Improving dengue virus diagnosis in rural areas of Mexico


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Abstract

Evaluation of an “in-house system” for the diagnosis of dengue infection by detection of specific IgM and IgG antibodies showed that 25 out of 34 (73.53%) serum samples were positive for IgM antibodies; 6 (17.64%) were positive for IgG and 3 (8.8%) were negative for both IgM and IgG anti-DENV antibodies. Ten samples from “non-symptomatic” people were all negative. In order to evaluate the anti-DENV ELISA, 20 serum samples obtained from healthy individuals from a non-endemic region (Mexico City) and 20 serum samples previously classified as positive were tested. All 20 samples from healthy individuals proved to be negative for both IgM and IgG anti-DENV antibodies, whereas not all positive samples resulted as positive in our assay.

Keywords: In-house diagnosis kit; dengue; surveillance; endemic; Tamiahua; Mexico.

Introduction

Dengue virus infection in the Americas, as in the rest of the world, is increasing dramatically. Currently, Mexico could be considered as an endemic region for dengue since the mosquito vector, Aedes aegypti, is present in more than 85% of the country.[1] Dengue viruses are members of the Flaviviridae family, genus Flavivirus. Infection can lead to dengue fever (DF), a self-limiting flu-like illness, or to dengue haemorrhagic fever (DHF) with fatal consequences. Dengue fever is caused by any of the four dengue virus serotypes (DENV-1-4). [2] It is well-documented that primary infection with one of the four serotypes confers long-lasting immunity to that specific serotype. However, secondary infection with a different serotype is associated with an increased risk of developing DHF where an antibody-dependent enhancement (ADE) of infection is associated with the pathophysiological mechanisms of DHF.[3,4] In addition to DF and DHF, some other clinical outcomes, such as

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neurological manifestations and liver damage have also been reported.[5-8]

Since all the four serotypes are circulating in Mexico, there is a need to develop an efficient diagnosis system to improve case management of the patients. Until now, the incidence of dengue infection has been underestimated since most cases are not properly diagnosed, especially in small towns or villages where private or state laboratories for diagnosis are lacking. The diagnosis problem exists not only at this level, since several regional hospitals outside Mexico City have inadequate budgets to buy the required number of diagnostic kits to match the demand, making epidemiological surveillance, case management and outbreak investigation difficult. In order to set up a rapid and reliable diagnosis, some reference laboratories in Mexico use the Panbio Dengue Duo IgM and IgG Rapid Cassette test kits or the ELISA Dengue IgM capture kits. However, it is sometimes difficult to have access to these kits due to stock shortage in the market, import difficulties, and of course, high cost.

Dengue infection diagnosis can be achieved by several assays such as RT-PCR,[9] real time-PCR,[10] virus isolation,[11] and NS-1 protein detection.[12,13] However, the enzyme-linked immune assay (ELISA) has for a while been, due to its practicality, the routine diagnostic system for dengue infection serological confirmation.[14-17] Different kits are commercially available, such as Dengue IgM capture ELISA (MAC-ELISA) and IgG ELISA, which, in terms of sensitivity and specificity, are reliable with regard to anti-dengue antibodies detection, although they do not clearly distinguish between different Flaviviruses. There are some other commercial assays that use antigens from each dengue virus serotype (DENV-1 to DENV-4); these assays have a high sensitivity and specificity, typically ranging from 90% to 100%.[18]

The distinction between primary and secondary infections is currently assessed by measuring IgM and IgG responses to dengue antigens in paired serum samples taken from a febrile patient in the acute stage of disease and after convalescence.[19] Clearly, the availability of systems for the detection of dengue infections is a public health priority. In this study, we report a reliable “in-house system” for the diagnosis of dengue infection that was field-tested in a small village (Tamiahua) in the state of Veracruz, Mexico.

