Flavivirus Infections in Travelers:
Insights into Prevention of Japanese Encephalitis
and Diagnostics of Dengue

Elina Erra

ACADEMIC DISSERTATION

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Helsinki 2013
Supervisor
Docent Anu Kantele, MD, PhD
Haartman Institute, Faculty of Medicine, University of Helsinki, and
Division of Infectious Diseases, Department of Medicine, Helsinki University Central Hospital
Helsinki, Finland

Reviewers
Hanna Nohynek, MD, PhD
Department of Vaccines and Immune Protection
National Institute for Health and Welfare
Helsinki, Finland

Docent Tytti Vuorinen, MD, PhD
Department of Virology, Faculty of Medicine
University of Turku
Turku, Finland

Opponent
Associate Professor Leo Visser, MD, PhD
Department of Infectious Diseases
Leiden University
Leiden, the Netherlands

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A journey of a thousand miles starts with a single step.

-Laozi
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I

II

III

IV

* The authors contributed equally to the study.

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<td>adverse event</td>
</tr>
<tr>
<td>ALT</td>
<td>alanine transaminase</td>
</tr>
<tr>
<td>AST</td>
<td>aspartate transaminase</td>
</tr>
<tr>
<td>C</td>
<td>capsid protein</td>
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<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
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<td>DENV</td>
<td>dengue virus</td>
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<td>DHF</td>
<td>dengue hemorrhagic fever</td>
</tr>
<tr>
<td>E</td>
<td>envelope protein</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>GI</td>
<td>genotype I</td>
</tr>
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<td>GII</td>
<td>genotype II</td>
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<tr>
<td>GIII</td>
<td>genotype III</td>
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<tr>
<td>GIV</td>
<td>genotype IV</td>
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<tr>
<td>GV</td>
<td>genotype V</td>
</tr>
<tr>
<td>GAMM</td>
<td>generalized additive mixed model</td>
</tr>
<tr>
<td>GMT</td>
<td>geometric mean titer</td>
</tr>
<tr>
<td>JE</td>
<td>Japanese encephalitis</td>
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<tr>
<td>JE-MB</td>
<td>inactivated, mouse brain–derived Japanese encephalitis vaccine</td>
</tr>
<tr>
<td>JE-VC</td>
<td>inactivated, Vero cell–derived Japanese encephalitis vaccine</td>
</tr>
<tr>
<td>JEV</td>
<td>Japanese encephalitis virus</td>
</tr>
<tr>
<td>M</td>
<td>membrane protein</td>
</tr>
<tr>
<td>NS1</td>
<td>non-structural protein 1</td>
</tr>
<tr>
<td>prM</td>
<td>precursor to membrane protein</td>
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<tr>
<td>PRNT</td>
<td>plaque-reduction neutralization test</td>
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<tr>
<td>RT-PCR</td>
<td>reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>SPR</td>
<td>seroprotection rate</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
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<tr>
<td>TBE</td>
<td>tick-borne encephalitis</td>
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<td>YF</td>
<td>yellow fever</td>
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Abstract

The mosquito-borne flavivirus infections Japanese encephalitis (JE) and dengue pose a considerable disease burden in the tropical and subtropical regions of the world. Sometimes these infections also affect international travelers visiting areas endemic for the diseases. This thesis addresses the prevention of Japanese encephalitis and diagnostics of dengue, with a focus on traveler’s health. The main aim was to find solutions to some practical clinical questions, and thus provide clinically important new data on travel-associated flavivirus infections.

The first three studies assessed the immunogenicity of two inactivated JE vaccines, the new Vero cell–derived (JE-VC) and the traditional mouse brain–derived (JE-MB) preparation, in 120 Finnish and Swedish adult travelers, by determining the pre- and post-vaccination titers of JE virus neutralizing antibodies with plaque-reduction neutralization test (PRNT). A PRNT$_{50}$ titer ≥10 was considered protective.

Study I addressed the boosting capacity of the SA14-14-2–based JE-VC vaccine in subjects previously primed with the Nakayama strain–based JE-MB preparation. The response rates were 91% after a homologous (JE-MB) and 95%-98% after a heterologous booster dose (JE-VC). Among those with no seroprotection at baseline, a higher proportion of JE-MB–primed (100%) than non-primed (40%) subjects seroconverted after a single JE-VC dose. The data suggest that a single JE-VC dose suffices for boosting JE-MB immunity, and call for re-evaluation of guidelines recommending two JE-VC doses for JE-MB–primed subjects.

Study II demonstrated that both JE-VC and JE-MB elicit neutralizing antibodies, not only against the vaccine genotype, but also against heterologous JE virus genotypes. The seroprotection rates against the heterologous strains were 93%-97% after JE-VC, and 83%-92% after JE-MB primary series. The data imply that the two vaccines are expected to confer seroprotection against all major JE virus genotypes circulating.

Study III evaluated long-term seroprotection after JE-VC primary and booster vaccinations. Two years after primary immunization, 93% of vaccinees were seroprotected against the vaccine strain but only 73% against the emerging genotype GI. JE-MB–primed vaccinees were seroprotected against both vaccine (100%) and non-vaccine (89%-95%) genotype strains two years after the heterologous JE-VC booster dose, further supporting the use of a single JE-VC dose for boosting JE-MB immunity.
Study IV explored the diagnostic markers of dengue in 93 Finnish traveler patients. The duration of viremia (9 days, 95% CI: 8-10) and NS1 antigenemia (15 days, 95% CI: 12-20) were longer than reported in endemic settings, presumably due to a high proportion of primary infections among travelers. The data support use of test combinations, e.g. antibody and NS1 detection, for efficient diagnostics. The relative levels of viremia and NS1 antigenemia were associated with some central clinical parameters, suggesting these virologic markers as predictors of the clinical manifestations in travelers’ dengue.
Preface

A total of 26 virus species of the genus Flavivirus, family Flaviviridae, are known to cause disease in humans\textsuperscript{1,2}. While many of these viruses only have a limited impact worldwide, the virus group also includes pathogens of major global significance, such as dengue, Japanese encephalitis, tick-borne encephalitis, West Nile, and yellow fever viruses.

Recent decades have witnessed remarkable changes in the epidemiology of flaviviral diseases. One striking example is the recent introduction and rapid spread of West Nile virus, a close relative of Japanese encephalitis virus, in North America\textsuperscript{1}. Over the past 50 years, the geographical distribution of dengue has expanded substantially, and today, more than 100 countries are affected\textsuperscript{3}. In 2010, autochthonous dengue cases were reported in Europe for the first time since the epidemic in Greece in the late 1920s\textsuperscript{4-7}. In 2012, a dengue epidemic with over 2000 cases occurred in Madeira\textsuperscript{8}. Interestingly, genomic sequences of Japanese encephalitis virus were detected from mosquitoes in Italy in 2012, suggesting for the first time introduction of the virus to Europe\textsuperscript{9}. Thus far, no autochthonous cases of Japanese encephalitis have been reported.

International travel has increased substantially in recent decades. In 2012, over one billion people traveled outside their countries’ borders, with Asia and the Pacific showing the greatest increase in international arrivals\textsuperscript{10}. The growing numbers of international travel have also been reflected as an emergence of travel-acquired dengue infections. Increasing mobility of humans thus exposes previously naïve populations to new pathogens.

In addition to endemic data, study of flaviviral pathogens in non-endemic populations is needed. Endemic and non-endemic populations differ in many essential background characteristics, such as the average age at the time of primary infection, genetic background, and pre-existing immunity to other flaviviruses – all of these factors may significantly affect the outcome of the infection\textsuperscript{11}.

This thesis addresses two globally important flavivirus infections, Japanese encephalitis and dengue, with a focus on traveler’s health. Prevention of Japanese encephalitis by vaccination is a frequent clinical question at pre-travel appointments among travelers heading to Asia, while dengue is one of the leading post-travel challenges in ill travelers returning from the tropics and subtropics around the world. Of the four original studies, three explore Japanese encephalitis vaccine immunity, and one focuses on the diagnostics of dengue infections in travelers from Northern
Europe. The main aim of this thesis is to find solutions to some practical clinical questions, and thus provide clinically important new data on travel-associated flavivirus infections.
1. REVIEW OF THE LITERATURE

1.1 Brief introduction to flaviviruses

The genus *Flavivirus* of the family *Flaviviridae* comprises over 50 species, 26 of which are known human pathogens\(^1,2\). The majority of these viruses are transmitted to vertebrate hosts by mosquitoes or ticks, and are therefore referred to as arthropod-borne, or arboviruses. Some members of the genus have no known vector, and some only appear to infect mosquitoes\(^2\). Table 1 lists the flavivirus species known to cause disease in humans.

Flavivirus virions are small, approximately 50 nm in diameter. The genome is a single-stranded positive-sense RNA molecule of \(~11\) kb. The core of the virion, the capsid, is composed of the viral genome and copies of the capsid (C) protein. The capsid is surrounded by a host-derived lipid envelope, in which copies of membrane (M) and envelope (E) proteins are inserted. In addition to the three structural proteins (C, prM [precursor to M], E), the viral genome also encodes seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, NS5) which are not incorporated into the virion.

![Flavivirus virion structure](image)

**Figure 1.** A schematic presentation of the flavivirus virion structure.
Table 1. Flavivirus species pathogenic to human (adapted from\textsuperscript{1,2})

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<th>Clinical manifestation</th>
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<td></td>
<td></td>
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<tr>
<td><em>Kyasanur Forest disease virus</em></td>
<td>India, Arabian Peninsula?</td>
<td>hemorrhagic fever</td>
</tr>
<tr>
<td><em>Langat virus</em></td>
<td>Malaysia, Thailand, Siberia</td>
<td>encephalitis</td>
</tr>
<tr>
<td><em>Louping ill virus</em></td>
<td>UK, Ireland</td>
<td>encephalitis</td>
</tr>
<tr>
<td><em>Omsk hemorrhagic fever virus</em></td>
<td>Western Siberia</td>
<td>hemorrhagic fever</td>
</tr>
<tr>
<td><em>Powassan virus</em></td>
<td>Russia, USA, Canada</td>
<td>encephalitis</td>
</tr>
<tr>
<td><em>Tick-borne encephalitis virus</em></td>
<td>Europe, Asia</td>
<td>encephalitis</td>
</tr>
<tr>
<td><strong>Mosquito-borne virus group</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Aroa virus group</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Bussuquara virus</em></td>
<td>Brazil</td>
<td>fever</td>
</tr>
<tr>
<td><em>Dengue virus group</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Dengue virus</em></td>
<td>Tropics, subtropics</td>
<td>(hemorrhagic) fever, rash</td>
</tr>
<tr>
<td><em>Japanese encephalitis virus group</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Japanese encephalitis virus</em></td>
<td>Asia</td>
<td>encephalitis</td>
</tr>
<tr>
<td><em>Koutango virus</em></td>
<td>Senegal</td>
<td>fever, rash</td>
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<tr>
<td><em>Murray Valley encephalitis virus</em></td>
<td>Australia, New Guinea</td>
<td>encephalitis</td>
</tr>
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<td><em>St Louis encephalitis virus</em></td>
<td>South and Central America</td>
<td>encephalitis</td>
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<tr>
<td><em>Usutu virus</em></td>
<td>Europe, Africa</td>
<td>fever, rash</td>
</tr>
<tr>
<td><em>West Nile virus</em></td>
<td>Worldwide</td>
<td>encephalitis</td>
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<tr>
<td><em>Ntaya virus group</em></td>
<td></td>
<td></td>
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<td><em>Bagaza virus</em></td>
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<td>fever</td>
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<tr>
<td><em>Ilheus virus</em></td>
<td>South and Central America</td>
<td>fever, encephalitis</td>
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<td><em>Ntaya virus</em></td>
<td>Africa</td>
<td>fever</td>
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<td>fever, rash</td>
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<td><em>Yellow fever virus group</em></td>
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<tr>
<td><em>Sepik virus</em></td>
<td>New Guinea</td>
<td>fever</td>
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<tr>
<td><em>Wesselbron virus</em></td>
<td>Africa, Asia</td>
<td>unknown</td>
</tr>
<tr>
<td><em>Yellow fever virus</em></td>
<td>Africa, South America</td>
<td>pantropic</td>
</tr>
<tr>
<td><strong>Probably Mosquito-borne virus group</strong></td>
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<td></td>
</tr>
<tr>
<td><em>Edge Hill virus group</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Banzi virus</em></td>
<td>Africa</td>
<td>fever</td>
</tr>
<tr>
<td><strong>No Known Vectors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Modoc virus group</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Apoi virus</em></td>
<td>Japan</td>
<td>encephalitis</td>
</tr>
<tr>
<td><em>Modoc virus</em></td>
<td>USA</td>
<td>encephalitis</td>
</tr>
<tr>
<td><em>Rio Bravo virus group</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Dakar bat virus</em></td>
<td>Africa</td>
<td>fever</td>
</tr>
<tr>
<td><em>Rio Bravo virus</em></td>
<td>USA, Mexico</td>
<td>fever</td>
</tr>
</tbody>
</table>
1.2 Japanese encephalitis

1.2.1 Etiology

*Japanese encephalitis virus* (JEV), the causative agent of Japanese encephalitis (JE), is a member of the Japanese encephalitis virus group in genus *Flavivirus*, family *Flaviviridae*. Other species of the group include the widely dispersed *West Nile virus*, *St Louis encephalitis virus* in the Americas, *Murray Valley encephalitis virus* in Australia and New Guinea, *Cacipacore virus* in South America, *Usutu virus* in Europe and Africa, as well as *Koutango* and *Yaounde viruses* in Africa\(^2,12\).

![Japanese Encephalitis Virus Distribution Map](www.nature.com)

*Figure 2.* The geographical distribution of four major members of the Japanese encephalitis virus group: *Japanese encephalitis virus* (JEV), *Murray Valley encephalitis virus* (MVEV), *St Louis encephalitis virus* (SLEV), and *West Nile virus* (WNV). Reprinted by permission from Macmillan Publishers Ltd: Nature Medicine\(^12\), copyright (2004). ([www.nature.com](www.nature.com))
1.2.2 Japanese encephalitis virus genotypes

JE viruses are divided into five phylogenetically distinct genotypes (GI-GV)\(^\text{13}\). The relationships between different JEV strains were first established by analyses based on limited sequences of the C/prM gene region, while more recent studies have utilized the E gene or full-length genomic sequences for phylogenetic investigations\(^\text{12,14}\).

The most divergent JEV genotypes, GIV and GV, are regarded the oldest lineages\(^\text{13}\). There is no evidence on these genotypes causing human disease at present. Only three strains of GV have ever been found: the first one (Muar strain) from the brain of a Malaysian patient in 1952, and the two others from mosquito pools in China in 2009 (XZ0934 strain), and in South Korea in 2010 (10-1827 strain)\(^\text{15,16}\). As for GIV, five strains of this type have been identified (JKT-6468, JKT-7003, JKT-7887, JKT-8442, JKT-9092), all isolated in Indonesia (in Java, Bali, and Flores) in the beginning of the 1980s. All of these GIV isolates originated in mosquito pools, and their ability to cause human disease remains unproven\(^\text{17}\).

