISCUSSION

With the emergence of dengue, and more recently Zika virus, coupled with the expansion in the distribution of many MBVs, the urgent need for specific diagnostics and surveillance of the circulation of these viruses has been highlighted. Traditionally, a venous blood sample has been used to diagnose cases of both dengue and Zika. This has limitations, however, since collecting blood samples from small children can be difficult, and collecting and storing venous blood from patients in remote areas, may be impractical. To date it has been observed that other sample materials are suitable and in some cases even superior to serum in DENV and ZIKV diagnostics [255,301-303]. The kinetics of the markers of DENV infections have been mainly studied in endemic settings, representing also secondary infections and severe disease cases. Travellers usually experience mild primary infections and thus their samples provide another insight into the variation of viral markers and their kinetics in a different study population.

The observation in 2015 in Brazil of the increased number of microcephaly cases interrelated to the epidemics of ZIKV was alarming and caused World Health Organization (WHO) to declare the Public Health Emergency of International Concern [304] The causality of congenital ZIKV infection and malformations in the development of the central nervous system of the foetus was soon under heated investigation. Travellers, as sentinels, often provide an opportunity to discover viruses from new areas or to observe an outbreak in its early phase.

In this thesis, samples obtained from returning travellers, including a unique collection of all Finnish suspected dengue cases from the last 18 years, were used to explore the kinetics of the markers of DENV infection.In addition, the potential of different sample materials for use in the diagnostics of DENV and ZIKV infections were exploredfrom a mainly flavivirus naïve population. These samples also facilitated the study of molecular epidemiology of the DENV strain responsible for the Madeiran outbreak, and revealed the circulation of ZIKV in the Maldives. Finally, congenital ZIKV infection with a mother's prolonged viremia was characterized and infection of the foetal brain was confirmed by the isolation of ZIKV from the affected tissue. This provided evidence for the causality between mother's ZIKV infection and brain abnormalities of the foetus.

Insights into diagnostics of DENV and ZIKV

Kinetics of markers of DENV infection

Studying the kinetics of the infection offers tools for assessing efficient diagnostics for each phase of the disease. More comprehensive data including patients with complicated DENV infections, for example with haemorrhages, are required in order to confirm the factors underlying the severe disease outcome and identifying the risk factors.

The kinetics of viral markers in primary and secondary dengue infections differs, as many previous studies have shown. The detection span of NS1 antigen seems to be notably longer in primary [228,305] than in secondary cases [248,306]. In secondary cases IgG antibodies are detected in a very early phase of the disease [179] and IgM may even remain negative. In primary cases IgM class antibodies will be detected first, starting from days 4-5 while IgG antibodies become measurable only after day 7 [179] This implies that more studies covering the kinetics, in populations both living in the endemic and non-endemic areas (e.g. travellers), are needed to cover the entirety of the kinetics of markers of DENV infections. Travellers differ from the populations living in endemic areas not only by having usually primary infections versus the typically secondary infections of populations from endemic areas but also by the genetic background. Different recommendations for methods of choice in diagnostics may also be needed for populations living in endemic and non-endemic regions.

It has previously been observed that the DENV NS1 Ag test detects the four DENV serotypes with unequal sensitivity, favouring the detection of NS1 Ag from DENV-1 infections [238,307]. Whether this is due to unequal recognition of the antigens by the anti-NS1 antibodies used in the antigen detection assays or differences in production rates of NS1 antigens in infections of different DENV-serotypes, genotypes or lineages is unclear. It has also been observed that NS1 antigen tests perform better in primary infections than in secondary [238,246,307]. The prevailing theory is, that in secondary infections, the existing antibodies against NS1 protein form immunocomplexes with the antigen and thus inhibit the recognition of the antigen by the anti-NS1-antibodies of the antigen capture tests [247]. Even though our patient material included only three cases suspected as secondary cases, all of these actually were negative in NS1 Ag test while positive for viral RNA.

Besides the detection rate of NS1 Ag, the DENV serotype has been reported to affect the level and duration of viremia. Both in primary and secondary cases it has been observed that viremia is higher in DENV-1 infections and the duration is longer when compared to infections of other DENV serotypes [248]. This has an impact, not only on diagnostics but also on the transmission of the virus.