Materials and methods

Serum samples

A total of 84 serum samples were included in the evaluation of the “in-house system” for the diagnosis of dengue virus infection. Forty-four samples were collected from a dengue endemic area in Tamiahua, which is located in the Gulf of Mexico, in the state of Veracruz between the Pánuco and Tuxpan rivers, at 21°06’ latitude north and 97°46’ longitude (Figure 1); and classified as shown in Table 1. Blood samples were collected from the 44 Tamiahua participants after obtaining parental consent in cases where it was needed. The participants answered a short list of questions that included whether they suffered from higher than 37.5 °C fever, headache, retro-orbital and abdominal pain, vomiting, skin rash, nose or any other type of haemorrhage over the last three months.

Children who neither had any of the above-mentioned manifestations nor had any clinical manifestation of dengue over the previous three months but who had said that someone in their household did have some of the clinical manifestations were also included in the study (Table 1). In addition, 20 dengue virus-negative serum samples
were obtained from healthy undergraduate students from Mexico City, a city officially reported as a non-endemic area for dengue. Their negative quality was confirmed by the absence of cytopathic effect on C6/36 cell culture. Twenty DENV-2-positive serum samples, previously classified as such by the Instituto de Diagnóstico y Referencia Epidemiológicos (InDRE) on the basis of virus isolation, RT-PCR, NS-1 detection (Bio-Rad kit) and IgM/IgG determination (PanBio), following the manufacturers’ standard protocols, were also included in the “in-house” dengue diagnostic system.

Sample collection and transport from Tamiahua to Mexico City

Five ml of blood were collected by venopuncture in 13×100 vacutainer tubes. After a 60-minute incubation period at room temperature, the tubes were centrifuged in a small portable centrifuge at 2000 rpm for 10 minutes. The serum was transferred to propylene eppendorf vials and stored in a fridge (4 °C). The sera were transported in ice boxes back to the research laboratory in Mexico City (travel time of about seven hours) where samples were stored at −70 °C until testing.

Evaluation of the in-house dengue diagnostic system

To evaluate the in-house-made diagnostic system, a WHO serum panel for dengue, composed of DENV-1, 2, 3, 4 and negative sera (anti-dengue, WHO Reference Panel, Code: 05/248, NIBSC, Potters Bar Hertfordshire, UK), as well as the positive and negative controls included in the Platelia dengue NS1 commercial kit (Bio-Rad) were used, along with the 84 serum samples mentioned in the serum samples section.

Table 1: Characterization of 44 individuals from Tamiahua included in the IgM/IgG anti-dengue virus detection trial by using the in-house ELISA system

<table>
<thead>
<tr>
<th>Average age</th>
<th>Group I Elementary school (n=17)</th>
<th>Group II Secondary school (n=18)</th>
<th>Group III Adults (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 years</td>
<td>14 years</td>
<td>29 years</td>
</tr>
<tr>
<td>Gender</td>
<td>Female=11 Male=6</td>
<td>Female=10 Male=8</td>
<td>Female=7 Male=2</td>
</tr>
<tr>
<td>High fever</td>
<td>Female=8 Male=6</td>
<td>Female=8 Male=6</td>
<td>Female=5 Male=1</td>
</tr>
<tr>
<td>Aches and pain</td>
<td>Female=8 Male=6</td>
<td>Female=8 Male=6</td>
<td>Female=1 Male=1</td>
</tr>
<tr>
<td>Nausea and vomiting</td>
<td>Female=3 Male=2</td>
<td>Female=4 Male=2</td>
<td>Female=3</td>
</tr>
<tr>
<td>Bleeding</td>
<td>Female=2 Male=2</td>
<td>Female=3 Male=1</td>
<td>Female=2 Male=1</td>
</tr>
<tr>
<td>“Healthy”</td>
<td>Female=3</td>
<td>Female=2</td>
<td>Female=2 Male=1</td>
</tr>
</tbody>
</table>
Dengue diagnosis by an in-house system