Of the three more recent JEV genotypes, reports on GII isolates are the rarest. The earliest known GII isolate (Bennett strain) was identified in Korea before the year 1951\(^\text{18}\). Between 1970 and 1999, several GII strains were isolated in Southern Thailand, Malaysia, Indonesia, Papua New Guinea, and Northern Australia\(^\text{12,19-21}\). In the 21\(^{\text{st}}\) century, no reports of GII encephalitis have been published.

While the clinical importance of GII, GIV, and GV appears minor at present, the remaining two genotypes, GI and GIII, have been associated with recent JE outbreaks\(^\text{22-25}\).

Since the first isolation of a JE virus (Nakayama strain, GIII) in 1935, strains belonging to GIII have been recognized as a major cause of clinical encephalitis. Until the latter part of the 20\(^{\text{th}}\) century, GIII was the dominant genotype circulating in large areas of Asia\(^\text{12}\). Since then, however, GI has been emerging, and many Asian countries have reported the replacement of GIII by GI strains\(^\text{12,14}\). The genotype shift from GIII to GI occurred in Japan and South Korea in the early 1990s\(^\text{26-30}\), in Thailand and Vietnam by the beginning of the 21\(^{\text{st}}\) century\(^\text{31-33}\), and in Taiwan around the year 2009\(^\text{34-36}\). In 2000, the appearance of GI was also demonstrated in the Torres Strait of Australia\(^\text{37}\). In China and India, both GI and GIII co-circulate\(^\text{23-25,38-41}\).

Despite existence of multiple genotypes, it is believed that all JEV strains belong to a single serotype. An analysis of complete E gene regions of different JEV strains showed that the divergence between strains was up to 12% (at the amino acid
level), which is less than the minimum difference between established serotypes of other positive-strand RNA viruses studied; polio (18%) and dengue (22%)\textsuperscript{42}.

### 1.2.3 Transmission

Pigs and ardeid birds are the main vertebrate hosts of JEV\textsuperscript{43}. Virus transmission occurs principally through Culex mosquitoes, of which Cx. tritaeniorhynchus is the main vector species. It breeds in water pools and flooded rice fields, and feeds outdoors at night time\textsuperscript{43}. Important secondary vectors include, for instance, Cx. gelidus, Cx. fuscocephala, and Cx. annulirostris\textsuperscript{14}.

Humans acquire the virus when bitten by an infected vector mosquito. In rare occasions, transplacental transmission from mother to fetus has been described\textsuperscript{45}. Humans are only incidental and dead-end hosts of JEV; they do not contribute to the natural transmission cycle due to low and transient viremia\textsuperscript{46}.

### 1.2.4 Epidemiology

According to current data, a total of twenty-five countries have areas with risk of JEV transmission\textsuperscript{47}. The affected countries include Australia, Bangladesh, Bhutan, Brunei Darussalam, Burma (Myanmar), Cambodia, China, India, Indonesia, Japan, Laos, Malaysia, Nepal, North Korea, Pakistan, Papua New Guinea, the Philippines, Russia, Singapore, South Korea, Sri Lanka, Taiwan, Thailand, Timor-Leste, and Vietnam.

JE is predominantly a rural disease, yet cases may also occur in peri-urban areas where the elements required for the transmission cycle are available. Two epidemiological patterns are characteristic for the disease; seasonal summer epidemics in temperate regions, and a year-round transmission with rainy season epidemics in tropical areas\textsuperscript{12}.

Reliable data on the incidence of JE are lacking. In rural hyperenzootic settings, almost all the population is exposed to the virus by early adulthood\textsuperscript{48}. The vast majority of human JEV infections are asymptomatic, and only around 0.1%-4% of infected persons develop a clinical illness\textsuperscript{16}. According to estimates, JEV causes approximately 68,000 clinical cases each year, of which around 14,300-27,200 lead to long-term disability and 13,600-20,400 to death\textsuperscript{49}. JE is largely a childhood disease;
approximately 75% of the cases affect children below 15 years of age\textsuperscript{49}. A second peak in incidence is seen in the elderly\textsuperscript{50}, probably due to waning immunity.

1.2.5 Clinical features

1.2.5.1 Symptoms

JEV has an incubation period of 5 to 15 days. A non-specific viral prodrome often precedes the neurologic illness which may involve the brain parenchyma (encephalitis), meninges (aseptic meningitis), or spinal cord (myelitis)\textsuperscript{50}. A typical JEV meningoencephalitis presents with fever, vomiting, headache, nuchal rigidity, focal neurologic signs, and a reduced level of consciousness. Seizures are more frequent in children than in adults\textsuperscript{46}. Motor weakness and movement disorders are common. The classic Parkinsonian syndrome associated with JE is characterized by dull facies, cogwheel rigidity, and tremor.

1.2.5.2 Laboratory and radiological findings

Routine blood laboratory findings may include mild leukocytosis and hyponatremia. Cerebrospinal fluid (CSF) analysis typically reveals a pleocytosis of 10 to 500 /mm\textsuperscript{3} with lymphocytes predominating, a normal or slightly elevated protein concentration, and normal glucose levels. The CSF opening pressure may be elevated\textsuperscript{50}.

When exploring the radiological findings in JE, magnetic resonance imaging (MRI) is more sensitive than computed tomography (CT)\textsuperscript{51}. Bilateral thalamic lesions are characteristic\textsuperscript{51-53}. Typical findings may include lesions in the basal ganglia, midbrain, cerebellum, pons, and cerebral cortex\textsuperscript{51-53}. Hemorrhagic lesions are common\textsuperscript{52,53}.

1.2.6 Microbiological diagnostics

In clinical settings, the microbiological diagnostics of JE mostly relies on serological methods. Demonstration of JEV-specific antibody in CSF by IgM capture enzyme-linked immunosorbent assay (ELISA) is usually regarded as confirmatory for the
In many endemic areas, the possibilities to obtain a laboratory-confirmed diagnosis are limited. As lumbar puncture is not feasible in all settings, serum samples are also used for detection of virus-specific IgM antibodies, even though the positive predictive value of the IgM capture ELISA is lower in serum than in CSF. If the early serum sample proves negative for anti-JEV IgM antibodies, assessment of a second sample at around the 10th day of illness is recommended.

Due to antigenic similarities among flaviviruses, cross-reactive responses are often seen in the serologic assays. This should be taken into account in the interpretation of serologic tests, as a positive test result may not always specify the etiologic agent. In JEV endemic areas, other relevant flaviviruses include, for instance, dengue and West Nile viruses. In temperate areas of Europe and Asia, immune responses to tick-borne encephalitis virus should be considered. Similarly, the history of flavivirus vaccinations may be of relevance. Notably, because of the shortness and low level of JEV viremia, efforts to isolate the virus or detect its genome in serum or CSF usually fail.

The differential diagnosis of JE includes other viral and flaviviral encephalitides, bacterial, fungal, and parasitic infections, as well as non-infectious etiologies. Special attention should be paid to central nervous system infections for which specific treatment are available, such as Herpes simplex virus infections, bacterial meningitis, and cerebral malaria.

1.2.7 Treatment and prognosis

While infection with JEV is usually asymptomatic, clinical cases often have a severe outcome. Currently, no specific therapy exists for JE. Treatment consists of supportive care, and management of infection complications, including seizures, raised intracranial pressure, and secondary infections.

Reported case-fatality rates vary between 8% and 33%57-60, and approximately half of the survivors suffer from persistent sequelae, such as motor deficits, seizures, cognitive impairment of varying severity, as well as changes in behavior and personality59,61,62. Various predictors of poor outcome have been reported, including female sex, advanced age, successful virus isolation from CSF, low Glasgow coma score, seizures, presence of focal neurological deficits, and a prolonged illness course57,59-61,63.
1.2.8 Japanese encephalitis in travelers

Accurate data on the incidence of travel-associated JE are lacking. The overall incidence is estimated to be extremely low, less than 1 case per 1 million travelers to Asia\textsuperscript{64,65}. Among Finnish and Swedish travelers to Thailand, the incidence has been estimated to be in the range of 1 case per 250,000-400,000 visits\textsuperscript{66,67}. The above mentioned crude incidence estimates have been calculated by retrospectively relating the number of published JE cases to the number of travelers to a given destination. The number of published cases probably underestimates the true number of JEV infections in travelers, as mild cases may go unrecognized, and not all diagnosed cases are published. Moreover, the overall number of travelers to a given destination unlikely represents the true at-risk population, as the risk of acquiring the disease varies based on the itinerary, season and duration of travel, activities of the traveler, and preventive measures taken, including possible immunization against the disease before departure. For persons staying prolonged periods in rural areas with on-going transmission, the monthly risk of acquiring JE has been estimated to be around 1 per 50,000\textsuperscript{48}. One Australian prospective study aimed at exploring the incidence of travel-associated JEV infections by determining virus-specific antibodies in travelers before and after their trip to Asia\textsuperscript{68}. In that study, none of the 387 travelers showed a diagnostic seroconversion against JEV.

Between 1973 and 2011, 61 cases of travel-acquired JE were published\textsuperscript{65,69-73}, including two cases in Finnish travelers\textsuperscript{66,74}. Among those with known travel history, the infection was most commonly acquired in Thailand, followed by Indonesia, China, and the Philippines [Table 2]. The infection was most frequently acquired during long-term trips (duration ≥4 weeks), although cases in short-term travelers were also reported\textsuperscript{66,75,76}. The age span of the patients ranged from 1 to 91 years, highlighting the fact that in previously naïve populations, JE may affect persons at any age. Of the cases with known outcome, 24% made full recovery, 53% developed sequelae, and 24% died. Notably, none of the traveler patients had reported previous vaccination against JE.
Table 2. Background characteristics of the travel-associated JE cases, 1973-2011.

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<th>median</th>
<th>range</th>
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<tbody>
<tr>
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<td>(1-91)</td>
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<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>54 %</td>
<td>(31/57)</td>
</tr>
<tr>
<td>Female</td>
<td>46 %</td>
<td>(26/57)</td>
</tr>
<tr>
<td>Duration of travel</td>
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<td></td>
</tr>
<tr>
<td>&lt;4 weeks</td>
<td>37 %</td>
<td>(15/41)</td>
</tr>
<tr>
<td>≥4 weeks</td>
<td>63 %</td>
<td>(26/41)</td>
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<tr>
<td>Travel destination</td>
<td></td>
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<tr>
<td>Thailand</td>
<td>37 %</td>
<td>(21/57)</td>
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<tr>
<td>Indonesia</td>
<td>16 %</td>
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<tr>
<td>China</td>
<td>12 %</td>
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<td>the Philippines</td>
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<tr>
<td>Japan</td>
<td>7 %</td>
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<tr>
<td>Vietnam</td>
<td>5 %</td>
<td>(3/57)</td>
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<tr>
<td>Cambodia</td>
<td>2 %</td>
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<tr>
<td>Papua New Guinea</td>
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<td>Malaysia</td>
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<td>Singapore</td>
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<td>Hong Kong</td>
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<td>Burma (Myanmar)</td>
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1.2.9 Control

Several interventions have been proposed for reducing the incidence of JE in humans, including control of mosquito and pig populations, personal protective measures to avoid mosquito bites, and so far the most effective means, human immunization.77

The current strategies to control vector mosquito populations in rural areas have proven insufficient.77 Biological control agents, such as larvicidal bacteria, larvivorous fish, or different natural products have been proposed as environmental-friendly alternatives for chemical insecticides.78 Alternate wet and dry irrigation, a vector control approach of a different type, has shown to decrease Cx. tritaeniörhynchus populations in rice fields, while simultaneously increasing the crop yield and decreasing water consumption.78 As for control of pig populations, vaccination of swines appears impractical and ineffective in preventing human
disease, whereas relocation of domestic pigs distant from human habitats might have a positive effect in reducing JE incidence\(^4^4\).

Personal protective measures, besides active immunization, focus on reducing the risk of vector exposure by using mosquito repellents and protective clothing, by avoiding outdoor activities in the evening, and by sleeping in screened rooms or under permethrin-impregnated mosquito bed nets\(^7^9\). These interventions provide, however, only a limited solution.

**1.2.10 Active immunization against Japanese encephalitis**

Active immunization of humans is currently considered the most effective means to reduce JE incidence\(^7^7\). After implementation of routine childhood vaccinations in the late 1960s and 1970s, several endemic countries have experienced a remarkable decline in disease incidence\(^4^8\). Humans only being dead-end hosts of the virus, the JE vaccines do not confer herd immunity. Moreover, as several host and vector species are involved, complete eradication of the virus is unlikely.

**1.2.10.1 Vaccination of endemic populations**

In endemic areas, universal childhood immunization against JE is considered desirable\(^4^8\). The World Health Organization (WHO) recommends integration of JE vaccination into immunization programs in all areas where the disease constitutes a public health problem\(^8^0\).

Due to economic reasons, competing health priorities, and differences in disease risk, the immunization schedules and quality of JE vaccination programs vary widely from one country to another. Some countries have well-developed immunization programs, while others only have a partial program or no program at all. In 2012, 12 of the 25 JEV endemic or epidemic countries had a vaccination program against the disease\(^4^7\). Eight of the programs were implemented in all risk areas, and four only in selected regions. Countries with a relatively high incidence but less developed or non-existent vaccination programs include, for example, Burma (Myanmar), Indonesia, Laos, Malaysia, and the Philippines\(^4^9\).
1.2.10.2 Vaccination of travelers

At present, vaccination against JE is not recommended routinely for all travelers heading to JEV endemic areas. When evaluating whether an individual traveler should be vaccinated, several factors are considered: the actual risk of getting infected and the severity of the potential disease; efficacy, potential adverse effects and costs of the vaccine; and the traveler’s individual preferences.

According to WHO, vaccination against JE is recommended for “travelers with extensive outdoor exposure (camping, hiking, working, etc.) during the transmission season, particularly in endemic countries or areas where flooding irrigation is practiced”\(^81\). The U.S. Centers for Disease Control and Prevention (CDC) currently recommends JE vaccination for travelers who plan to spend at least one month in endemic areas during the transmission season\(^82\). According to their guidelines, vaccination should also be considered for those traveling for a shorter period of time if the risk of JEV exposure is regarded high, for those who go to an area with an ongoing outbreak, and for those uncertain of their specific travel plans. Short-term travelers visiting only urban areas or traveling outside a well-defined JEV transmission season are not considered to need a vaccine. According to the current Finnish guidelines, active immunization can be considered in travelers residing a longer period of time (in general over four weeks) at rural endemic areas during the transmission season and in those planning to engage in extensive outdoor activities\(^83\).