Association of viral markers to disease outcome and other laboratory parameters in DENV infection

Identifying the factors affecting the outcome of the illness provides insights into the pathogenesis of the infection, but may also help in determining the patients at risk for complications. The complicated disease forms require hospital care, thus identifying the patients at risk could have practical implications for patient monitoring needs. The knowledge of the factors affecting the disease outcome is still incomplete and this fact hampers also the development of the methods of treatment. Our patients with DENV infections mainly presented with primary infections with no complications. The duration of hospitalization varied and longer hospitalization was considered as sign of more severe disease. Our patient sample showed that NS1 antigenemia and high-level viremia correlated with disease outcome in terms of length of hospitalization. The observations suggest that there is a gradient of disease severities within the "non-complicated, mild dengue fever" of travellers. Also the markers predicting a more severe disease in the mild disease forms seem actually to be the same markers that have been identified in the endemic populations to predict more severe disease outcomes of also more complicated dengue, including dengue haemorrhagic fever [308,309]. The hallmarks of DF such as leukopenia, thrombocytopenia and elevated liver transaminases were also observed in traveller patients. The elevated hematocrit, known to reflect the plasma leakage in severe forms of dengue [310] was also observed in some of the studied patients with uncomplicated dengue fever.

ZIKV diagnostics

Current diagnostic recommendations by the US CDC include the testing of ZIKV RNA from serum or urine during the first 14 days after the onset of illness [311]. The viremic phase in ZIKV infections, or at least the length of detectability of viral RNA after onset of symptoms, seems to be longer as compared to those observed in DENV infections. Notably, the detection of viral RNA from blood samples of patients with ZIKV infection seems to follow the path of WNV. In WNV infections the period where RNA is

detectable from serum seems to be slightly longer than in DENV infections but notably longer from whole blood samples. [233,245,312,313].

The transmission of ZIKV from mother to foetus has been a totally new and alarming feature of flavivirus infections. The infection has, in most cases, been asymptomatic for the mother and yet the virus has been transmitted to the foetus and caused severe developmental disorders, emphasizing the need to carefully monitor pregnant women at risk of ZIKV infection. We demonstrated prolonged viremia, possibly due to the release of the virus from persistent infection in the foetoplacental system, into the mother's circulation. This assumption was later supported by results of Suy et al and Meaney-Delman et al who also reported prolonged viremia in pregnant women [265,266]. This feature was unexpected but has now been supported by several studies, and ultimately has changed the diagnostic recommendations of ZIKV RNA sampling and testing of pregnant women. The persisting viremia enables the screening of mothers whose babies have an increased risk of ZIKV derived developmental disorders. This observation has already impacted the CDC, who has recommended nucleic acid testing for pregnant women up to week 12 after exposure [311]. The rapid change in emergence of Zika virus and the alarming transmission routes, from mother to foetus and via sexual transmission, and associated complications shows the capability of the viruses to obtain new features that can have an impact in their virulence, epidemiology or transmission. As mosquito-borne viruses are relatively rapidly evolving RNA viruses, these mutations can take place over a short period of time.

The associations of the mother's prolonged viremia to zika congenital infection and its outcomes are important aspects of ongoing and future studies. The diversity of the outcomes of ZIKV congenital infections in the developing foetus and also the effects that may become visible after birth and later during the childhood are currently not well known. The most severe forms of congenital infections including microcephaly, once detected, are reasons for considering termination of pregnancy. Detection of congenital ZIKV infection by measuring the mother's viremia may facilitate earlier diagnosis of the congenital infection compared to ultrasonography which is currently relied upon. It is still unclear, however, when during maternal infection, or which aspect of infection leads to these

congenital defects in the foetus, making the interpretation of maternal test results problematic without further research.

Differentiating DENV and ZIKV

It is well known that antibodies against different flaviviruses cross-react and that causes challenges in diagnostics, especially in the later phases of the disease when viremia and NS1 antigenemia have passed and these virus specific tests are not choices for the diagnostics. DENV IgM ELISA has been reported to give positive results in ZIKV infection by us and also by others [240,314]. The commercial ZIKV combined IgM/IgG test (Euroimmun) has been reported to be quite specific but with lower sensitivity when compared to IgM-capture ELISA assays (InBios and CDC) [315,316]. There may be variation in the specificity of the tests of different manufacturers. The DENV NS1 Ag ELISA test (Bio-Rad) has not been reported to give false positive; cross-reaction neither suspected results in our studies nor reported by others yet a positive DENV NS1 ag test may not rule out ZIKV infection, as double infections are also possible. However, we observed that Bioline DENV NS1 Ag rapid test (Standard Diagnostics Ink., Republic of Korea) gave positive results when tested with culture supernatants from different Zika virus isolations [317]. Although the tested sample did not represent a clinical sample, it demonstrated that antibodies utilized in the detection of DENV NS1 also recognized ZIKV NS1. In studies IV and V cross-reaction derived false positive results were observed in DENV serology when patient samples of ZIKV infections were tested. Currently, as the diagnostics of DENV in many countries is based on clinical findings and possibly serology, the risk of misdiagnoses and delayed observation of the outbreaks is notable.