All serum samples were tested by an indirect ELISA method performed as follows: 96 well plates were coated with 100 µl of carbonate buffer (pH 9.6) containing the antigen (DENV-2, New Guinea strain, propagated in C6/36 cells). After an overnight 4 °C incubation, the plates were washed five times with PBS-T (0.01% Tween-20 in phosphate buffer solution), five minutes per wash, and blocked with 3% skimmed milk in PBS for 45 minutes at room temperature. Serum samples were serially diluted; the dilution which generated an OD value three times higher than that from negative samples was thoroughly used in the rest of the study. All serum samples were diluted 1:50, and 100 µl of each one was added to individual wells by triplicate and incubated for two hours at 37 °C. After washing three times with PBS-T, 100 µL of peroxidase-conjugated protein-A (invitrogen; 1:10 000 dilution) or 100 µL of anti-human IgM-peroxidase (serotec; 1:2000 dilution) was added per well. Plates were incubated for one hour at 37 °C, and after washing three times, 200 µL of ortophenylenediamine (OPD) solution (0.020 g of OPD dissolved in 10 ml of citrate solution, pH 5.0 and 10 µL 30% H₂O₂) was added to each well. Plates were incubated for 30 minutes (kept away from light); the reaction was stopped by adding 30 µl of 0.2 N H₂SO₄, and ODs were read in an ELISA plate reader with a 492 nm filter (Labsystems).

Cross-reactivity test for other Flaviviridae family viruses

In order to test the in-house dengue virus ELISA for cross-reactivity with other flaviviruses, four human serum samples positive for West Nile virus (virus isolation and PCR), kindly provided by the InDRE, were tested (it proved impossible to procure more samples). It is worth noting that yellow fever and Japanese encephalitis are diseases not found in Mexico; so, samples of these two infections were not included in the study due to non-availability of the serum samples.

Data analysis

Mean and confidence intervals (95% CI) from OD ELISA data were calculated using GraphPad Prism version 5.02 (GraphPad software, Inc).

Results

The in-house ELISA system for the detection of IgM and IgG dengue-specific antibodies allowed us to distinguish between the likely primary and secondary dengue infections using serum samples collected in a dengue endemic region in Mexico’s countryside. 25 out of 34 (73.53%) samples obtained from both children and adults that reported having symptoms comparable with a dengue virus infection within the previous three months to the time of sampling presented high OD values for IgM dengue-specific antibodies, whereas six (17.64%) did so for IgG dengue-specific antibodies and three (8.8%) scored negative for both IgM and IgG anti-dengue as shown in Figure 2 and Table 2. Taken together, these results indicate that 91% of the selected (based on referral symptoms) serum donors were positive for anti-dengue antibodies; this correlates with the endemicity of the geographical region. The 10 Tamiahua samples obtained from people that reported no symptoms were negative both for IgM and IgM anti-dengue as shown in Figure 2 and Table 2. The evaluation results of the in-house system showed that it scored 100% for specificity and 77% sensitivity for IgM and 83% for IgG,
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There was no cross-reactivity (data not shown) with West Nile virus samples, suggesting a high specificity for our in-house anti-dengue ELISA. However, a higher number of West Nile-positive samples would have to be tested for a more reliable sensitivity and specificity assessment of the dengue virus diagnostic system.

In-house dengue system evaluation

The evaluation of our in-house system with the anti-dengue WHO panel resulted in a clear difference between the negative serum and the positive for DENV-1, 2, 3, and 4 sera. Also, the OD values for DENV-2 were higher than those for the other serotypes. However, some cross-reactivity was observed between the four serotypes (data not shown). Positive and negative controls included in the Platelia dengue NS1 commercial kit (Bio-Rad) were clearly positive and negative, respectively, when tested by the in-house ELISA system (data not shown).