In 2007, the JE vaccination status of US travelers was evaluated by interviewing nearly 1700 airline passengers heading to JEV endemic countries during the transmission season\(^84\). While 25% of the study participants were considered as potentially having a high risk for JEV exposure according to the CDC definitions, only 11% of them had received a vaccine against the disease. Another study analyzed the use of JE vaccines in US travel medicine practices in 2009-2012\(^85\). Of the 671 long-term (≥30 days) travelers heading to JEV endemic areas during the transmission season, 187 (28%) received a JE vaccine or had previously been immunized against the disease. According to the multivariable analyses, the clinicians were less likely to offer active immunization for travelers visiting friends and relatives (VFR), and for those traveling to India.
1.2.11 Assessment of Japanese encephalitis vaccine efficacy

Vaccine efficacy is ideally assessed through randomized, double-blind, placebo-controlled trials with reduction in disease incidence as the primary endpoint. Having internationally registered JE vaccines available, acquisition of direct efficacy data through placebo-controlled trials is considered unethical. Comparator vaccine-controlled trials, on the other hand, are too laborious to conduct since the relatively low incidence of the disease would imply extremely large study populations. As an alternative for efficacy studies, case-control studies can be used to assess vaccine effectiveness, i.e. the ability of the vaccine to reduce disease incidence in routine circumstances. These studies require a significantly smaller study population than efficacy trials; however, the major challenge with case-control studies is to ensure that the vaccinated and unvaccinated study participants are alike in all relevant characteristics other than vaccination. Due to the above mentioned limitations, JE vaccine studies, similarly to several other virus vaccine studies, have adopted immunological endpoint measurements as surrogate markers of protection\textsuperscript{86}.

Antibodies represent an important part of the protective immunity against JEV and other flavivirus infections\textsuperscript{87}. According to early data with limited numbers of subjects, laboratory workers who passively received JEV antibodies after accidental exposure to the virus did not develop a clinical disease\textsuperscript{88}. In animal models, passive transfer of virus-specific antibodies or immune sera confers protection against a subsequent challenge with JEV\textsuperscript{89-92}. Moreover, the level of protection has shown to correlate positively with the input antibody titer\textsuperscript{91}. The JEV E protein is considered to be a major target of the antibody response\textsuperscript{93}.

A WHO expert panel recommends determination of neutralizing antibodies by the plaque-reduction neutralization test (PRNT) for assessing the immunogenicity of JE vaccines\textsuperscript{86}. A PRNT\textsubscript{50} titer of $\geq 10$ is accepted as an indicator of protective immunity\textsuperscript{86}.

PRNT is a functional assay measuring virus neutralization \textit{in vitro}. It is regarded the most virus-specific test among all the serological flaviviral tests\textsuperscript{94}. Figure 3 illustrates the principle of the test. In order to determine JEV-neutralizing antibodies in the test serum, a predetermined amount of virus is mixed with the heat-inactivated serum sample, and, after incubation, the serum-virus mixture is added to virus-susceptible cells. If the serum contained neutralizing antibodies, virus-antibody interactions occur and the amount of infectious virus is reduced, as indicated by the formation of fewer viral plaques in a cell monolayer. The number of plaques is compared to that observed after a virus-only inoculation, and the test serum titer
resulting in a given reduction in plaque number is calculated. A PRNT titer is the reciprocal of the serum dilution that reduces the virus plaque count by a certain percentage compared to the virus-only controls. The PRNT protocols may vary in many essential respects, including the choice of the test virus strain, cell line, and cell culture conditions, as well as the percentage of plaque reduction required. The differing protocols may not be readily comparable, which should be taken into account in the interpretation and comparison of results derived with various protocols.

**Figure 3.** The principle of the plaque-reduction neutralization test (PRNT). Serial dilutions of test sera are mixed with a predetermined amount of virus. The serum-virus mixture is incubated. If the serum contained test virus-neutralizing antibodies, virus-antibody interactions occur and the amount of infectious virus is reduced. The serum-virus mixture is added to virus-susceptible cells. After incubation, the numbers of plaques are counted. A PRNT_{50} titer is the reciprocal of the test serum dilution that reduces the virus plaque count by 50% compared to a virus-only control.
1.2.12 Japanese encephalitis vaccines

JE vaccines in current use include inactivated, attenuated, and chimeric attenuated preparations. The traditional inactivated mouse brain-derived JE vaccines are in use in some endemic countries, including Malaysia, South Korea, Taiwan, Thailand, and Vietnam. Until recently, the mouse brain-derived vaccines were also the only preparations available for immunization of non-endemic populations. Two preparations were available internationally. The principal Japanese manufacturer, Biken, ceased the production of its own product (trade name Je-Vax) in 2005, whereas the South Korean Green Cross still manufactures a corresponding preparation under the trade name Japanese Encephalitis Vaccine GCC. Inactivated mouse brain-derived vaccines are also produced by national manufacturers in India, Taiwan, Thailand, and Vietnam. The internationally available vaccines are based on Nakayama strain, whereas for domestic markets, either Nakayama or Beijing-1 has been used as the vaccine strain.

Inactivated vaccines propagated in Vero cells (a cell line derived from the kidney epithelial cells of African green monkey) appeared in markets around 2009. A European company, Valneva, produces the SA14-14-2 strain-based product which is licensed in Europe, Canada, the U.S., Hong Kong, Singapore, and Israel (under the trade name Ixiaro), as well as in Australia and New Zealand (under the trade name Jespect). An Indian company (Biological E. Ltd.) also manufactures a corresponding preparation for use in domestic markets (under the trade name Jeev). Two Japanese (Biken and Kaketsuken) and two Chinese manufacturers (Beijing Tiantan Biological Products Co., Ltd. and Liaoning Chengda Biotechnology Co., Ltd) produce Beijing-1 and Beijing-3 strain–based inactivated Vero cell vaccines for domestic markets in Japan and China.

Besides the inactivated preparations, two attenuated vaccines are available for immunization against JE. The SA14-14-2–based attenuated preparation is currently the most widely used JE vaccine in endemic areas. The vaccine is manufactured in China (by Chengdu, Lanzhou, and Wuhan Institutes of Biological Products). It is used in China, India, Sri Lanka, Nepal, South Korea, North Korea, Thailand, Laos, and Cambodia. Sanofi Pasteur manufactures an attenuated chimeric vaccine which is licensed in Australia and Thailand (under the trade name Imojev).

The internationally available inactivated and attenuated JE vaccines are discussed in more detail in the following sections.
1.2.12.1 Inactivated mouse brain–derived Nakayama-based vaccine

The first versions of the inactivated, mouse brain–derived JE vaccines were developed as early as the 1930s[68]. Later, the production was refined, and more purified preparations became available. In the current manufacturing process, the mouse brain–propagated vaccine virus is inactivated with formaldehyde, purified, stabilized with gelatin and sodium glutamate, and preserved with thiomersal. Before use, the freeze-dried product is reconstituted with sterile water. The vaccine is administered subcutaneously. The following sections discuss the immunogenicity and safety of the internationally available Nakayama strain–based preparation (JE-MB).

Immunogenicity and protective efficacy

In endemic areas, the JE-MB vaccines are usually administered in two doses one to four weeks apart, with subsequent boosters at one to three years’ intervals. Efficacy of the two-dose primary schedule was demonstrated in a large, placebo-controlled, randomized trial in Thai children in the 1980s[95]. The study showed an efficacy of 91% (95% CI: 54-98) against symptomatic JE both for a monovalent (Nakayama strain) and a bivalent (Nakayama and Beijing-1 strain) preparation. Furthermore, the trial provided some evidence on the vaccines reducing the severity of subsequent dengue infections.

In non-endemic populations, no efficacy trials have been conducted. The immunogenicity studies conducted in non-endemic adult populations suggest that the seroprotection provided by a two-dose primary schedule might be insufficient for persons with less natural exposure to circulating flaviviruses. In one US study, 77% of vaccinees seroconverted after two doses, and only 29% were seroprotected six to 12 months later[96]. In another US study, the seroprotection rate was 80% at two months, and 33% at six months following a two-dose primary series[97]. By contrast, a three-dose primary regimen has been shown to elicit neutralizing antibodies in 91% to 100% of vaccinees[98-100], and consequently, three doses, given usually on days 0, 7, and 30, are recommended for primary immunization of non-endemic populations[82].

Data on the duration of JE-MB–induced immune protection are scarce. One study in 293 US laboratory workers showed a 50% seroprotection rate around two years after a three-dose primary series[100], while a small-scale follow-up study in 17 US soldiers showed protective levels of neutralizing antibodies up to three years after a three-dose primary series in the majority (94%) of subjects[101]. Among 219 children and adults living in Badu island (Torres Strait, Australia), only 70 (32%) were
seroprotected around three years after primary or booster vaccination\textsuperscript{102}. Of note, in that study, the neutralizing antibody responses were assessed by a PRNT\textsubscript{80} protocol which requires a greater reduction (80\%) in plaque count than the more widely used PRNT\textsubscript{50}.

**Safety**

Local side effects, such as soreness or redness at the injection site, have usually been reported in around 20\% of vaccine recipients\textsuperscript{96,97,99,103}. Systemic adverse events (AE) occur in approximately 10\% to 40\% of vaccinees, and may include, for instance, headache, fatigue, myalgia, influenza-like illness, or nausea\textsuperscript{96,97,99,103,104}.

The mouse brain–derived JE vaccines have been associated with hypersensitivity reactions, including generalized urticaria, angioedema, and in a few cases, anaphylaxis\textsuperscript{105-109}. In addition, sporadic cases of neurologic complications temporally related to vaccination have been reported\textsuperscript{109-112}. In 1999-2009, the U.S. surveillance system for vaccine adverse events received 23 reports of serious AEs, corresponding to a reporting rate of 1.8 serious AEs per 100,000 doses distributed\textsuperscript{113}. The reported cases included, among others, one fatality (sudden death after JE-MB and typhoid vaccinations in a male soldier using over-the-counter pseudoephedrine\textsuperscript{114}), nine hypersensitivity events, and three neurologic events (Bell’s palsy, generalized weakness, and ataxia)\textsuperscript{113}.

**1.2.12.2 Inactivated Vero cell–derived SA14-14-2–based vaccine**

The internationally available inactivated, Vero cell–derived JE vaccine (JE-VC), is based on an attenuated JEV strain, SA14-14-2. The vaccine virus is grown in Vero cells, purified, inactivated with formaldehyde, and adsorbed to aluminum hydroxide adjuvant. A single dose contains 6 \(\mu\)g of the virus\textsuperscript{115}.

**Immunogenicity**

The primary vaccination series with JE-VC consists of two intramuscular injections into the deltoid muscle on days 0 and 28\textsuperscript{115}. The immunogenicity of the primary regimen was demonstrated in a non-inferiority, randomized controlled trial in which 98\% of the adult subjects developed neutralizing antibodies against the vaccine strain
The longevity of the JE-VC–elicited immune response has been studied in different non-endemic populations\textsuperscript{116,119,120}. One study in Austrian, German, and Romanian adults recorded a 95% seroprotection rate at six months, and 83% at 12 months\textsuperscript{119}, whereas another study in Northern Irish and German adults showed lower rates of long-term seroprotection: 83% at six months, 58% after one year, and 48% after two years following primary immunization\textsuperscript{116}. The longer duration of seroprotection observed in the first study might be due to a priming effect of preceding tick-borne encephalitis (TBE) vaccinations\textsuperscript{121}; a majority of the subjects in the first study had previously been immunized against TBE, compared to none with a history of TBE vaccinations in the second study\textsuperscript{116}. A third study in Austrian and German adults with no reports on preceding flavivirus vaccinations recorded a 69% seroprotection rate 15 months after the primary series\textsuperscript{120}.

According to manufacturer’s guidelines, the first JE-VC booster dose should be given 12 to 24 months after the primary series, whereas no recommendations exist on the need of subsequent boosters\textsuperscript{115}. A booster dose given at 11, 15, or 23 months has proven immunogenic\textsuperscript{116,120}. The majority of those who received a booster at 11 or 15 months were still protected one year later\textsuperscript{116,120}. According to model predictions on antibody kinetics, a majority of vaccinees will maintain protective titers of neutralizing antibodies against the vaccine strain for at least four years after the first booster dose\textsuperscript{120}.

**Safety**

The safety profile of JE-VC was evaluated in a pooled analysis with data from 4043 adult vaccinees\textsuperscript{122}. Local AE:s were reported by 54% of subjects, the most common local symptoms being pain (33%) and tenderness (33%), followed by redness (9%), hardening (8%), swelling (5%), and itching (4%). Approximately 40% of vaccine recipients experienced at least one systemic AE related to vaccination, the most common side effects being headache (19%), myalgia (13%), fatigue (10%), influenza-like illness (9%), and nausea (5%). Two percent of vaccinees in the pooled safety
population experienced at least one serious AE; however, none of these were considered vaccine related. Safety of JE-VC has not been studied in pregnant, breastfeeding, or immunosuppressed persons.

As for post-marketing surveillance data, four serious AE:s were passively reported during the first year after licensure, corresponding to a reporting rate of 1.6 serious AE:s per 100,000 doses distributed\textsuperscript{122}. The four serious AE:s included neuritis, meningism, oropharyngeal spasm, and iritis.

### 1.2.12.3 Live attenuated SA14-14-2 vaccine

The live attenuated JE vaccine is based on JEV strain SA14-14-2. The attenuated vaccine strain was obtained from its parental strain, SA14, by serial passage in laboratory animals and cell cultures\textsuperscript{123}. Primary hamster kidney cells are used for vaccine virus production. The vaccine contains gelatin and sucrose as stabilizers. Before use, the freeze-dried product is reconstituted with sterile water.

A single subcutaneous dose of the attenuated SA14-14-2 vaccine has been shown to be highly immunogenic. In one study, 96% of South Korean children seroconverted after vaccination\textsuperscript{124}. In another study with Chinese children, the seroconversion rates to different dilutions of the vaccine varied between 83.3%-100\%\textsuperscript{125}.

Several case-control studies have evaluated the effectiveness of the vaccine in children living in JEV endemic or epidemic areas. One study in India demonstrated a vaccine effectiveness of 94.5% (95% CI: 81.5-98.9) for the first six months following immunization\textsuperscript{126}. In Nepal, in an area with yearly JEV epidemics, the estimated effectiveness of a single dose was 99.3% (95% CI: 94.9-100) for the first transmission season\textsuperscript{58}, 98.5% (95% CI: 90.1-99.2) for the second season\textsuperscript{127}, and 96.2% (95% CI: 73.1-99.9) even five years later\textsuperscript{128}. One study in rural China estimated the effectiveness of one dose at 80% (95% CI: 44-93), and that provided by two doses at 97.5% (95% CI: 86-99.6)\textsuperscript{129}.

In the international medical literature, only few studies have addressed the safety of the attenuated SA14-14-2 vaccine. In endemic areas, however, over 300 million vaccine doses have been administered with no apparent complications being reported\textsuperscript{48}. The vaccine is not recommended for immunosuppressed or pregnant individuals. Definitive data on the transmission potential of the vaccine virus are lacking.
1.2.12.4 Chimeric, live attenuated vaccine

The chimeric, live attenuated JE vaccine (JE-CV) is based on a genetically engineered vaccine virus, which contains the viral “backbone” of an attenuated yellow fever virus strain (17D), but the structural genes (E, prM) of an attenuated JEV strain (SA14-14-2)\textsuperscript{130,131}. The vaccine virus is produced in Vero cells. Before use, the freeze-dried vaccine is reconstituted with sterile sodium chloride diluent. The vaccine is administered subcutaneously.