Non-invasive diagnostics

The use of non-invasive sample materials and capillary blood samples have been studied widely in endemic populations but to lesser extent in traveller

populations [253,255,301,306,318-323]. In dengue infections NS1 Ag, viral RNA and also antibodies against DENV are shown to be detectable from saliva and urine [253,301,320,322,323].

The use of urine as a diagnostic material in DENV & ZIKV infections (studies III and IV)

Although it may be suspected that DENV and ZIKV, related viruses would have similar profiles regarding diagnostic procedures, these have been observed to differ. In both infections the kinetics of the secretion of RNA to urine seems to differ from that of serum. In DENV infections the peak of the levels in urine takes place notably later (from day 6 on after the onset of symptoms) than in ZIKV infections, and moreover, the viral loads, in terms of viral RNA, seem to be higher in urine than in serum in light of recent studies [255,301]. In ZIKV infections, the evidence is more controversial than in DENV infections, where naturally more research data is available. Some studies show longer period and higher levels of the ZIKV RNA in urine than in serum [240,303] but another exhaustive study reports longer duration of ZIKV RNA detection from serum than urine, two and one weeks after onset of symptoms respectively [233]. In case of Zika, further investigations of the kinetics of infection and the secretion of markers of viral infection to different sample materials are needed. Even though it has been shown that the serotype of DENV has impact on the level and duration of viremia and so to the detection span of the viral RNA from serum [248] it remains to be explored whether this is also valid for detection of DENV RNA from urine.

The observation that the time frame and peaks of viremia (or RNAemia) from sera and urine differs, is interesting also from the viewpoint of the course of the infection in the human body, which is currently not well known. The unsuccessfulness of the urine sample DENV isolation in comparison to the success from the serum was also a surprise and interestingly, there are no other reports either of isolation of DENV from these sample materials. This may be related to the chemical properties of urine. However, isolation of other mosquito- and tick-borne flaviviruses, including ZIKV, from urine has been reported [324-326].

Urine as a sample material represents features that should be standardized in order to get comparable results, for instance incubation time in bladder. It would be easily an assumption that the dilution, and thus the amount of

fluids a patient has drunk and the incubation period of the urine in the bladder, would have impact on the results. Yet, the lacking correlation between urine osmolality and both urine RNA and NS1 Ag levels [255] suggest, that this might not play a major role in using urine as a test material.

Saliva as a diagnostic material in DENV diagnostics

Saliva as a diagnostic material offers interesting possibilities for diagnostics e.g. in terms of rapid tests. However, it seems (for at least now) that saliva as a diagnostic sample material is complex as compared to serum and urine. The detection of viral RNA in saliva seems to be infrequent when comparing to serum and urine in both DENV and ZIKV infections [233,255]. The detection rates may be influenced by the saliva as a sample material, e.g. the used RNA extraction methods may not be optimal for saliva due to composition and enzymatic activities of components saliva. Additionally, saliva may contain inhibitors that may hamper RNA detection. Also the detection of NS1 from saliva resulted not in as consequential conclusions as from urine. This may be due to differences in sampling or sample quality, if the person has ate just before sampling, the inhibitors in saliva, the composition of the sample etc that may affect NS1 ag detection from saliva. Most of these could theoretically be avoided with a specifically developed rapid test that would just be stick-to-mouth principle (this also would standardize the sampling). To increase the usability of saliva in diagnostics, developing specific buffers and reagents may be required. If the methodological challenges in saliva diagnostics could be overcome, saliva as a sample material may provide wide usability.

