Dengue: a trilogy of people, mosquitoes and the virus. Current epidemiology and pathogenesis in (non-)endemic settings
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INCIDENCE OF PRIMARY DENGUE VIRUS INFECTIONS IN SOUTHERN VIETNAMESE CHILDREN AND REACTIVITY AGAINST OTHER FLAVIVIRUSES

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Objectives: To study the incidence of asymptomatic primary dengue infections among children and reactivity against other flaviviruses.

Methods: A total of 216 children who had no dengue specific IgG antibodies during a sero-survey in 2003, were re-examined 23 months later, in 2005, to determine if sero-conversion had occurred. Dengue specific IgG was demonstrated with ELISA and reactivity patterns against other flaviviruses were assessed by using immunofluorescence assay (IFA).

Results: Sixty-six children showed sero-conversion for dengue virus specific IgG; the true annual incidence of primary dengue was thus 17.3% (95% CI: 13.8 - 21.4). Japanese Encephalitis virus (JEV) specific IgG antibodies were detected by IFA among 3 (4.6%) samples that showed sero-conversion in the dengue ELISA, due to cross-reactivity.

Conclusions: Our findings highlight the high incidence of dengue among Vietnamese children; JEV infections are rare. The true annual incidence of dengue can be estimated with a single cross-sectional sero-prevalence survey.

1. INTRODUCTION

Mosquito-borne flavivirus infections such as dengue have rapidly become important emerging diseases globally, in terms of morbidity and mortality. Dengue is transmitted by mosquitoes, mainly Aedes aegypti and Aedes albopictus. It is a public health problem with growing global incidence and geographical distribution to almost all tropical and subtropical regions; its transmission is changing from epidemic to endemic. World Health Organization (1997) estimates that more than 2.5 billion (two-fifths) of the world population are at risk of dengue, of whom 50 million (2%) are infected annually. Dengue fever (DF) can be caused by any of the four distinct serotypes of dengue virus (DENV). Immunity against dengue is mainly based on production of neutralizing antibodies. IgG antibodies become detectable 10–14 days after infection and remain lifelong. Primary infection with any one of the four serotypes provides lifelong immunity against that serotype but confers only partial or transient immunity against the other serotypes. In endemic areas dengue is typically a disease of children and young adults with a mean age of 5–10 years. A correct diagnosis is essential for case management of individual patients and for control programs that rely on accurate incidence figures. The diagnosis of dengue by primary healthcare workers is usually a clinical diagnosis, but this is notoriously inaccurate. Therefore, serological confirmation of DF, by demonstrating dengue-specific antibodies, is a very important tool. However, most tests do not distinguish between the four dengue virus types and show more or less cross-reactivity with other flaviviruses, such as West Nile virus (WNV), Yellow Fever virus (YFV) and Japanese Encephalitis virus (JEV). The serological distinction between DENV and JEV especially is difficult where DENV and JEV are endemic, such as in Southeast Asia. Binh Thuan, a rural province in southern Vietnam, is highly endemic for DF. In 2003, a cross-sectional study among primary school children of two rural villages showed that the dengue IgG sero-prevalence increased by age, corresponding to an annual incidence primary dengue infections of 11.7%. The true incidence of primary dengue infection, measured by sero-conversion rates, has not been studied. Therefore, we repeated the sero-survey at the primary schools of two rural communes, to measure the true incidence of primary dengue infection by sero-conversion, while controlling for cross-reactivity with other flaviviruses, notably JEV.