Similarly to the attenuated yellow fever (YF) vaccine, JE-CV induces a low-level viremia in vaccinees\textsuperscript{132}. A single dose has proven immunogenic, with approximately 94% of Australian and Northern American adult vaccinees seroconverting against the vaccine virus by day 14, and 99% by day 30\textsuperscript{104,133}.

The co-administration of JE-CV and the attenuated YF vaccine was addressed in an Australian study with 108 adult participants\textsuperscript{134}. Seroconversion to JE-CV was 100% when the vaccine was administered 30 days before YF vaccine, 96% when the two preparations were given simultaneously, and 91% when YF vaccine was given first and JE-CV 30 days thereafter. The anti-JEV geometric mean titers (GMT) were significantly higher in subjects who received JE-CV first, than in those who had received attenuated YF vaccine 30 days before JE-CV, or in those who received both vaccines simultaneously. The seroconversion rates to the attenuated YF vaccine were 100% with all vaccination regimens.

As to duration of protection, a follow-up study in Australian adults demonstrated long-lasting immunity after a single-dose primary regimen, with 93% of subjects seroprotected five years after vaccination\textsuperscript{133}. According to model predictions on antibody kinetics, over 80% of adult vaccinees will retain protective levels of JE-CV neutralizing antibodies for at least 10 years, the estimated median duration of seroprotection being around 21 years\textsuperscript{135}.

The most common AE:s include injection site pain and erythema, fatigue, malaise, headache, myalgia, and diarrhea\textsuperscript{104,132,133}. The use of a chimeric live virus in the vaccine has raised some speculative concerns, including possible reversion to virulent strains, hypothetical spread of recombinant viruses in nature, and a possible risk of viscerotropism due to the YF virus component\textsuperscript{131}. The vaccine is not recommended for immunosuppressed, pregnant, or breast-feeding individuals.
1.2.13 Cross-protection within the Japanese encephalitis virus group

1.2.13.1 Cross-protection between Japanese encephalitis virus genotypes

All the JE vaccines currently in use are based on JEV strains belonging to a single genotype (GIII) which used to be the dominant genotype circulating for decades. The recent emergence of GI strains has raised a concern on the ability of the JE vaccines to confer cross-protection against heterologous JEV genotypes.

The vaccine-elicited cross-protection against other JEV genotypes has mainly been addressed by mouse studies. In these studies, active immunization with GIII vaccines or passive transfer of GIII vaccine antisera has usually provided at least partial protection against a subsequent challenge with heterologous JEV genotypes\(^ {91,136} \). In one study, high-titer sera from human JE-VC and JE-MB vaccinees gave complete protection against peripheral challenge with GI, while lower-titer sera protected only 0%-44% of subjects\(^ {91} \). In another mouse study, both the attenuated SA14-14-2 and an inactivated P3-strain vaccine provided protection against peripheral GI challenge, but when exposed via the intracerebral route, only the attenuated vaccine gave acceptable protection against GI\(^ {136} \). In a third mouse study, hyperimmune ascitic fluid raised against JE-MB or JE-CV provided variable levels of protection against a stringent challenge with GII-GIV strains, and only weak protection against GI challenge\(^ {137} \).

Some studies in humans have evaluated the capacity of the GIII vaccine-elicited antibodies to neutralize test strains of heterologous JEV genotypes. In two studies with Thai children and Australian adults, 70%-97% of vaccinees developed protective levels of GI-GIV neutralizing antibodies after primary immunization with JE-CV\(^ {133,138} \). One Taiwanese study explored the long-term seroprotection in children immunized with JE-MB (three-dose primary immunization at 1.5-2.5 years of age and booster at around 7-8 years of age). In that study, less than 50% of 5-15 year-old children had neutralizing antibodies against the GI test strain, while a significantly higher percentage were seroprotected against the vaccine strain Nakayama\(^ {139} \).
1.2.13.2 Cross-protection between different species of Japanese encephalitis virus group

Data on cross-protection between JEV and other species of the JEV group come mainly from animal experiments. A preceding West Nile or Murray Valley encephalitis virus infection has been shown to prevent or suppress subsequent JEV viremia in pigs, blackbirds, and hamsters\textsuperscript{92,140-142}. Immunization with a heterologous virus species has usually provided at least a partial survival advantage against a challenge with another member of the virus group\textsuperscript{143-145}. Some studies have shown no cross-protection between the different virus species, however\textsuperscript{146}. Interestingly, some challenge studies have documented disease enhancement between JEV and Murray Valley encephalitis virus, especially when low-dose vaccination regimens or low-titer antisera have been administered before challenge to heterologous virus\textsuperscript{145,147-149}.

Human data on potential cross-protection within the JEV group are scarce. In one study, low levels of West Nile virus neutralizing antibodies were observed in a small number of subjects immunized with JE-MB and an attenuated YF vaccine\textsuperscript{150}. Two other studies failed to detect protective levels of West Nile virus neutralizing antibodies after a natural JEV infection, and after immunization with either JE-MB or the attenuated SA14-14-2 vaccine\textsuperscript{151,152}. At present, no evidence exists on disease enhancement between heterologous members of the JEV group in humans.
1.3 Dengue

1.3.1 Etiology

The four antigenically distinct serotypes of dengue virus (DENV) comprise the dengue virus group in genus *Flavivirus*, family *Flaviviridae*. Infection with any of the serotypes (DENV-1 to DENV-4) can cause a wide spectrum of clinical disease in humans, ranging in severity from a mild undifferentiated fever to a severe life-threatening systemic illness.

1.3.2 Transmission

DENV is transmitted between humans by *Aedes* mosquitoes. The urban-adapted *Aedes aegypti* is the most important vector. It feeds primarily on humans during the daytime, both indoors and outdoors, and is capable of biting several people in a short period of time. The mosquito oviposits in diverse natural and artificial water containers. The viruses can also be transmitted by other species of the genus *Aedes* mosquitoes, including *Aedes albopictus*, *Aedes polynesiensis*, and members of the *Aedes scutellaris* complex.

Non-vector borne transmission of the virus is rare but possible through exposure to infected blood or tissues. Some case reports have described vertical transmission of the virus from mother to child during pregnancy or delivery.
1.3.3 Epidemiology

According to recent estimates, DENV causes each year approximately 390 million (95% credible interval: 284-528) infections, of which nearly 100 million (95% credible interval: 67-136) manifest clinically\textsuperscript{160}.

The geographical distribution of dengue has expanded substantially in recent decades, and the disease is today present in all tropical and many subtropical areas worldwide. Several factors have been suggested to contribute to the current dengue pandemic, including increasing urbanization, climate change, human movement between population centers, global trade, and lack of effective vector control\textsuperscript{11,160}. The majority (70%) of clinical cases are estimated to occur in Asia (34% in India alone), followed by Africa (16%), the Americas (14%, over half of which in Brazil and Mexico), and countries of Oceania (<0.2%)\textsuperscript{160}. The disease mostly affects populations living in urban and peri-urban settings where overcrowding and high abundance of vector breeding sites promote virus transmission. Figure 4 shows the estimated geographical distribution and burden of dengue in 2010.

Infection with any of the four DENV serotypes confers long-term protection only against strains of the same serotype, and consequently, an individual may get infected up to four times. Presence of pre-existing heterotypic immunity is a well-recognized risk factor for a severe illness course upon re-infection with another serotype (see classification of disease severity below)\textsuperscript{161-165}. Other epidemiological risk factors for severe illness include infection with certain virus strains and genotypes, young age, female sex, high body mass index, pre-existing co-morbidities, and certain genetic determinants of the host\textsuperscript{164,166-170}. 
Figure 4. The distribution and burden of dengue in 2010. Evidence consensus on presence of dengue (a), probability of dengue occurrence (b), and annual number of infections (c) in different geographical areas. Reprinted by permission from Macmillan Publishers Ltd: Nature 160, copyright (2013). (www.nature.com)
1.3.4 Clinical features

1.3.4.1 The clinical course

DENV has an incubation period of 3 to 10 days. Infection can be subclinical, or cause a variety of clinical manifestations ranging in severity from a benign febrile illness to life-threatening complications\textsuperscript{171}.

Characteristically, the illness course follows three phases: the febrile, the critical, and the recovery phase. The initial febrile phase starts with an abrupt onset of high-grade fever. Other typical symptoms include severe headache and retro-orbital pain, nausea and vomiting, flushed facies, and skin erythema\textsuperscript{172,173}. Patients often complain of myalgia, arthralgia, or generalized body ache. Some patients have mild respiratory symptoms or conjunctival injection. The febrile phase usually lasts 2 to 7 days. Primary infections have a longer-lived febrile period than secondary infections\textsuperscript{165,174}.

While the vast majority of patients recover spontaneously after a benign illness course, some are at risk for developing a severe vascular leakage syndrome which becomes apparent around the time of defervescence. These patients may experience an increase in capillary permeability, as evidenced by a rising hematocrit, hypoproteinemia, pleural effusions, or ascites\textsuperscript{11}. The critical phase typically lasts 1 to 2 days\textsuperscript{155}. The degree of extravasation varies; if a critical plasma volume is lost, the patient passes into a state of shock. During the initial stage, physiological compensatory mechanisms are able to maintain a normal systolic blood pressure. The patient has tachycardia, cold extremities, and a delayed capillary refill time. As a result of increased vascular resistance, the pulse pressure narrows. Later, as the compensatory mechanisms become insufficient, the systolic blood pressure decreases rapidly. A profound hypotensive shock has a difficult clinical course and may lead to multiple organ failure and advanced disseminated intravascular coagulation. Development of a clinically significant vascular leakage syndrome is often preceded by warning signs, such as acute abdominal pain, persistent vomiting, lethargy and restlessness, or increase in hematocrit concurrently with a rapid decrease in platelet count\textsuperscript{175}.

Rare manifestations of severe disease include hepatitis\textsuperscript{176}, myocarditis\textsuperscript{177}, and neurological manifestations\textsuperscript{178}. Neurological complications associated with DENV infection include encephalopathy, DENV encephalitis, post-infectious immune-mediated syndromes, cerebrovascular complications, muscle dysfunction, and neuro-ophthalmic complications\textsuperscript{178}. 
Hemorrhagic symptoms are characteristic for the critical period but may also be present during the febrile phase\textsuperscript{11}. Patients often have a positive tourniquet test (over 10-20 petechiae per 2.5 cm\textsuperscript{2} on the forearm after having an inflated blood pressure cuff on the upper arm for 5 minutes). Hemorrhagic tendency may also manifest as bleeding at the venipuncture site, easy bruising, gingival bleeding, epistaxis, or hematuria. Severe hemorrhagic complications, such as massive gastrointestinal or vaginal bleeding, are most common in patients with shock\textsuperscript{155}.

During the early recovery phase, the extravascular compartment fluid is gradually reabsorbed. Hypervolemia may complicate the illness course if intravenous fluids have been administered in excess. Bradycardia and changes in the electrocardiogram are common\textsuperscript{155}. Some patients develop a macular rash or generalized pruritus. Especially adults may experience prolonged fatigue and depression after the acute infection\textsuperscript{11}.

1.3.4.2 Classification of disease severity

The previous WHO classification system grouped DENV infections into three clinical categories: (1) undifferentiated fever, (2) dengue fever, and (3) dengue hemorrhagic fever (DHF)\textsuperscript{179}. Cases were classified as DHF if the patients presented with fever, at least one sign indicative of hemorrhagic tendency, thrombocytopenia (100,000 cells/mm\textsuperscript{3} or less), and evidence of plasma leakage. DHF cases were further divided into four severity grades on the basis of the hemorrhagic manifestations and the hemodynamic status of the patient. The most severe DHF grades (III and IV) were defined as dengue shock syndrome (DSS).

Due to criticism of previous case definitions, the WHO classification criteria were revised in 2009\textsuperscript{155,180}. The new classification comprises three clinical categories: (1) dengue without warning signs, (2) dengue with warning signs, and (3) severe dengue\textsuperscript{155}. The following manifestations are considered warning signs: abdominal pain or tenderness, persistent vomiting, clinical fluid accumulation, mucosal bleed, lethargy, restlessness, liver enlargement >2 cm, and increase in hematocrit concurrent with rapid decrease in platelet count. A severe disease is defined by the presence of severe plasma leakage, severe bleeding, or severe organ involvement.
1.3.4.3 Routine blood laboratory findings

Leucopenia with lymphopenia is usually the earliest abnormality detected in routine blood laboratory tests\textsuperscript{155}. Thrombocytopenia appears later than leucopenia, and is typically observed between illness days 3 and 8.

Hematocrit is the single most important routine laboratory test during the course of illness\textsuperscript{155}. It should first be measured early in the illness to establish the patient’s baseline value. When a significant plasma leakage syndrome develops, hematocrit rises, typically in parallel with a rapidly decreasing platelet count. Hematocrit should be monitored repeatedly to assess the stage and severity of illness, as well as to evaluate the response to possible fluid therapy. In addition to a rising value associated with vascular leakage, a decrease in hematocrit should alert the clinician as this may indicate severe hemorrhage.

Apart from full blood cell count, the clinical status of the patient may require determination of other laboratory values, such as serum electrolytes, glucose, lactate, C-reactive protein, creatinine, urea, liver transaminases, and cardiac biomarkers. A mild elevation in liver transaminases is a common finding\textsuperscript{181}.

1.3.5 Microbiological diagnostics

The clinical diagnosis of a suspected DENV infection can be confirmed by isolation of the virus, detection of viral genome or antigens, or by determination of virus-specific antibodies in paired serum samples\textsuperscript{166}. The choice of the diagnostic approach depends on the purpose of testing, the time of sample collection, and resources available.

In clinical settings, laboratory diagnostics of dengue is often based on serological tests. Enzyme immunoassay–based methods are commonly used, as they are relatively easy to perform, inexpensive, and allow differentiation between primary and secondary infections. The disadvantages of serological testing include the requirement of serial samples for confirmed diagnosis, potential cross-reactivity with other flaviviruses, and possible false positivity in some other conditions\textsuperscript{182}. Detection of virus-specific IgM antibodies or a high titer of anti-DENV IgG in a single serum sample indicates probable DENV infection, while a confirmed diagnosis requires demonstration of seroconversion (IgM or IgG) or at least a four-fold rise in IgG titer\textsuperscript{155,166}.
Patients with primary infections typically show DENV-specific IgM antibodies by the 5th day of illness, and the vast majority by day 9. After reaching peak levels at around 2 weeks since illness onset, serum anti-DENV IgM antibody level gradually declines and becomes undetectable over the following 2 to 3 months. In primary infections, virus-specific IgG antibodies start to increase slowly after the first week of illness, and remain detectable for long periods of time, even for life. While in primary infections the level of anti-DENV IgM antibodies typically exceeds that of anti-DENV IgG, the opposite is true for secondary cases which are characterized by rapidly rising IgG titers and a significantly lower or even undetectable IgM level. The anti-DENV IgM:IgG ratio can be used to determine the immune status of the patient.