Emergence and complications of ZIKV

As the ZIKV epidemics hit Brazil a question emerged of associations between ZIKV infection of pregnant women and children born with microcephaly due to a major increase in microcephaly cases in the region

[327]. We were able to show in the studied patient case the causality between foetal infection with ZIKV and abnormalities in brain development. We also showed the prolonged viremic phase of the mother due to the congenital infection. Prolonged viremia of pregnant women is currently noted as a common phenomenon [265,266,311]. In addition neurological complications, including GBS and meningoencephalitis, of adults were observed since French Polynesian outbreak [214,216,289]. As ZIKV was previously considered as a moderately harmless virus with mild symptoms and low occurrence, the current epidemic with new and striking disease associations caused WHO to declare the Public Health Emergency of International Concern (PHEIC) on February 1, 2016.

Besides the alarming complications, transmission routes not typical for flaviviruses, namely sexual and prenatal transmission, were associated with ZIKV infections [149,151,157,328-330]. The complications associated with ZIKV infection may, however, have become observable simply because of the high case numbers in the current outbreak. Prior to outbreak of Yap island, less than 20 human cases had been reported for five decades.

Global epidemiology of DENV and ZIKV

With help of case studies of the travellers the molecular epidemiology and circulation of both ZIKV and DENV were studied. This resulted in observations of new areas for ZIKV circulation and provision of data for molecular epidemiology of Madeiran dengue epidemic in 2012.

The case of a Finnish traveller revealed the presence of ZIKV in Maldives; our observation was the first one reported from the country. The nearest reports of the ZIKV infections previously were from mainland of Southeast Asia. After our notification for authorities in the Maldives, the authorities of Maldives have taken action to survey the situation. CDC has now announced the finding of mosquitoes infected by ZIKV reported by public health officers of the Maldives [331]. Yet, no further reports of the human cases from Maldives are found in PubMed in the time of writing this thesis. As most of the ZIKV infections are asymptomatic or the clinical picture is really

mild and resembles dengue and CHIKV infections, known to circulate in Maldives this patient case would have easily been misdiagnosed on the basis of symptoms alone. With serology testing the case showed characteristics of an acute dengue infection. Without PCR testing, which was initially performed to find out the infecting serotype of DENV, this case would not have been identified as ZIKV.

The outbreak in Madeira was the largest autochthonous one Europe has had to face so far. The vector in this outbreak was *Ae. aegypti* which presence has been reported from Madeira (Funchal) 2005 [332]. The virus causing the outbreak was shown to be DENV-1, American/African genotype with greatest probabilities of originating from Venezuela [43,333]. Considering the expanding distribution areas of *Ae. albopictus*, this outbreak most probably will not be the last one. Viremic travellers returning to their homelands with infestation of suitable vector species may create a risk for outbreak as it has been suggested to happen in Madeira based on peak of dengue importation by travellers and the timing of the outbreak [43].

One interesting viewpoint in European dengue outbreaks is that majority of them have so far been shown to be caused by a single serotype, namely DENV-1. Knowing that all four serotypes are circulating throughout tropics and subtropics, a question arises of why DENV-1 seems to be dominating type in European cases. Is it due to its prevalence in the areas European travellers like to travel or could it be that DENV-1 produces highest level of viremia or more severe symptoms-leading to seeking medical care during primary infections and would in that way be most probable cause of outbreaks when imported to previously unaffected regions by viremic travellers? Also the question of competence of European vectors for DENV can be raised. As the mosquito species in different geographical areas differ from each other, the vector competence for different DENVs might vary. However, within the Finnish travellers, viremic patients of all serotypes have been detected. As cell culture virus isolation is considered less sensitive than mosquito inoculation, we would expect these patients to be infectious for suitable mosquito vector species. Taking into account that in last few years DENV-2 has displaced DENV-1 as the most common serotype circulating in South East Asia, Europe might experience more outbreaks caused by DENV-2 in future. On the other hand it has been observed that longer viremia and higher viral loads are often associated with DENV-1

infections compared to infections caused by either DENV-2 or DENV-3 [248]. This could at least partly explain the DENV-1 outbreaks in Europe as the travellers returning from endemic areas with DENV-1 infection stay viremic longer, thus increasing the possibility of transmission by the local vectors.

Cases from the Madeiran outbreak were diagnosed across Europe and also in Finland [44,334]. Madeira lies near to North African coast and its subtropical climate has enabled the establishment of *Aedes aegypti* which was first recorded there in 2005 [335]. The onset of the outbreak was in the beginning of October 2012 and it continued to February 2013. More than 2000 cases were reported, out of these 78 imported to 13 countries in Europe, 7 cases to Finland [44,334]. The origin of this outbreak has been proposed to be in South America and specifically Venezuela on the basis of importation index and also viral phylogeny [43,333,334].