2. MATERIAL AND METHODS

2.1. STUDY SITE AND POPULATION

Binh Thuan Province is located along the south-eastern coast of Vietnam, 150 km north-east of Ho Chi Minh City. It covers 7828 km2, spread over a rather flat coastal zone, where small-scale agricultural activities prevail, and the forested Truong Son Mountains, an extension of the Anamite mountains ridge. In 2004, the estimated population was 1.1 million inhabitants. This study was conducted at the primary schools of Ham Kiem and Ham Hiep, 5 km west and 15 km south of the provincial capital Phan Thiet. In 2002, the population of the two communes counted 6,467 and 11,131 members, respectively. The study cohort included all children >7 years of age attending the primary schools of Ham Kiem and Ham Hiep. This accounted for approximately 55% of the entire children’s population in Ham Kiem and 26% in Ham Hiep. Children who had no dengue virus-specific IgG serum antibodies in a sero-survey of 2003 were retested in 2005, provided they still attended school. Dengue virus-specific IgG serum antibodies were determined by enzyme-linked immunosorbent assay (ELISA). Children who showed sero-conversion between 2003 and 2005 were also tested for other flaviviruses with an immunofluorescence assay (IFA). In cooperation with the People’s Committee of the village, the local health post-staff and school teachers, all pupils of the primary school and their parents were informed about the study and consent was obtained from all. The study was approved by the Provincial Health Services, the community health centres of Ham Kiem and Ham Hiep and the Scientific Committee of Cho Ray Hospital, Ho Chi Minh City. Blood collection and dengue virus ELISA Approximately 1 ml of blood was collected by finger puncture into plain vials (Greiner; Minicollect™). Vials were centrifuged on the spot; serum was transferred to a sterile vial and transported immediately to the laboratory of the Provincial Centre for Malaria and Goiter Control, where it was stored at -20 °C. After collecting all specimens, they were transported on ice packs in a cooling box to the Virology Laboratory of Cho Ray Hospital, Ho Chi Minh City, where they were stored at -70 °C. A commercial direct IgG ELISA (Focus Diagnostics Inc., Cypress, CA, USA) was used to demonstrate dengue virus-specific IgG serum antibodies. The tests were performed according to the manufacturer’s instructions. Optical density (OD) values were measured at 450 nm with 620 nm as a reference with a Benchmark microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, USA). OD results were corrected by subtracting the OD values of blank samples which are included in every test kit. The ratio between the sample OD value and
the OD of the kit calibration serum is expressed as optical density ratio (ODR), by the manufacturer referred to as ‘index value’. ODR >1 was considered positive. There were no samples with ODR = 1.00.

2.2. FLAVIVIRUS IFA
Serum samples taken in 2005 from children who showed sero-conversion in the dengue IgG ELISA between 2003 and 2005 were tested with commercially available IFA slides (Panbio Diagnostics Inc., Baltimore, MD, USA), coated with antigens of WNV, Venezuelan Equine Encephalitis virus (VEE), JEV, YFV and as a control DENV. These slides were used for the detection of IgG serum antibodies to the respective virus antigens. In brief, the serum samples were first screened at dilutions 1:16 and 1:32 in veronal buffer supplemented with 5% guinea-pig serum. Serum was applied on the IFA slides, which were incubated in a moist chamber for 2h at 37 °C. Slides were then washed in phosphate-buffered saline (PBS) twice and rinsed twice with deionized water. After air-drying of the slides, anti-human IgG fluorescein-labelled conjugate (Dako Denmark A/S, Glostrup, Denmark) was applied, and slides were incubated in a moist chamber for 30 min at 37 °C, followed by a second wash in PBS and rinsed with de-ionized water. The IFA slides were evaluated at x 400 using a fluorescence microscope (Axioskop 40; Carl Zeiss AG, Jena, Germany). Fluorescence was graded using the following scale: cytoplasmic fluorescence was graded from moderate to intense (apple green), 2–4. Low intensity or ‘dim’, but definite, cytoplasmic fluorescence was graded as 1. Samples with grade 2 or more were considered positive. Samples which were found positive to DENV and to other flaviviruses were further tested at dilutions 1:32, 1:64, 1:128, 1:256, 1:512 and 1:1024.