Isolation of the virus is possible during the early viremic phase of illness. DENV is often isolated from patient sera, but other samples, including tissue specimens, may be used as well. The Aedes albopictus mosquito cell line C6/36 usually serves as the host cell for virus culture. Virus isolation provides a specific diagnosis and allows for identification of the infecting serotype; however, it is not usually used for routine diagnostics due to the amount of time, expertise, and resources required.

Many nucleic acid amplification tests have been developed for the diagnostics of dengue. DENV RNA can be detected in serum samples early in the illness, usually during the first 5 to 7 days. The clearance of plasma viremia is slower in primary than in secondary infections. The advantages of DENV RNA detection include high specificity, and possibility for identifying the infecting serotype.

The DENV non-structural protein 1 (NS1) can be found in the blood of infected individuals during the acute phase of disease. Detection of virus-specific NS1 by an antigen capture ELISA has proven effective for the diagnostics of dengue early in the illness course. Studies conducted in endemic areas have reported an average duration of 5 to 7 days for NS1 antigenemia. In primary infections, NS1 antigen is detected for a longer period of time than in secondary infections.

Differential diagnosis of a dengue-like illness includes non-dengue flaviviral diseases and other viral infections, such as influenza, infectious mononucleosis, measles, acute HIV infection, chikungunya, and Pogosta disease. Other conditions to be considered include a variety of bacterial and other infectious, as well as non-infectious causes of fever. Malaria should be ruled out if the patient has resided in a malaria endemic area.
1.3.6 Treatment and prognosis

Currently, no effective antiviral therapy exists for dengue. Uncomplicated cases can be treated at home but should be monitored daily until the critical period is over\textsuperscript{155}. Symptomatic treatment of these patients consists of paracetamol and adequate intake of oral fluids. Non-steroidal anti-inflammatory drugs (NSAIDs) should be avoided due to their antiplatelet properties.

Development of any of the warning signs or severe manifestations indicates a need for hospitalization. During the phase of plasma leakage, restoration of plasma volume by adequate fluid resuscitation is essential. Isotonic crystalloid solutions are preferred for treatment of compensated shock; in hypovolemic shock, colloid solutions may be needed. Excessive administration of intravenous fluids should be avoided due to the risk of hypervolemia which is a common complication during the recovery phase. In addition to administration of intravenous fluids, patients with severe manifestations may require adjuvant therapy, such as vasopressor and inotropic agents, blood transfusion, renal replacement therapy, or further treatment of organ impairment. Severe cases should be treated in high-dependency or intensive care units. The case-fatality rate may be as high as 12\% but can be reduced to less than 1\% with adequate treatment\textsuperscript{155}.

1.3.7 Pathogenesis

Monocytes, macrophages, and dendritic cells have been proposed as the primary target cells of DENV infection\textsuperscript{191-193}. After inoculation of the virus into the skin through the bite of an infected mosquito, keratinocytes and skin dendritic cells are potentially the first cells to become infected\textsuperscript{191,194}. It is believed that the activated dendritic cells then migrate to lymph nodes, cells of the macrophage-monocyte lineage become infected, and the infection is disseminated to other sites through the lymphatic system and blood.

The pathogenesis of dengue remains largely unresolved. The absence of good animal models has hampered efforts to understand the mechanisms underlying the clinically important disease manifestations. According to current understanding, the pathogenesis of severe dengue is a complex interplay between host immune response, other host factors, and virulence of the virus\textsuperscript{195}.
Several studies have identified secondary DENV infection as a risk factor for a complicated illness course\textsuperscript{161-165}. In addition, primary infections of infants have been associated with a severe illness\textsuperscript{161,196}. Pre-existing, non-neutralizing antibodies have been proposed to explain the more severe illness seen in secondary infections and in primary infections of infants\textsuperscript{197,198}. According to the theory of antibody-dependent enhancement (ADE), non-neutralizing cross-reactive antibodies bind the virus and facilitate its entry into Fc receptor-bearing cells, consequently increasing the number of virus-infected cells. A high level of viremia has been shown to be associated with a severe illness\textsuperscript{199,200}.

Recent studies have suggested that immature virus particles may play an important role in ADE\textsuperscript{201,202}. DENV-infected cells are known to secrete high levels of immature virus particles which contain precursor forms of the M protein (prM)\textsuperscript{203}. Antibodies against DENV prM are known to be highly cross-reactive, but non-neutralizing even at high concentrations\textsuperscript{202}. In vitro studies have demonstrated that anti-prM antibodies are able to mediate ADE, allowing otherwise non- or less-infectious immature virus particles to enter Fc receptor-bearing cells and to become infectious\textsuperscript{201,202}. Pathogenic memory T-cell responses, increased expression of certain pro-inflammatory cytokines, and activation of the complement system have also been associated with the pathogenesis of severe illness\textsuperscript{195,204}.

The levels of DENV NS1 antigen in patient sera have been found to correlate with the severity of illness\textsuperscript{205}. The glycoprotein may be involved in the pathogenesis of severe infections, although the mechanism remains unclear. NS1 has been proposed to protect DENV from complement-dependent neutralization\textsuperscript{206,207}, and to elicit autoantibodies that cross-react with platelet and extracellular matrix antigens\textsuperscript{208,209}.

1.3.8 Control

Despite active research in the field, no licensed vaccine is as yet available against dengue. The prevention of human cases relies on efforts to control vector mosquito populations. The most important means is elimination and management of containers that provide breeding sites for \textit{Aedes aegypti}\textsuperscript{175}. Chemical insecticides have a limited effect, and should only be used complementary to a more comprehensive environmental management approach. Biological control of mosquitoes by larvivorous fish or predatory copepods has proven effective in some settings\textsuperscript{210,211}. Novel vector control strategies are actively studied. One such approach is to introduce a DENV infection inhibiting bacterium (intracellular \textit{Wolbachia}) into \textit{Aedes aegypti}.
populations. Another strategy is to eliminate vectors by releasing genetically engineered variants into the target population. Personal protective measures to reduce human-vector contact include use of protective clothing, insect repellents, insecticide-treated mosquito nets, and mosquito screening on windows and doors.

1.3.9 Dengue in travelers

Many of the DENV endemic areas are popular tourist destinations, and concomitantly with the global emergence of the disease, reports on travel-acquired cases have increased. In specialized travel and tropical-medicine clinics, dengue has become the most common cause of a systemic febrile illness in travelers returning from Southeast Asia, South Central Asia, South America, and the Caribbean.

The risk for acquiring dengue during travel has been estimated by mathematical models and prospective serological studies. Serological studies have recorded DENV infection attack rates of 0.2%-6.7% in Swiss, Australian, Dutch, and Israeli travelers to endemic countries. Some of these studies may, however, slightly overestimate the true rate of infections because of possible false positive test results caused by cross-reactive immune responses to other flaviviruses.

DENV infection risk has been shown to vary greatly based on the season, duration, and destination of travel. In travelers visiting Thailand for one week, the estimated risk of acquiring the virus was 0.2% during the peak transmission season, and only 0.00008% during the dry season. In Singapore, during the high transmission season in 2005, the infection risk was 0.17% for travelers staying one week, and 4.57% for those staying six months.

In different case series, Southeast Asia is often one of the most common regions to acquire the disease. Among 888 dengue patients presenting to 18 clinics of the global GeoSentinel network in 2000-2010, the most common travel destinations were Southeast Asia (50%) and South-Central Asia (17%). Similarly, among 219 European travelers (2003-2005), the infection was most commonly acquired in Southeast Asia (35%), or in the Indian subcontinent (29%). Of the Swedish patients diagnosed with dengue in 2005-2008, over 75% had been infected in Southeast Asia, most of them in Thailand. In Australian traveler patients from years 1999-2012, more than half had acquired the disease in Indonesia, mainly in Bali. Among US travelers diagnosed with dengue in 1996-2005, the most commonly visited regions were the Caribbean, Central America, and Mexico (around 60% of the cases with a known travel history), followed by Southeast Asia (24%). As evident, the case series
not only provide data on the risk of contracting dengue in each region but also reflect the popularity of each area as a travel destination.

Typically, travelers from non-endemic countries present with primary infections and a benign illness course. Approximately 23%-57% of infected adult travelers develop a clinical illness$^{21,28,20,24}$. Severe forms of disease are rare. Of the 334 dengue cases reported in the U.S. in 1996-2005, only two (0.5%) met the former criteria for DHF$^{229}$. In one European retrospective study, DHF was diagnosed in 0.9% (2/219) of the patients$^{226}$. Among 132 Dutch dengue patients from the years 2006-2011, no-one fulfilled the former criteria of DHF, while 33% (44/132) of the cases were retrospectively classified as dengue with warning signs according to the current WHO criteria$^{230}$.

**Figure 5.** Number of travel-acquired dengue cases in Finland, 1985-2012. During the time period, the laboratory diagnostics of DENV infections was based on detection of virus-specific IgM antibodies, or demonstration of at least a four-fold rise in anti-DENV IgG antibodies.
2. AIMS OF THE STUDY

The main aim of this thesis was to find solutions to some practical clinical questions, and thus provide clinically important new data on travel-associated flavivirus infections.

Studies I-III addressed the immunogenicity of the inactivated Japanese encephalitis vaccines, JE-VC and JE-MB, in Finnish and Swedish adult travelers.

Study IV examined the diagnostics and the clinical picture of dengue in Finnish traveler patients.

The specific aims were to:

- assess the immunogenicity of a single dose of the SA14-14-2–based JE-VC vaccine in travelers primed with a heterologous preparation, the Nakayama-based JE-MB vaccine (I, III)

- evaluate the immune responses elicited by the two inactivated JE vaccines (JE-VC, JE-MB) against heterologous JEV strains and genotypes (II, III)

- examine the duration of the specific and the cross-protective immune responses after primary and booster immunization with JE-VC (III)

- study the kinetics of viremia and NS1 antigenemia in travel-acquired dengue (IV)

- analyze the associations between virologic markers (viremia, NS1 antigenemia) and clinical parameters in traveler dengue patients (IV)
3. MATERIALS AND METHODS

3.1 Studies I-III: Immunogenicity of the inactivated Japanese encephalitis vaccines in Finnish and Swedish adult travelers

3.1.1 Subjects and serum samples

Studies I-III examined the immunogenicity of two inactivated JE vaccines, JE-VC and JE-MB, in Finnish and Swedish adult travelers. The Vero cell–derived vaccine (JE-VC; trade name Ixiaro) is based on JEV strain SA14-14-2. The mouse brain–derived preparation (JE-MB; trade name Japanese Encephalitis Vaccine GCC) is based on JEV strain Nakayama.

The studies were investigator-initiated, open-label, and non-randomized. The study participants, Finnish and Swedish adult travelers, received one of the two vaccines as primary or booster immunization at a travel clinic in Helsinki, Finland (Aava Travel Clinic, Aava Medical Centre, former MediCity Travel Clinic) or in Stockholm, Sweden (Wasa Vaccination / CityAkuten Vaccination och Resemedicin).

The vaccinees were grouped into four study groups based on the vaccination regimen received (Table 3). Travelers with no previous history of JE vaccination or diseases received a primary series with JE-VC or JE-MB, while those previously primed with JE-MB received one of these two vaccines as a single booster dose. The choice of the vaccine type depended on travelers’ preferences and availability of the vaccines. The exclusion criteria were the following: age under 18 years, acute disease at the time of enrollment, pregnancy or lactation, clinically significant immunodeficiency or immunosuppressive treatment, known history of clinical JE, alcohol or drug abuse, or known or suspected hypersensitivity to any of the vaccine components.

Participants provided serum samples before and four to eight weeks after vaccination (study I, II). In addition, a subgroup of volunteers (48/120) provided a late follow-up serum sample around two years post-vaccination (study III).

 Originally, 158 volunteers were enrolled for the studies. Thirty-eight subjects were excluded before analyses due to protocol violations\(^1\), and the final study population comprised of 120 travelers in study I, 41 in study II, and 48 in study III.
Table 3. Study groups.

<table>
<thead>
<tr>
<th>Prior history of JE vaccinations</th>
<th>Group MB</th>
<th>Group VC</th>
<th>Group MB-MB</th>
<th>Group MB-VC</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>none</td>
<td>primary series with JE-MB (JE Vaccine GCC)</td>
<td>primary series with JE-MB (JE Vaccine GCC)</td>
<td></td>
</tr>
<tr>
<td>JE vaccines given at travel clinic</td>
<td>primary series with JE-MB (JE Vaccine GCC)</td>
<td>primary series with JE-VC (Ixiaro)</td>
<td>a booster of JE-MB (JE Vaccine GCC)</td>
<td>a booster of JE-VC (Ixiaro)</td>
</tr>
<tr>
<td>JE vaccination schedule</td>
<td>3 doses (day 0 + 7 + 30)</td>
<td>2 doses (day 0 + 28)</td>
<td>single dose</td>
<td>single dose</td>
</tr>
</tbody>
</table>

3.1.2 Laboratory analyses

The immunogenicity of the vaccines was assessed by determining the serum titers of JEV neutralizing antibodies with plaque-reduction neutralization test (PRNT). The neutralization tests were carried out in a WHO reference laboratory (Center for Vaccine Development, Mahidol University, Bangkok, Thailand). Table 4 lists the various JEV test strains used in the studies. In study I, neutralizing antibodies were determined using both vaccine strains, Nakayama and SA14-14-2, as PRNT test strains. In study II and III, neutralizing antibodies were determined using the vaccine strains, as well as different non-vaccine strains as test strains.

The principle of PRNT has been described above. In brief, serial dilutions of heat-inactivated sera (1:10, 1:100, and 1:1000) and an equal volume of diluted JE test virus (40–60 plaque-forming units/0.2 mL) were mixed and incubated. Next, an aliquot of each was inoculated in duplicate on 6-well plates with confluent LLC-MK2 cells (Rhesus monkey kidney epithelial cells). After incubation, the monolayers were overlaid with 3.0% carboxymethyl Q4 cellulose / minimum essential medium. Plates were incubated for 7 days at 37°C with 5% carbon dioxide. Plaques were counted, and PRNT<sub>50</sub> titers (the reciprocal of the serum dilution that reduced the virus plaque count by 50% compared with the virus-only controls) were determined by SPSS (IBM SPSS, Chicago, Illinois). In line with WHO guidelines, a PRNT<sub>50</sub> titer ≥10 was considered protective.⁸⁰
Table 4. JEV test strains used in studies I-III.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Isolation year</th>
<th>Isolation place</th>
<th>Isolation source</th>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>1991</td>
<td>G1</td>
<td>1991</td>
<td>Korea</td>
<td>mosquito</td>
<td>III</td>
</tr>
<tr>
<td>SM-1</td>
<td>G1</td>
<td>2002</td>
<td>Thailand</td>
<td>pig</td>
<td>II, III</td>
</tr>
<tr>
<td>1034/8</td>
<td>GII</td>
<td>1983</td>
<td>Thailand</td>
<td>mosquito</td>
<td>II, III</td>
</tr>
<tr>
<td>Nakayama</td>
<td>GIII</td>
<td>1935</td>
<td>Japan</td>
<td>human (fatal case)</td>
<td>I, II, III</td>
</tr>
<tr>
<td>Beijing-3</td>
<td>GIII</td>
<td>1949</td>
<td>China</td>
<td>human (fatal case)</td>
<td>III</td>
</tr>
<tr>
<td>SA14-14-2*</td>
<td>GIII</td>
<td>1954 (SA14)</td>
<td>China (SA14)</td>
<td>mosquito (SA14)</td>
<td>I, II, III</td>
</tr>
<tr>
<td>9092</td>
<td>GIV</td>
<td>1981</td>
<td>Indonesia</td>
<td>mosquito</td>
<td>II, III</td>
</tr>
</tbody>
</table>

* SA14-14-2 is an attenuated strain. The parental strain SA14 was isolated from mosquitoes in China in 1954.