Ae. albopictus is known as highly invasive species and it is adapted for breeding in various environments. The first occurrence of this species in Europe was reported in 1979 from Albania [336] and to date it has been established in Southern Europe and Central Europe. In Eastern and Northern, Europe the winter temperature is likely to be the limiting factor for distribution of *Ae. albopictus*, as the winter temperatures are too low for the survival of either adults or eggs. In Southern Europe, the temperatures of the summers set the limits as the optimal development of *Ae. albopictus* requires temperatures between 25-30°C [336]. Yet it is not known in detail why this or other mosquito species survive in some environments and not in others- thus which are the critical factors behind establishment. It could be speculated if the micro environments, like greenhouses, would enable the spread of small communities more easily also to otherwise "unsuitable" regions. Surveillances of the greenhouses might be needed as it is known that in Netherlands populations in these environments have been observed. When flowers are traded from country to another, also mosquitoes might bet transported. Establishment of this mosquito species offers infrastructure for MBVs, such like Zika and dengue, which do not need other vertebrate hosts for amplification. Also *Ae. aegypti* has been established in Europe previously but has disappeared from the mainland (except the easternmost Europe; Georgia and Russia) whereas it is established in Madeira [85,332,337,338]. Re-establishment of this efficient vector of for example ZIKV, DENV and CHIKV to Continental Europe would

probably have an effect on the MBV virome and transmission dynamics in Europe.

Considering situation in Finland, there already exist possible vectors (for example *Culex pipiens*) for WNV and USUV. Knowing the implications of warming climate on many species of insects it is probable that also the occurrence and prevalence of mosquito species in Finland will change/expand in the future decades. This will enable entrance for also new mosquito-borne viruses. The active traffic of migratory birds has already probably enabled spreading of Sindbis virus to Finland in 1970s [339]. The expanding distribution areas of the vector mosquitoes does not affect only if introduced to Finland. The existence of vector species and MBVs in Europe increases greatly the volume of Finnish travellers exposed to these viruses and so highlights the need of accurate diagnostics and consciousness of possibilities of MBV infections also with travellers returning from Europe.

The expanding distribution areas of the vector mosquitoes and along that, the viruses will produce challenges in the future. Screening of the travellers offers tools not just in managing the care of that patient but also in monitoring the outbreaks and the global movement of the viruses. While the mosquito species establish in new areas it is highly important to monitor the viruses that are imported to these areas in terms of disease distribution control. The DENV outbreak of Madeira, the ZIKV case from Maldives (studies I and IV) show the role of travellers in both as a risk to distribute the virus into new, formerly naïve areas but also to uncover the circulation of the virus in areas that are not known to be endemic. As viremic travellers, returning from dengue endemic areas have been considered as a main route for the virus transmission to non-endemic areas, rapid diagnosis does not serve solely as managing the individual infection but also assists in controlling the spreading of the virus for example by guidance of the patients to avoid contact with the local mosquitoes. It has been reported that the only limitation for establishment of DENVs is the existence of suitable vectors[86] which emphasizes the necessity of recognition of viremic patients especially in areas infested with *Aedes albopictus* or *aegypti* to prevent subsequent transmission by local mosquitoes.

There is no special medication against Zika or dengue or vaccine against Zika. The dengue vaccine on market in the Americas has its disadvantages

which restrict its use for naïve populations like travellers. The importance of vector control as preventive action against these viruses, as well as against many other mosquito-borne pathogens, is major. Moreover, this emphasizes the necessity of monitoring the outbreaks and global circulation of these viruses and their vectors. Exact diagnostics is not just to manage the patient but also to benefit population and observing possible outbreaks as soon as possible in order to control them.

10 CONCLUDING REMARKS

The distribution, occurrence and prevalence of vector-borne diseases are under changes due to environmental changes causing the changes in distribution areas of the vectors and also the increase in mobility of humans and the global trade. This tendency can be seen both in tick-borne and mosquito-borne infections. Also new vector-borne diseases are emerging. This causes challenges in differential diagnostics and emphasizes the need for optimized laboratory diagnostic protocols including methodologies and sample materials used. Understanding the kinetics of the markers of the infection and secretion of them to different sample materials helps to find the effective combinations for diagnostics at different stages of the disease. Optimized early diagnosis helps also in patient management.