2.3. STATISTICAL ANALYSIS
The age-dependent sero-prevalence was modelled under the assumption that dengue is endemic in Southern Vietnam, that the force of infection was constant over the years and that antibodies remain detectable lifelong. Thus the proportion of sero-negative subjects that sero-converted per year is also constant:

\[
\frac{dy}{dt} = -\lambda y
\]

where y is the prevalence of sero-negative subjects and k is a measure of the force of infection. This can be rewritten as:

\[
y(t) = y(0) \exp(-\lambda t)
\]

The annual incidence rate is thus, when t = 1 year:

\[
1 - \exp(-\lambda)
\]

The true annual incidence of primary dengue was calculated using the prevalence of seronegatives in 2003 and 2005, where t was 23 months after the first measurement on t = 0 (March 2003). Statistical analysis was performed using the software package S-plus 2000 Professional (Release 2; Mathsoft Inc., Seattle, WA, USA). Overall, age-specific and gender specific sero-prevalence was calculated with 95% confidence intervals; chi-square tests were used for proportions. All tests were carried out at a significance level of 0.05.

3. RESULTS

SAMPLE POPULATION
In 2005, 831 children were registered as pupils at both schools, 459 in Ham Kiem and 372 in Ham Hiep. From the cross-sectional study in 2003, a total of 330 (34.3%, 330/961) serum samples of children tested negative for dengue virus-specific IgG. These children were included in this study except for 99 children (57 from Ham Kiem and 43 from Ham Hiep) who had graduated, 1 who had dropped out of school and 14 who were absent at the time of sampling blood. This left 216 (93.9%, 95% CI: 90.0–96.3) subjects for analysis, 124 from Ham Kiem and 92 from Ham Hiep, with a male:female ratio of 1:1.2. Sero-conversion of dengue IgG and incidence rate Of 216 serum samples, 66 (30.6%, 95% CI: 24.8–37.0) tested positive for IgG antibodies against dengue in 2005: 37 in Ham Kiem and 29 in Ham Hiep. The true annual incidence of primary dengue, calculated from the sero-conversion rate was 17.3% (95% CI: 13.8–21.4). IFA reactivity pattern
of serum samples from children Reactivity against other flaviviruses was tested with IFA in sera of children who showed sero-conversion for dengue virus-specific IgG in the ELISA. Sixty-two of the 66 samples (93.9%, 95% CI: 85.4–97.6) obtained in 2005 from children who sero-converted by ELISA also tested positive by IFA for DENV. Of these 62 samples, eight (12.1%) reacted to YFV (12.1%), three (4.6%) reacted to JEV and one (1.5%) to WNV. The antibody titres to YFV, JEV and WNV did not exceed the DENV antibody titres in the IFA and were thus interpreted as cross-reactivity because of a primary dengue infection (Table 1).

Table 1. Immunofluorescence reactivity against flaviviruses in serum of nine out of sixty-six primary school children who demonstrated sero-conversion by dengue IgG- ELISA.

<table>
<thead>
<tr>
<th>Individuals</th>
<th>Flavivirus specific serum IgG antibody serology</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ELISA (ODR1)</td>
</tr>
<tr>
<td>Sex / Age (y)</td>
<td>VEE</td>
</tr>
<tr>
<td>M / 11</td>
<td>4.7</td>
</tr>
<tr>
<td>M / 11</td>
<td>3.6</td>
</tr>
<tr>
<td>M / 10</td>
<td>7.8</td>
</tr>
<tr>
<td>M / 11</td>
<td>3.6</td>
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<td>M / 11</td>
<td>1.5</td>
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<tr>
<td>F / 11</td>
<td>4.3</td>
</tr>
<tr>
<td>M / 11</td>
<td>4.1</td>
</tr>
<tr>
<td>F / 9</td>
<td>7.4</td>
</tr>
<tr>
<td>F / 10</td>
<td>8.5</td>
</tr>
</tbody>
</table>

1 ODR: optical density ratio; ODR > 1 is considered positive; 2 IFA: immunofluorescence assay; titers are shown as reciprocal values; titers ≥ 1:64 were considered positive.