3.1.3 Statistical analyses

Statistical analyses were performed with the R 2.13.0 software\textsuperscript{231}. Two-sided $\chi^2$ tests and two-sided Wilcoxon exact tests were used for assessing the statistical significance of observed differences. $P$ values <0.05 were considered significant.

3.1.4 Research clearances

The study (EudraCT: 2010-023300-27; ClinicalTrials.gov: NCT01386827) was approved by the local ethics committees in Helsinki (Ethics Committee, Department of Medicine, Hospital District of Helsinki and Uusimaa) and in Stockholm (the Regional Ethical Review Board in Stockholm). All participants provided informed consent.

3.1.5 Funding of the studies

Study I was supported in part by Novartis Vaccines and Diagnostics (assay expenses), by a specific Finnish governmental subsidy for health science research, and by Wiipurilaisen Osakunnan Stipendisäätiö. Shailesh Dewasthaly and Katrin Dubischar-Kastner (Intercell) commented on a late-stage draft of the original article I, and Lisa DeTora (Novartis Vaccines and Diagnostics) provided linguistic revisions for the
manuscript of the original article I. Study II was supported by Oskar Öflunds stiftelse, the Finnish governmental subsidy for health science research, and the Centre for Clinical Research, Sörmland County Council, Sweden. Study III was supported by Finska Läkaresällskapet, the Maud Kuistila Memorial Foundation, and the Finnish Foundation for Research on Viral Diseases.

The funders had no role in study design, data collection, analysis, interpretation of the data, or decision to submit the articles for publication. Apart from providing comments and linguistic revisions on a late-stage manuscript of original article I (as indicated above), the funders had no role in preparation of the manuscripts.
3.2 Study IV: Viremia and NS1 antigenemia in travelers’ dengue

3.2.1 Subjects

In Finland, the laboratory diagnostics of dengue infections is currently centralized in one single laboratory (HUSLAB, Helsinki). In this study, we retrospectively analyzed data on all the patients who had proven positive for anti-DENV IgM antibodies in Finland in 1999-2008 (154 patients). Those with insufficient clinical information (57 patients), or suspicion of a false positive IgM (four patients) were excluded. Consequently, a total of 93 cases were included in the study population.

3.2.2 Laboratory analyses

From the 93 study patients, we collected all available serum samples taken within the first 21 days after illness onset (a total of 139 samples, 1-3 per patient). Existing data archives of the diagnostic laboratory provided the serological test results obtained with a commercial anti-DENV IgM enzyme immunoassay (Focus Technologies) and an in-house IgG immunofluorescence assay (IFA). The RT-PCR and NS1 tests (Platelia Dengue NS1 Ag EIA, Bio-Rad) were run separately from frozen aliquots of sera. A subgroup of samples had been tested previously as a part of a separate study. The test protocols have been described in detail previously.

3.2.3 Collection of clinical data

Retrospective examination of the medical records provided data on the patients’ background characteristics, travel history, clinical manifestations, laboratory findings, diagnosed co-infections, and hospitalization. The severity of the cases was classified according to the WHO criteria valid at the time of data collection. Illness day 1 was defined as the date of symptom onset.
3.2.4 Statistical analyses

The kinetics of diagnostic markers were studied with generalized additive mixed models\textsuperscript{234}. We analyzed both the probability of test positivity and the relative levels of serum diagnostic markers as a function of time. In addition to a single test approach, we also studied all the possible combinations of two diagnostic tests.

The associations between diagnostic markers and clinical parameters were examined with regression models. The following potential confounding factors were included in the analyses: age, sex, day of illness, presence of co-infections, and presence of chronic diseases. Patients with insufficient data on the controlled factors (n=4) were excluded from the association analyses. All statistical analyses were carried out with the R software\textsuperscript{231}. The statistical analyses are presented in detail in the original article\textsuperscript{4}.

3.2.5 Research clearances

The study protocol received approval from the research boards of the Department of Internal Medicine of Helsinki University Central Hospital, Helsinki University Hospital laboratory (HUSLAB), and the Ministry of Social Affairs and Health.

3.2.6 Funding of the study

Study IV was supported by the Finnish governmental subsidy for health science research, the Academy of Finland, and the Finnish Medical Foundation. The funders had no role in study design, data collection, analysis, interpretation of the data, preparation of the manuscript, or decision to submit the article for publication.
4. RESULTS

4.1 Studies I-III: Immunogenicity of the inactivated Japanese encephalitis vaccines in Finnish and Swedish adult travelers

4.1.1 Characteristics of the study population

A total of 120 adult travelers participated in the studies exploring the immunogenicity of JE-VC and JE-MB. The background characteristics of the study population are presented in Tables 5 and 6.

The study population consisted of both males (40%) and females (60%). Median age was 31.0 years (range 18-72 years). The majority of volunteers were generally healthy (94%), and of Finnish or Swedish origin (97%). None of the subjects with underlying chronic diseases had a clinically significant immunosuppression. Of those with a history of JE-MB primary immunization, 39% had received a two-dose series and 61% a three-dose regimen. The median time from the latest JE-MB primary dose to the booster was 5.0 years (range 1-20.5 years). Sixty-three percent of the travelers received other vaccines, or a prescription for them, at the same visit. The most common concomitant vaccination was that against typhoid fever (48% of travelers).

Table 5. Background characteristics of the volunteers in the primary vaccination groups.

<table>
<thead>
<tr>
<th></th>
<th>Group VC</th>
<th>Group MB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I (n=31)</td>
<td>II (n=29)</td>
</tr>
<tr>
<td>Age, years (median, range)</td>
<td>26 (18-69)</td>
<td>26 (18-61)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>39 % (12/31)</td>
<td>41 % (12/29)</td>
</tr>
<tr>
<td>Female</td>
<td>61 % (19/31)</td>
<td>59 % (17/29)</td>
</tr>
<tr>
<td>Ethnic origin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Finnish/Swedish</td>
<td>100 % (31/31)</td>
<td>100 % (29/29)</td>
</tr>
<tr>
<td>Other</td>
<td>0 % (0/31)</td>
<td>0 % (0/29)</td>
</tr>
<tr>
<td>Chronic diseases</td>
<td>3 % (1/31)</td>
<td>3 % (1/29)</td>
</tr>
<tr>
<td>History of YF vaccination</td>
<td>32 % (10/31)</td>
<td>31 % (9/29)</td>
</tr>
<tr>
<td>History of TBE vaccination</td>
<td>19 % (6/31)</td>
<td>14 % (4/29)</td>
</tr>
</tbody>
</table>
Table 6. Background characteristics of the volunteers in the booster vaccination groups.

<table>
<thead>
<tr>
<th></th>
<th>Group MB-VC I (n=42)</th>
<th>Group MB-VC III (n=19)</th>
<th>Group MB-MB I (n=32)</th>
<th>Group MB-MB III (n=14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years (median, range)</td>
<td>32 (19-63)</td>
<td>41 (21-67)</td>
<td>33 (19-72)</td>
<td>36 (22-65)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>33 % (14/42)</td>
<td>37 % (7/19)</td>
<td>41 % (13/32)</td>
<td>43 % (6/14)</td>
</tr>
<tr>
<td>Female</td>
<td>67 % (28/42)</td>
<td>63 % (12/19)</td>
<td>59 % (19/32)</td>
<td>57 % (8/14)</td>
</tr>
<tr>
<td>Ethnic origin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Finnish/Swedish</td>
<td>98 % (41/42)</td>
<td>95 % (18/19)</td>
<td>94 % (29/31)</td>
<td>93 % (13/14)</td>
</tr>
<tr>
<td>Other</td>
<td>2 % (1/42)</td>
<td>5 % (1/19)</td>
<td>6 % (2/31)</td>
<td>7 % (1/14)</td>
</tr>
<tr>
<td>Chronic diseases</td>
<td>12 % (5/42)</td>
<td>16 % (3/19)</td>
<td>3 % (1/32)</td>
<td>7 % (1/14)</td>
</tr>
<tr>
<td>History of YF vaccination</td>
<td>33 % (14/42)</td>
<td>53 % (10/19)</td>
<td>28 % (9/32)</td>
<td>29 % (4/14)</td>
</tr>
<tr>
<td>History of TBE vaccination</td>
<td>21 % (9/42)</td>
<td>32 % (6/19)</td>
<td>0 % (0/32)</td>
<td>0 % (0/14)</td>
</tr>
</tbody>
</table>

4.1.2 Immunogenicity of primary vaccination

Seroprotection against the vaccine strains (Study I)

Study I included 31 participants who received a primary series with the SA14-14-2-based JE-VC vaccine, and 15 volunteers who received a primary immunization regimen with the Nakayama-based JE-MB preparation. The sera of vaccinees were evaluated for the presence of neutralizing antibodies against the vaccine strains SA14-14-2 and Nakayama. Figure 6 shows the individual PRNT<sub>50</sub> titers and their geometric means in both groups four to eight weeks after the last vaccine dose.

Four to eight weeks after primary immunization with JE-VC, 97% (30/31) and 94% (29/31) of the subjects had protective levels of neutralizing antibodies against the JEV test strains SA14-14-2 and Nakayama, respectively. The post-vaccination PRNT<sub>50</sub> titers were higher against the strain present in JE-VC than against that in JE-MB (GMT 499 vs. 120 against SA14-14-2 and Nakayama, P<.001).

Among those immunized with JE-MB, the corresponding post-vaccination seroprotection rates were 100% (15/15) against Nakayama and 87% (13/15) against SA14-14-2. Also in this vaccine group, the PRNT<sub>50</sub> titers were higher against the test strain homologous to the vaccine strain (GMT 304 vs. 46 against Nakayama and SA14-14-2, P<.001).
Figure 6. Titers of neutralizing antibodies after primary immunization. Individual PRNT$_{50}$ titers and their geometric means 4-8 weeks after receiving the primary series with JE-VC (group VC; n=31) or JE-MB (group MB; n=15). The grey horizontal line indicates the threshold for protection, a PRNT$_{50}$ titer of 10.

Seroprotection against strains of non-vaccine genotypes (Study II)

In study II, sera of the primary vaccinees were evaluated for neutralizing antibodies using five different test strains, including three strains belonging to non-vaccine genotypes (GI, GII, and GIV). After excluding subjects with pre-existing antibodies to any of the test strains (n=5), the study involved 29 travelers primed with JE-VC and 12 primed with JE-MB. Figure 7 shows the post-vaccination titers of neutralizing antibodies in each individual. Table 7 summarizes the post-vaccination seroprotection rates and geometric mean titers against the five different test strains.

Four to eight weeks after immunization, 93% (27/29) to 97% (28/29) of travelers primed with JE-VC, and 83% (10/12) to 100% (12/12) of those primed with JE-MB had protective levels of neutralizing antibodies against the various GI-GIV test strains (Table 7). With both vaccines, the highest post-vaccination titers were observed against the GII test strain (GMT 811 and 580 after primary series with JE-VC and JE-MB, respectively) (Table 7). The second highest titers were recorded against the test strains homologous to the vaccines (GMT 559 against SA14-14-2 after JE-VC primary series, GMT 293 against Nakayama after JE-MB primary series).
Figure 7. Titers of neutralizing antibodies against heterologous JEV genotypes. The individual PRNT50 titers against each target strain 4-8 weeks after a primary vaccination series with a SA14-14-2-based (group VC; n=29) or a Nakayama-based (group MB; n=12) vaccine. The titer values of each individual are connected by a thin black line. The grey horizontal lines indicate the threshold for protection, a PRNT50 titer of 10. Following test strains were used: SM-1 (GI), 1034/8 (GII), Nakayama (GIII Nak), SA14-14-2 (GIII SA), and 9092 (GIV) [Table 4].

Table 7. Short-term seroprotection after primary immunization. Seroprotection rates (SPR) and geometric mean titers (GMT) against various test strains 4-8 weeks after primary immunization with JE-VC (group VC; n=29) or JE-MB (group MB; n=12). The volunteers with pre-vaccination anti-JEV antibodies were excluded from the analyses.

| test strain | Group VC | | | Group MB | | |
|-------------|----------|-------------|-------------|----------|----------------|
| test strain | SPR | GMT | Spray | GMT | Spray | GMT |
| SM-1 (GI) | 97 % (28/29) | 55 | 92 % (11/12) | 50 |
| 1034/8 (GII) | 97 % (28/29) | 811 | 92 % (11/12) | 580 |
| SA14-14-2 (GIII) | 97 % (28/29) | 559 | 83 % (10/12) | 37 |
| Nakayama (GIII) | 93 % (27/29) | 118 | 100 % (12/12) | 293 |
| 9092 (GIV) | 93 % (27/29) | 116 | 92 % (11/12) | 46 |
Follow-up data (Study III)

In order to evaluate the duration of protection provided by JE-VC, follow-up serum samples were collected from study participants 2.1 (SD 0.1) years after primary vaccination. In addition to using a test strain homologous to the vaccine strain (SA14-14-2), the neutralizing antibodies were also determined against six heterologous target strains to assess the cross-neutralization capacity of the sera [Table 4]. Only subjects with no seroprotection before primary vaccination were included in the analyses. Table 8 provides the two-year seroprotection rates and geometric mean titers.

Of the subjects available for the two-year follow-up, 93% (14/15) showed protective levels of neutralizing antibodies against the vaccine strain, SA14-14-2. Eighty-seven percent (13/15) of the subjects had protective levels of neutralizing antibodies against the two other GIII test strains, Beijing-3 and Nakayama. The seroprotection rates against strains of heterologous JEV genotypes were 73% (GI), 93% (GII), and 87% (GIV).

Table 8. Long-term seroprotection after primary immunization with JE-VC. Seroprotection rates (SPR) and geometric mean titers (GMT) against various test strains 2.1 (SD 0.1) years after primary immunization with JE-VC (n=15). The volunteers with pre-vaccination anti-JEV antibodies were excluded from the analyses.