The changing and expanding distribution areas of vector-borne flaviviruses will probably also effect to the epidemiology of these diseases in the future. As increasing number of people live in areas with available predisposition to some of these viruses, it will mean that large populations have old flaviviral immunity. This will have implications to interpretation of serological test results, but may also have other effects. It remains to be seen how the pre-existing, potentially cross-reactive and non-protective immunity will affect to disease outcome and virus transmission. Old immunity against DENV is thought to cause more severe course of infection with Zika virus via mechanisms of antibody-dependent enhancement. Whether this is the case, will remain to be properly investigated. In Europe, Usutu and West Nile are endemic in large areas. Within decades, there probably will be high seroprevalence against these viruses in the endemic regions. Whether the

population immunity against these viruses would protect against or enhance potentially emerging heterologous flavivirus infections, such as DENV or ZIKV in Europe will remain to be seen.

Infected travellers provide a possibility to identify a causative viral agent from local circulation perhaps already before the outbreaks and spread distribution areas of the MBVs. In the endemic areas the resources do not often allow thorough surveillance of MBVs and the limited resources may be targeted to outbreak situations. This however does not allow the understanding of the complete picture, as the increase of symptomatic patient cases is often only the tip of the iceberg that has become visible. The information about the global circulation of the MBVs gained from traveller cases promotes the predictions about future distribution areas and disease burden of these viruses.

DENV and ZIKV infections are transmitted by the same vector so the distribution areas naturally overlap. The symptoms can be similar and as they are closely related viruses the risk of misdiagnosis is obvious. Antibodies cross-react and then again in many endemic areas the facilities do not enable the detection of viral nucleic acids. Though there are shortcomings in specificity of the DENV NS1 Ag rapid tests, NS1 Ag ELISA tests have been shown to be highly specific. The NS1 Ag ELISA detection provides a promising method to differentiate DENV from ZIKV. What would be needed would be a ZIKV specific ZIKV-NS1 ag test. This is not yet on the market, but probably under development by various diagnostic companies. The potential of this kind of test is however depending on the kinetics, secretion and detectability of ZIKV NS1 Ag in patient samples, which is currently not known. As it has been seen in other infection markers, such as the viral RNA detection, DENV and ZIKV infections are not alike- this may be the case also with NS1 Ag. Currently many different test types are needed in order to detect and reliably confirm DENV or ZIKV infection. Overall the diagnostic method selection for flaviviruses has developed during the recent years and is now boosted to respond to the current needs for ZIKV diagnosis the problems with cross-reactions. When the research data so suggests, also the diagnostic guidelines will be changed. The accumulating evidence of the effects of Zika virus infection to foetus, and the detectability of ZIKV markers in pregnant women have already forced to re-evaluate the testing protocol for this specific group of

patients. Populations living in endemic regions might benefit from different procedures than travellers, who are often naïve for flavivirus infections. In future there may be specific diagnostic recommendations for different patient populations.

To date we are heading towards an era when more and more sequence data will be available by NGS methods including whole genome sequences. Most probably this will reveal new viruses circulating and possibly some new disease associations. Yet, finding a virus sequence from a patient sample with NGS does not mean that it is associated to an acute infection. The symptoms of many arboviral infections resemble each other and traditionally specific diagnostics have been required for their detection. The detection has further been hampered the correct timing of the sampling if e.g. only serological methods have been available. Among flaviviruses and other viruses prone for cross-reactivity in serology, there also is risk of indistinct or even misdiagnosis. As NGS methodologies are in principle nucleic acid detection, also similar issues need to take into account as in traditional diagnostic viral nucleic acid detection, such as the timing of sampling in regard to onset of symptoms, sample type and the methodologies used for viral nucleic acid extraction.

If fever patients suspected for arboviral infections patients were screened with NGS methods would we find viruses not known to circulate in certain areas? Another interesting insight would be to screen animals, for example encephalitic horses, as they might be the first sign of the circulating WNV. The lesson learned from Zika is that in very short period of time, a virus that we have neglected or at least not considered to have a big impact on public health may turn into an epidemic with high morbidity and symptoms that have not been seen previously. This emphasizes the need for efficient surveillance and diagnostic tools.

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