### 4. DISCUSSION

This study confirms that dengue is a very common disease in southern Vietnam, with an annual incidence of first infections as high as 17.3%. This is only slightly higher than the incidence of first DENV infections that we estimated in our first sero-survey: 11.7% in children aged 7–14 years.272 This result confirms that the analysis that we used in our first survey, i.e. binary regression of sero-prevalence to age, using a complementary log–log link function, yields a rather accurate estimate of the incidence of DENV infection. Much has been written about the sero-prevalence, but few studies in south-eastern Asia have investigated the incidence of DENV infection in healthy children. The incidence found in our study was similar to that reported in Thailand and Indonesia among healthy children in prospective cross-sectional studies.85,281 Other data in this region showed variable (overall) incidences of DENV infection in different endemic areas for dengue.84,252 These incidences are lower than results from our study, because they were based on individuals with minimally symptoms to severe cases of DENV infection seeking for medical aid. A more recent study that also investigated sero-conversion in children in Nicaragua reported similar incidence rates, but, surprisingly, found a much higher prevalence of DENV antibodies.12 The sample size in sero-epidemiological studies is a crucial aspect, because sample size can influence precision or power of the study. Using a sero-prevalence of 65.7%, as observed previously272, and assuming a precision of 5% and a confidence level of 95%, the calculated sample size for our study would be 179 children. We included 330 children of whom 216 were retested for serum-specific IgG antibodies against dengue. This sample size was sufficient and could be used for calculation of the annual incidence. Serological technique is the method of choice for routine diagnosis of flavivirus infections. Many commercially available immunoassays are available for the diagnosis of DENV and other flavivirus infections.85,151 Although the indirect IgG-ELISA for DENV, used in this study, is a suitable tool for detecting dengue IgG antibodies with high sensitivity and high specificity; cross-reactivity may occur with other flavivirus antibodies.153 In areas where multiple flaviviruses, e.g. DENV and JEV, are endemic, samples may show reactivity to different antigens. This may be not only due to cross-reactivity but also due to the presence of specific antibodies acquired by different flavivirus infections. In this study, we found some reactivity to different flaviviruses with IFA but because the titres were lower than those to DENV, this was interpreted as cross-reactivity. Although southern Vietnam is endemic for JEV and Japanese encephalitis cases occur sporadically throughout the year, its public health importance is minor.287 Secondly, as YFV and WNV infections have never been reported in south-eastern Asia, the IFA reactivity to those viruses should be considered as cross-reactivity, whose degree also confirmed previous findings.153,181 Knowledge of the sero-prevalence and incidence of DENV infections is essential for planning public health interventions such as vector control or possible future use of a vaccine. But, it is also important information to clinicians who are involved in individual case management. In areas without facilities and laboratory confirmation of common infectious diseases doctors base their clinical diagnosis on signs and symptoms. Clinical diagnosis of dengue is highly inaccurate, and the WHO classification system for clinicians is not very accurate either.53,219 However, it is not yet clear which case definition could better identify cases of (potentially) severe dengue at the primary healthcare level. This study shows that the large burden of dengue disease presented to primary health facilities is underpinned by a much greater incidence of asymptomatic to mild dengue infection in the general population. Inappropriate, too sensitive case detection could lead to unaffordable intervention policies that may not always be necessary. Knowledge of the incidence of largely asymptomatic infections does not need to reflect the incidence of symptomatic infections presented to the health services, but this knowledge may help sensitizing primary healthcare workers to making the presumptive diagnosis of dengue. In conclusion, our data established a high incidence rate of DENV infections. Although DENV and JEV are both endemic in Binh Thuan province, JEV infection is of minor importance in children. Using a binary regression with a
log–log link function, applied on sero-prevalence in cross-sectional sero-survey data, yields a rather accurate estimate of the true annual incidence in endemic areas such as Vietnam.

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