<table>
<thead>
<tr>
<th>test strain</th>
<th>SPR</th>
<th>GMT</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM-1 (GI)</td>
<td>73 % (11/15)</td>
<td>52</td>
</tr>
<tr>
<td>1991 (GI)</td>
<td>73 % (11/15)</td>
<td>61</td>
</tr>
<tr>
<td>1034/8 (GII)</td>
<td>93 % (14/15)</td>
<td>62</td>
</tr>
<tr>
<td>SA14-14-2 (GIII)</td>
<td>93 % (14/15)</td>
<td>26</td>
</tr>
<tr>
<td>Nakayama (GIII)</td>
<td>87 % (13/15)</td>
<td>24</td>
</tr>
<tr>
<td>Beijing-3 (GIII)</td>
<td>87 % (13/15)</td>
<td>30</td>
</tr>
<tr>
<td>9092 (GIV)</td>
<td>87 % (13/15)</td>
<td>37</td>
</tr>
</tbody>
</table>
4.1.3 Immunogenicity of homologous and heterologous booster vaccines

Booster capacity of homologous and heterologous vaccines (Study I)

Study I addressed the booster capacity of the SA14-14-2–based JE-VC and Nakayama-based JE-MB vaccines in subjects primed with JE-MB. Tables 9 and 10 summarize the seroprotection rates and geometric mean titers in both groups before and after booster vaccination. Figure 8 shows the individual PRNT\(_{50}\) titers in both groups four to eight weeks after receiving the booster vaccine.

Among travelers primed with JE-MB, a homologous booster dose resulted in seroconversion or at least a two-fold increase in titers of neutralizing antibodies in 91% (29/32) of subjects. When examining only those unprotected at baseline, the response rate to the homologous booster was 100% (9/9). The response rates for the homologous booster were comparable with those seen after the heterologous booster: 95% (40/42) to 98% (41/42) of JE-MB–primed travelers responded to the heterologous JE-VC booster with seroconversion or at least a two-fold increase in titers of neutralizing antibodies. A subgroup analysis including only those with no protection at baseline revealed that 100% (17/17) of JE-MB–primed subjects seroconverted after a single dose of JE-VC, while the corresponding rate in non-primed subjects was only 40% (10/25) (p<.001).

Table 9. Seroprotection rates before and after booster immunization. Seroprotection rates in JE-MB–primed subjects before booster immunization (day 0), four to eight weeks after receiving a single dose of a homologous (JE-MB) or a heterologous (JE-VC) booster vaccine, and approximately two years later.

<table>
<thead>
<tr>
<th></th>
<th>day 0</th>
<th>4-8 wks</th>
<th>2 yr</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>group MB-VC</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>test strain SA14-14-2</td>
<td>48 %</td>
<td>98 %</td>
<td>100 %</td>
</tr>
<tr>
<td></td>
<td>(20/42)</td>
<td>(41/42)</td>
<td>(18/18)</td>
</tr>
<tr>
<td>test strain Nakayama</td>
<td>60 %</td>
<td>100 %</td>
<td>100 %</td>
</tr>
<tr>
<td></td>
<td>(25/42)</td>
<td>(42/42)</td>
<td>(18/18)</td>
</tr>
<tr>
<td><strong>group MB-MB</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>test strain SA14-14-2</td>
<td>53 %</td>
<td>97 %</td>
<td>93 %</td>
</tr>
<tr>
<td></td>
<td>(17/32)</td>
<td>(31/32)</td>
<td>(13/14)</td>
</tr>
<tr>
<td>test strain Nakayama</td>
<td>72 %</td>
<td>100 %</td>
<td>93 %</td>
</tr>
<tr>
<td></td>
<td>(23/32)</td>
<td>(32/32)</td>
<td>(13/14)</td>
</tr>
</tbody>
</table>
**Figure 8.** Titers of neutralizing antibodies after booster vaccination. Individual PRNT$_{50}$ titers and their geometric means in JE-MB–primed subjects four to eight weeks after receiving a single booster dose with JE-VC (group MB-VC; n=42) or JE-MB (group MB-MB; n=32). The grey horizontal line indicates the threshold for protection, a PRNT$_{50}$ titer of 10.

**Table 10.** Geometric mean titers before and after booster immunization. Geometric mean titers in JE-MB–primed subjects before booster immunization (day 0), four to eight weeks after receiving a single dose of a homologous (JE-MB) or a heterologous (JE-VC) booster vaccine, and approximately two years later.

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 0</th>
<th>4-8 Wks</th>
<th>2 Yr</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group MB-VC</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test strain SA14-14-2</td>
<td>19</td>
<td>504</td>
<td>101</td>
</tr>
<tr>
<td>Test strain Nakayama</td>
<td>23</td>
<td>523</td>
<td>103</td>
</tr>
<tr>
<td><strong>Group MB-MB</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Test strain SA14-14-2</td>
<td>19</td>
<td>398</td>
<td>97</td>
</tr>
<tr>
<td>Test strain Nakayama</td>
<td>54</td>
<td>1017</td>
<td>93</td>
</tr>
</tbody>
</table>
Follow-up data (Study III)

In order to study the longevity of the antibody response, follow-up serum samples were collected around two years after booster vaccination. Group MB-MB provided serum samples 2.2 (SD 0.3) years and group MB-VC 2.1 (SD 0.3) years after receiving the booster dose. The neutralizing antibodies were determined against seven different test strains (Table 4). Tables 9 and 10 summarize the follow-up seroprotection rates and geometric mean titers against the test strains homologous to the vaccines, while Table 11 presents the corresponding data against non-vaccine test strains.

Of those primed and boosted with JE-MB, 93% (13/14) had protective levels of neutralizing antibodies against the vaccine strain (Nakayama) and the two other GIII test strains (Beijing-3, SA14-14-2) at the two-year follow-up. The corresponding seroprotection rates against the test strains of heterologous genotypes were 93% (GI), 100% (GII), and 100% (GIV). Of those primed with JE-MB and boosted with JE-VC, 100% (18/18) had protective levels of neutralizing antibodies against the vaccine strains (Nakayama, SA14-14-2) and a heterologous GIII strain (Beijing-3) at follow-up. The two-year seroprotection rates against test strains of other genotypes were 89% (GI), 95% (GII), and 95% (GIV).

Table 11. Seroprotection against non-vaccine JEV strains and genotypes. Seroprotection rates (SPR) and geometric mean titers (GMT) in JE-MB–primed subjects approximately two years after receiving a heterologous (group MB-VC) or a homologous (group MB-MB) booster vaccine.

<table>
<thead>
<tr>
<th>test strain</th>
<th>Group MB-VC</th>
<th></th>
<th>Group MB-MB</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SPR</td>
<td>GMT</td>
<td>SPR</td>
<td>GMT</td>
</tr>
<tr>
<td>SM-1 (GI)</td>
<td>89 % (16/18)</td>
<td>118</td>
<td>93 % (13/14)</td>
<td>573</td>
</tr>
<tr>
<td>1991 (GI)</td>
<td>89 % (17/19)</td>
<td>142</td>
<td>93 % (13/14)</td>
<td>582</td>
</tr>
<tr>
<td>1034/8 (GII)</td>
<td>95 % (18/19)</td>
<td>217</td>
<td>100 % (14/14)</td>
<td>361</td>
</tr>
<tr>
<td>Beijing-3 (GIII)</td>
<td>100 % (19/19)</td>
<td>122</td>
<td>93 % (13/14)</td>
<td>109</td>
</tr>
<tr>
<td>9092 (GIV)</td>
<td>95 % (18/19)</td>
<td>98</td>
<td>100 % (14/14)</td>
<td>145</td>
</tr>
</tbody>
</table>
4.2 Study IV: Viremia and NS1 antigenemia in travelers’ dengue

4.2.1 Clinical data

The majority of the 93 dengue patients were Finnish adult travelers with no chronic diseases. Of the patients with a known travel history, 72% (66/92) had acquired the infection in Asia, 21% (19/92) in the Americas, and 8% (7/92) in Africa. The vast majority of cases were considered primary; a secondary infection was suspected in only three patients with considerable anti-DENV IgG titers, a negative IgM, and a positive RT-PCR result in the acute phase.

All the cases were classified as uncomplicated according to the WHO criteria valid at the time of data collection. The frequency of various symptoms is presented in Figure 9. Table 12 lists the routine laboratory findings in the study population. 79% (73/92) of the patients were admitted to hospital and 19% (17/91) had also another simultaneous infection, most commonly a bacterial gastroenteritis.

![Figure 9. The frequency of various symptoms in the study population.](image-url)
Table 12. Laboratory findings in study patients*.

<table>
<thead>
<tr>
<th>Finding</th>
<th>%</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anaemia (&lt;134/117 g/L)</td>
<td>9%</td>
<td>(5/56)</td>
</tr>
<tr>
<td>Elevated hemoglobin (&gt;167/155 g/L)</td>
<td>13%</td>
<td>(11/87)</td>
</tr>
<tr>
<td>Low hematocrit (&lt;39%/35%)</td>
<td>9%</td>
<td>(5/56)</td>
</tr>
<tr>
<td>Elevated hematocrit (&gt;50%/46%)</td>
<td>8%</td>
<td>(7/87)</td>
</tr>
<tr>
<td>Leukopenia (&lt;3.4 x10⁹/L)</td>
<td>67%</td>
<td>(59/88)</td>
</tr>
<tr>
<td>Thrombocytopenia (&lt;150 x10⁹/L)</td>
<td>78%</td>
<td>(68/87)</td>
</tr>
<tr>
<td>Elevated AST (&gt;45/35 U/L)</td>
<td>78%</td>
<td>(49/63)</td>
</tr>
<tr>
<td>Elevated ALT (&gt;70/45 U/L)</td>
<td>61%</td>
<td>(51/83)</td>
</tr>
<tr>
<td>Elevated creatinine (&gt;100/90 µmol/L)</td>
<td>25%</td>
<td>(10/40)</td>
</tr>
</tbody>
</table>

* Reference values for males and females in parentheses

4.2.2 Associations between virologic markers and clinical parameters

The regression analyses revealed significant associations between the relative levels of virologic markers (viremia, NS1 antigenemia) and several clinical parameters. The results of the association analyses are presented in detail in the original article IV.

A high relative amount of serum DENV RNA in the first serum sample predicted lower leukocyte and platelet nadirs in follow-up, as well as an increased probability and duration of hospitalization. Overall initial RT-PCR positivity was associated with a higher probability of leukopenia (OR 10.0, 95% CI 2.9-34.1), thrombocytopenia (OR 22.6, 95% CI 5.1-101.5), and elevated alanine transaminase (ALT) (OR 15.6, 95% CI 2.9-84.8) and aspartate transaminase (AST) levels (OR 12.7, 95% CI 1.8-90.0) during the illness, as well as an increased probability of hospitalization (OR 6.4, 95% CI 1.9-22.2).

Relative levels of NS1 antigen in the first serum sample correlated negatively with leukocyte and platelet nadirs, and positively with maximum levels of hematocrit, hemoglobin, and liver transaminases in follow-up. Overall initial NS1 positivity was associated with a higher probability of leukopenia (OR 4.3, 95% CI 1.1-17.1), thrombocytopenia (OR 10.9, 95% CI 2.6-45.2), and elevated ALT (OR 4.4, 95% CI 1.1-17.6) and AST levels (OR 13.2, 95% CI 2.2-77.5) during the illness, as well as occurrence of certain symptoms; fatigue (OR 7.4, 95% CI 1.6-33.1), and abdominal pain or diarrhea (OR 5.5, 95% CI 1.2-24.5).
4.2.3 Kinetics of the diagnostic markers

According to model predictions, the probability of PCR positivity was 91% (95% CI: 82-96%) on illness day 2 and 65% (95% CI: 54-74%) on illness day 7. PCR positivity lasted on average until illness day 9 (95% CI: day 8-10).

As to the kinetics of NS1, the probability of positivity was 91% (95% CI: 80-96%) on illness day 2 and 80% (95% CI: 71-87%) on illness day 7. NS1 positivity lasted significantly longer than PCR positivity, on average until illness day 15 (95% CI: day 12-20).

In this patient population, the combinations of IgM and PCR, IgM and NS1, IgG and PCR, as well as IgG and NS1 tests showed a good sensitivity during the first three weeks after illness onset. The combination of IgM and IgG failed to detect cases in the early phase, whereas a combined approach with DENV RNA and NS1 detection became less sensitive during the later stages of disease.
5. DISCUSSION

5.1 Studies I-III: Immunogenicity of the inactivated Japanese encephalitis vaccines in Finnish and Swedish adult travelers

5.1.1 Primary immunization: seroprotection against vaccine strains

The first three studies (I-III) evaluated the immunogenicity of the inactivated JE vaccines, JE-VC and JE-MB, in Finnish and Swedish travelers. In study I, both JE-VC and JE-MB were shown to be highly immunogenic in Northern European travelers: a primary immunization with either preparation induced neutralizing antibodies against the vaccine strains in the vast majority (87%-100%) of study participants. The seroprotection rates recorded were in line with results from previous immunogenicity studies in non-endemic populations. In a large non-inferiority, randomized controlled trial, 98% of European and Northern American adults seroconverted after JE-VC primary series, and 95% after JE-MB primary immunization\textsuperscript{103}. An earlier study in British and Nepalese adults demonstrated a seroconversion rate of 89% for the three-dose primary series with JE-MB\textsuperscript{98}.

Many previous JE vaccine studies, including immunogenicity trials with JE-VC, have evaluated the vaccine-induced neutralizing antibodies using only one test strain which has usually been homologous to the vaccine strain\textsuperscript{99,102,116,120}. This single test strain-approach has even been used when comparing the immunogenicity of two heterologous vaccines\textsuperscript{103,119}. In study I, the JE-VC and JE-MB-elicited neutralizing antibodies were determined using both vaccine strains, SA14-14-2 and Nakayama, as test strains. This approach revealed notable differences in the levels of neutralizing antibodies; the titers were significantly higher against the vaccine strain than against the heterologous test strain. Higher antibody responses to homologous strains have also been reported previously\textsuperscript{133,235}. This observation is of importance, since it implies that a PRNT target strain homologous to one but heterologous to the other vaccine strain may introduce a bias in favor of the homologous vaccine. Our data thus indicate that comparative vaccine studies using vaccine strains as PRNT target strains should include analyses against both strains to avoid a test strain-dependent bias favoring either vaccine.
Study III addressed the duration of seroprotection after JE-VC primary series. At the two-year follow-up, the majority (93%) of JE-VC primed subjects were still seroprotected against the vaccine strain SA14-14-2. The seroprotection rate recorded in study III was higher than reported previously\textsuperscript{116,119,120}. A study in Northern Irish and German adults has reported the lowest rates of long-term seroprotection: 58% at one year, and 48% at two years after JE-VC primary immunization\textsuperscript{116}. Another study in Austrian, German, and Romanian adults reported a one-year seroprotection rate of 83\%\textsuperscript{119}, while a third one in Austrian and German adults reported a rate of 69% at 15 months after the primary series\textsuperscript{120}.

One possible explanation for the long duration of protection observed in the present study might be the TBE and YF vaccination history in some of the study participants. Differences in TBE vaccination status have been suggested to affect the duration of immunity after JE-VC primary series\textsuperscript{116}. In addition, as many volunteers visited endemic areas before providing follow-up serum samples, exposures to natural flaviviral boosters cannot be excluded, yet the possibility of such boosters appears only marginal since the incidence of flavivirus infections in travelers is relatively low.