Discussion

Dengue infection is a growing public health concern in endemic areas all over the world. Hyperendemic geographical areas have been defined as those with continuous presence of multiple viral serotypes and competent vectors, and a large population of susceptible hosts, as seems to be the case for Mexico,[1] so diagnostic systems for dengue are badly needed. Several serological diagnostic kits are commercially available; however, in practice they are not always accessible due to high cost and other constraints. In addition, some technical drawbacks have been reported.[17] As a result there is poor epidemiological surveillance, the number of cases is underestimated, and infected people do not always receive appropriate medical treatment.

Taking all this into account, we are working to improve the development of an in-house ELISA system for the detection of IgM and IgG antibodies specific for dengue virus. After testing it with samples known to be positive (by means of viral isolation, RT-PCR and ELISA) or negative for dengue, further field-testing was carried out in a small village in Veracruz, Mexico. Although paired samples (in the acute stage of the disease and after convalescence) are recommended for the diagnosis of dengue by IgM and IgG ELISAs, in...
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Table 2: Serological diagnosis of dengue infection, based on the in-house ELISA system

<table>
<thead>
<tr>
<th>Characteristics of the samples tested with the in-house-made system</th>
<th>Positive results for IgM</th>
<th>Positive results for IgG</th>
<th>Negative results for IgM or IgG</th>
<th>Specificity of the in-house-made assay</th>
<th>Sensitivity of the in-house-made assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Samples from individuals with dengue-like symptoms within three months before sampling (Tamiahua) n=34</td>
<td>25/34 (73.53%)</td>
<td>6/34 (17.64%)</td>
<td>3/34 (8.8%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Samples from healthy individuals from endemic region (Tamiahua) n=10</td>
<td>0/10</td>
<td>0/10</td>
<td>10/10</td>
<td>10/10 (100%)</td>
<td></td>
</tr>
<tr>
<td>Samples from healthy individuals from non-endemic region (Mexico City) n=20</td>
<td>20/20</td>
<td>20/20</td>
<td>20/20</td>
<td>20/20 (100%)</td>
<td></td>
</tr>
<tr>
<td>True positive samples for primary dengue infection (laboratory diagnosed in InDRE) n=20</td>
<td>14/20</td>
<td>0/20</td>
<td>6/20</td>
<td>77%</td>
<td></td>
</tr>
<tr>
<td>True positive samples for secondary dengue infection (laboratory diagnosed in InDRE) n=20</td>
<td>0/20</td>
<td>16/20</td>
<td>4/20</td>
<td>(83%)</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 3:** Assessment of IgM and IgG anti-dengue virus antibodies in positive and negative serum samples by using an in-house ELISA system [20 positive and 20 negative samples characterized as such by virus isolation, NS-1 detection and RT-PCR were used. Distribution, mean and confidence intervals for OD values for IgM and IgG are shown. The hatched line represents the cut-off OD values.]

In the present study this was not possible since donors did not show any clinical symptoms at the time of blood sampling but rather symptoms were reported during a period of time between two and three months before sampling. The threshold for an IgM- or IgG-positive test result was established at an OD value two times (IgM) or three times (IgG) higher than that of the negative controls taken from PanBio IgM and IgG capture ELISA kits.

To evaluate the specificity and sensitivity of the in-house ELISA system, 20 dengue-positive samples (laboratory characterized as such two years earlier) along with 20 samples from healthy donors that proved to be negative in C6/36 cytopathic effect assays were assayed.

All in all, the in-house detection system for dengue viruses seemed to perform well in terms of specificity, and seemed suitable
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for in-field diagnosis. We propose the use of the in-house IgM/IgG anti-dengue detection systems as a viable alternative in Mexico when commercial kits are not available, at least in terms of a first screening. Even if this system needs to be improved by increasing the number of samples, for example, especially from asymptomatic individuals from the endemic regions in Mexico, to increase its accuracy in the dengue virus diagnosis, it is important to keep working on the development of reliable diagnostic tools in order to establish an efficient surveillance system in dengue endemic areas.

Acknowledgements

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References