### 5.1.2 Primary immunization: seroprotection against non-vaccine strains

All the JE vaccines currently available are based on strains of a single JEV genotype (GIII). Due to the recent emergence of heterologous strains and genotypes in Asia\textsuperscript{33,36}, we wanted to evaluate the immunogenicity of JE-VC and JE-MB against test strains representing non-vaccine genotypes.

Study II demonstrated that a primary series with both JE-VC and JE-MB has the potential to elicit neutralizing antibodies against heterologous JEV strains and genotypes: the majority of subjects (83\%–97\%) were able to neutralize the heterologous GI-GIV test strains four to eight weeks after primary immunization with either preparation. Even though protective levels of neutralizing antibodies were elicited against all major genotypes, the relatively low antibody titers against the GI test strain raised a concern regarding the duration of cross-protection against this genotype currently dominating in large parts of Asia\textsuperscript{12,14}. Notably, strains of genotype V were not available for testing. However, as long as GV remains such a rare cause of encephalitis, this genotype appears to be of minor clinical significance.

Study III addressed the longevity of the cross-protective immunity after JE-VC primary immunization. At the two-year follow-up, the seroprotection rates against the non-vaccine GII-GIV test strains varied between 87\%–93\%, while the corresponding
rates against GI test strains were somewhat lower, 73%. These data suggest that seroprotection might wane earlier against GI than against strains of other genotypes. This finding is of particular concern since GI predominates in many areas. Whether the 73% seroprotection rate against GI is sufficient can be questioned.

Previously, only a few human studies have evaluated the antibody responses elicited by the GIII vaccines for their ability to neutralize strains of heterologous JEV genotypes\textsuperscript{133,138,139}. The cross-protective capacity of the live chimeric JE vaccine has been evaluated in two studies in Thai children and in Australian adults. In these studies, 70\%-97\% of vaccinees had neutralizing antibodies against GI-GIV test strains one month after primary immunization\textsuperscript{133,138}. Regarding studies on inactivated JE vaccines, one Taiwanese study explored the long-term seroprotection in JE-MB-immunized children. In that study, less than 50\% of 5-15 year-old children had neutralizing antibodies against the GI test strain, while a significantly higher percentage were seroprotected against the GIII vaccine strain Nakayama\textsuperscript{139}. Hence, similarly to our results with JE-VC, the study suggested that GIII-vaccine immunity might wane earlier against GI than against the vaccine genotype.

Studies II and III were the first human studies to address the seroprotection provided by the JE-VC primary series against strains of heterologous JEV genotypes. These studies demonstrated satisfactory short-term seroprotection rates against strains of all major JEV genotypes. The somewhat lower two-year seroprotection rates against the emerging genotype call for further studies. Future research should address the long-term cross-protection in larger study populations, and at different time points in order to determine the optimal timing of possible booster doses.

### 5.1.3 Seroprotection after heterologous and homologous booster vaccination

Prior to study I, no data were available on the potential of the new SA14-14-2–based JE-VC vaccine to boost immunity after a primary series with the Nakayama-derived JE-MB vaccines. Lack of data had resulted in guidelines, e.g. from the CDC, recommending a two-dose JE-VC primary series also for those previously primed with JE-MB\textsuperscript{82}.

Study I demonstrated that a single dose of JE-VC efficiently boosts immunity in travelers with a history of JE-MB primary immunization; the vast majority (95\%-98\%) of JE-MB–primed subjects responded to the heterologous booster vaccine with seroconversion or at least a two-fold increase in titers of neutralizing antibodies. Furthermore, 100\% of JE-MB–primed subjects with no protection at baseline
seroconverted after receiving a single JE-VC dose, compared to a corresponding rate of only 40% in non-primed subjects. The heterologous booster responses were comparable to those seen after a homologous booster dose. The results of the first study support the use of a single JE-VC dose for boosting JE-MB immunity.

At the same time as our study, another study on heterologous boosting was conducted in US military personnel by Woolpert and colleagues. In line with our results, this study also demonstrated the booster capacity of JE-VC in JE-MB-primed subjects. Of the 44 subjects with a history of ≥3 JE-MB doses, 68% were seroprotected before booster vaccination, and 100% one month after receiving a single JE-VC booster dose. The levels of neutralizing antibodies were significantly higher one month after booster immunization (GMT 315) than before administration of the booster vaccine (GMT 13).

In addition to examining the immunogenicity of a single JE-VC booster dose, Woolpert and colleagues also studied the effect of a two-dose booster regimen on short-term immune responses. In that study, administration of a second booster dose 28 days after the first booster did not increase the seroprotection rates or GMTs significantly (100% seroprotection already post-booster 1; GMT 315 post-booster 1, GMT 414 post-booster 2). Whether the two-dose booster regimen could have beneficial effects on long-term immune protection remains as yet unanswered.

While study I and the investigation by Woolpert et al. were the first to address the boosting capacity of JE-VC after a primary series with JE-MB, the principle of heterologous boosting has previously been evaluated with heterologous JE-MB vaccines. In one study, Japanese high school students from a JEV non-endemic area received a homologous or a heterologous booster with either a Nakayama- or a JaGArO1-based JE-MB vaccine. Similarly to our study, both heterologous and homologous booster doses induced high levels of neutralizing antibodies against both vaccine strains.

While study I only examined the short-term immunity, study III addressed the long-term seroprotection after heterologous booster immunization. In that study, all of those primed with JE-MB and boosted with the heterologous JE-VC vaccine still had neutralizing antibodies against the vaccine strains two years after the booster vaccination. Moreover, the majority of subjects (89%-95%) also had neutralizing antibodies against strains of other genotypes, including the emerging genotype GI, at the two-year follow-up. The two-year seroprotection rates after a heterologous booster were comparable to those observed after a homologous booster. Along with the results from study I, the long-term cross-protection data from study III support the use of a single JE-VC dose for boosting JE-MB immunity. While these data suggest that JE-MB-primed travelers are still seroprotected two years after a single JE-VC
booster, the question remains whether the second JE-VC booster dose could, in fact, be delayed beyond the two-year limit. Further longevity studies are needed to address the optimal timing of the second booster dose.

To our knowledge, the current CDC guidelines of giving two doses of JE-VC to those previously primed with JE-MB have been based solely on the lack of any data on heterologous boosting with JE-VC. It is not customary to change international guidelines based on single studies. However, our studies I and III, and the report by Woolpert and colleagues were independently conducted, yet provided similar results supporting the use of a single JE-VC dose for boosting JE-MB immunity. To date, no contradictory data have been reported. Moreover, cross-protection between the two vaccine strains appears immunologically sound in terms that these JEV strains, Nakayama and SA14-14-2, closely resemble one another. Some countries, including Finland, have already changed their recommendations to state that only one dose of JE-VC is needed for boosting JE-MB immunity.

5.1.4 Limitations of the studies

Studies I-III provide data on the immunogenicity of the JE vaccines when used in routine circumstances. The real-life setting of the studies can, on one hand, be considered as a strength, as the results will mostly be applied to travelers in similar situations. On the other hand, the study design introduces some limitations regarding generalization of the results to populations with different background characteristics.

The non-randomized design and the fairly small group sizes may be considered as the main limitations of studies I-III. The research questions would have ideally been addressed by a large, randomized, double-blind, controlled trial which would have potentially provided a more solid basis to validate the vaccination recommendations.

Many study participants received other vaccines concomitantly with JE immunization, and some traveled to Asia before providing the follow-up serum samples. In addition, some volunteers had previously been immunized against TBE or YF. All these factors may be considered as additional limitations of the studies. Although relatively unlikely, the possibility of natural flaviviral boosters cannot be excluded in subjects who visited endemic areas in-between serum samples. Regarding the history of immunizations against other flaviviruses, no clear differences were observed in the immune responses between subjects with or without preceding TBE or YF vaccinations. Yet, the studies were not designed to address the effect of other
flavivirus vaccines on JE vaccine immunity, and the small number of participants limits firm conclusions based on these data.

5.2 Study IV: Viremia and NS1 antigenemia in travelers’ dengue

5.2.1 Kinetics of viremia and NS1 antigenemia

Study IV examined the kinetics of diagnostic markers and associations between virologic (viremia, NS1 antigenemia) and clinical parameters in travel-associated dengue infections.

Kinetics analyses confirmed the high sensitivity of RT-PCR and NS1 antigen detection early in the illness course; the vast majority of patients were positive with both methods during the first days of illness, in line with results from previous studies

Viremia and NS1 antigenemia lasted longer in the traveler patients than usually reported in endemic settings. One possible explanation for the long detection span of DENV RNA and NS1 antigen can be the high percentage of primary infections among the study population, as the clearance of viremia and NS1 antigenemia has been suggested to be slower in primary than in secondary infections.

NS1 antigen was detected in serum samples for a significantly longer period of time than DENV RNA, possibly due to a slower rate of NS1 clearance from plasma compared to that of viral RNA. The early appearance and long duration of NS1 antigenemia, as well as the high specificity, rapidity, and easy performance of the test make the detection of NS1 a feasible tool in the diagnostics of traveler’s dengue.

In addition to individual diagnostic markers, we also examined different combinations of two diagnostic tests. These analyses supported a combined approach with serologic testing and DENV RNA or NS1 detection for efficient diagnostics, in line with results from other studies.

5.2.2 Associations between virologic markers and clinical parameters

In the Finnish traveler patients, several significant associations were found between the early relative levels of virologic markers and clinical parameters. High viral load
predicted deeper thrombocytopenia during the illness course, as well as greater likelihood and length of hospitalization. The early magnitude of NS1 antigenemia correlated negatively with platelet counts, and positively with hematocrit and liver transaminase levels. Notably, the day of illness was controlled in the statistical analyses to avoid a potential confounding effect on the observed associations.

In endemic patient populations, high viremia and NS1 antigenemia have been linked to a severe illness course\textsuperscript{165,199,200,205}. However, some studies have reported contradictory results\textsuperscript{190,196}. Based on the observed associations between virologic and clinical parameters (e.g. hematocrit and platelet count), this study suggests that viremia and NS1 antigenemia may serve as predictors of the clinical picture also in traveler patients.

5.2.3 Limitations of the study

The main limitations of study IV are related to the retrospective design of the investigation, leading to some heterogeneity of the data, lacking details in some patient records, etc. Unfortunately, a prospective study would not have been an option in Finland; despite increasing numbers, the relatively low incidence of travel-acquired dengue infections limits the possibilities to conduct a prospective study in traveler patients.

The use of IgM positivity as an eligibility criterion may be considered as an additional limitation. Since serologic testing was the main diagnostic method in Finland, we collected the patient data by tracking all the patients who had proven anti-DENV IgM positive during the study period. To improve diagnostic accuracy, cases strongly suspected to show false-positive anti-DENV IgM (presumed cross-reactivity with other flaviviruses) were excluded from study population. As using IgM positivity as an eligibility criterion might favor inclusion of patients presenting to hospital at a later stage of illness, patient sera from the IgM-negative stage of illness were also included in analyses and the day since illness onset was controlled in all the statistical analyses.
6. CONCLUSIONS

This thesis explores the immunogenicity of inactivated Japanese encephalitis vaccines and the diagnostics of dengue in Northern European travelers.

The first study confirmed the immunogenicity of JE-VC and JE-MB vaccines in priming, and showed that a single dose of JE-VC was able to efficiently boost immunity in adult travelers primed with a heterologous preparation, the traditional JE-MB vaccine. Even though no specific data were available prior to the study, a cross-reactive immunological memory between JE-MB and JE-VC was expected, since the strains in the vaccines closely resemble one another genetically. Previously, as no data were available on the use of JE-VC as a heterologous booster, the health authorities refrained from recommending the new vaccine for boosting JE-MB immunity but, instead, recommended a two-dose primary series for those previously primed with JE-MB. The demonstration of heterologous booster capacity for JE-VC has practical clinical consequences, since the results may call for vaccination recommendations to be re-visited. In Finland, for instance, the guidelines have already been updated accordingly.

In addition to providing data on heterologous boosting, the first study also demonstrated differences in the titers of neutralizing antibodies depending on the JEV test strain used in the assay. The neutralizing capacity was found to be significantly higher against the homologous than the heterologous target strain. This finding stresses the point that comparing the immunogenicity of heterologous vaccines requires testing with all relevant strains to avoid a bias favoring either vaccine – in contrast to what has been done in many previous JE vaccine immunogenicity studies.

The second study explored the seroprotection against non-vaccine strains and genotypes after primary immunization with JE-VC and JE-MB. This study demonstrated that a primary series with both vaccines has the potential to elicit neutralizing antibodies against heterologous strains of JEV genotypes GI, GII, and GIV, not only against those representing the vaccine genotype, GIII. This implies that the current JE-VC and JE-MB vaccines are expected to confer protection against all major JEV genotypes circulating.

The third study addressed the long-term seroprotection provided by JE-VC primary and booster vaccinations. After primary series with JE-VC, the seroprotection seemed to wane earlier against GI strains than against strains of other genotypes. This finding is of particular concern, since GI strains currently predominate in many endemic areas. The long-term cross-protection after JE-VC primary immunization
should be confirmed in larger study populations before the vaccination recommendations can be re-evaluated accordingly.

As to long-term immunity after heterologous booster vaccination, all of those primed with JE-MB showed neutralizing antibodies against the vaccine strains two years after receiving a single booster dose with JE-VC. Moreover, the majority of subjects also had protective levels of neutralizing antibodies against the heterologous JEV test strains at the follow-up. Along with results from study I, these data further support the use of a single dose of JE-VC for boosting JE-MB immunity.

The fourth study examined the diagnostic markers of dengue in traveler patients. The duration of viremia and NS1 antigenemia was found to be longer than usually reported in endemic settings, probably at least partly because of the high percentage of primary infections among the study patients. The differences highlight the importance of carrying out individual studies in different patient populations. In this traveler population, a combined approach with antibody and DENV RNA or NS1 detection appeared feasible for efficient diagnostics. With respect to clinical outcome, the relative levels of viremia and NS1 antigenemia were found to correlate with some central clinical parameters of dengue, such as the degree of thrombocytopenia, suggesting these virologic markers as predictors of the clinical manifestations in travel-acquired dengue infections.
7. FUTURE PROSPECTS

With several host and vector species available, flaviviruses are likely to remain as significant pathogens of mankind in the future. Research on these viruses in different patient populations will be needed. To reduce the disease burden, efficient prevention, diagnostics, and therapeutics are crucial. Currently, no anti-viral agents are available against any of the viruses. As to prevention, a safe and effective vaccine against the most common flaviviral pathogen, dengue, is urgently needed.

In addition to research on prevention and management, continued international surveillance of these viruses is essential, as proven by the recent expansion of major flaviviral pathogens, dengue and West Nile viruses. Several natural and man-made factors are regarded to affect the spread and resurgence of flaviviruses. To combat these pathogens, multidisciplinary co-operation is needed.
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