Kinetics of antibodies in sera, saliva, and urine samples from adult patients with primary or secondary dengue 3 virus infections


"Pedro Kouri" Tropical Medicine Institute, PAHO/WHO Collaborating Center for Dengue and its Vectors, Autopista Novia del Mediodía, Km 6 1/2, La Lisa, Havana City, Cuba

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Introduction

Dengue virus is a mosquito-borne virus of the Flaviviridae family. The four serotypes (dengue 1, 2, 3, and 4) are...
transmitted to humans through the bite of infected mosquitoes, *Aedes aegypti* and *Aedes albopictus* being the main vectors. Dengue virus infection causes a spectrum of syndromes ranging from mild febrile illness to classical dengue fever and severe and fatal hemorrhagic disease. Inapparent infection is very common.

Specific dengue IgM detection in serum by ELISA has become one of the most important and useful methods of dengue diagnosis. Serum is the preferred sample for serological studies, but its collection is difficult in infants and small children and in field conditions. The usefulness of saliva for dengue diagnosis has been partially evaluated by others, but we know of no report of the use of urine samples in dengue diagnosis.

To define the usefulness of saliva and urine for dengue diagnosis and to study the kinetics of specific dengue antibodies, the presence of dengue IgM, IgA, and IgG was determined in serum, saliva, and urine collected from dengue 3 virus infected during the Havana outbreak of 2001–2002. The total and specific dengue IgE antibodies response in serum samples were also studied, in view of previous reports of the increase of this antibody in dengue patients.

### Materials and methods

#### Clinical samples

A total of 411 clinical samples of serum, saliva, and urine (137 for each kind of sample) were collected from 22 hospitalized and confirmed adult cases of dengue at "Pedro Kouri" hospital. Dengue 3 virus was isolated and/or detected by RT-PCR in all patients. The samples were collected during the acute illness (from day 3 to 8 and 20 days later) early in the morning. Serum was obtained by conventional methods. All samples (serum, saliva, and urine) were kept at −20°C until study. Informed consent was obtained from all patients and subjects. This group of patients constituted 11 women and 11 men aged between 21 and 58 years (average: 36 years). Cases were classified as dengue fever (DF) or dengue hemorrhagic fever (DHF) according to the PAHO/WHO Guidelines for the Control and Prevention of Dengue and Dengue Hemorrhagic Fever in The Americas.

#### Viral antigens

The antigens used in the serological studies were dengue 1 (Hawaii strain), dengue 2 (New Guinea C strain), dengue 3 (H-87 strain), and dengue 4 (H-241 strain) prepared in mouse brains and extracted by acetone–sucrose extraction technique.

**Capture IgM (MAC-ELISA), IgA (AAC-ELISA), and IgE (EAC-ELISA) specific dengue antibodies by ELISA**

In-house tests with similar protocols to MAC, AAC, and EAC ELISA were used. Briefly, NUNC MaxiSorp plates were coated with goat IgG anti-human IgM (Sigma), goat IgG anti-human IgA (ImmunoAssay Center, Havana, Cuba), and goat IgG anti-human IgE (Sigma). After blocking, 50 μL of 1/20 serum dilution to detect IgM and IgA dengue antibodies and 1/5 serum dilution to detect IgE dengue antibodies in phosphate-buffered saline (PBS) plus 0.5% bovine serum albumin (BSA) were added. Saliva and urine samples (50 μL/well) without dilution were used. Positive (duplicate) and negative (quadruplicate) controls were included in each test. Samples were incubated for two hours at room temperature for IgM and IgA and at 37°C for IgE antibody detection. An antigen mixture of the four dengue serotypes was added. After overnight incubation, 50 μL of human conjugate IgG anti-dengue diluted 1/5000 was added. Orthophenylenediamine (OPD) and hydrogen peroxide were used as substrate. The reaction was stopped and the plates were read at 492 nm.

The optical density ratio (OD ratio) = P/N was calculated in all samples. P represents the OD of each serum sample and N represents the OD mean of the negative control, calculated with the four OD values. The negative controls were pools of sera, saliva, and urine from individuals without dengue background and negative to IgM, IgA, IgE, and IgG antibodies.

All samples with an OD ratio ≥2 were considered positive by MAC and AAC ELISA; for EAC ELISA, an OD ratio ≥1.4 was considered positive.

#### Total IgE antibody

To detect total IgE antibody the UMELISA IgE system developed by the ImmunoAssay Center, Havana, Cuba was used. The test was performed according to the manufacturer’s instructions. A total IgE concentration greater than or equal to 150 IU/mL was considered positive in serum samples from adult patients.

**ELISA inhibition method (EIM) to detect IgG anti-dengue**

The ELISA inhibition method (EIM) was followed as previously described. Briefly, polystyrene plates (Costar No. 3591) were adsorbed with human anti-dengue IgG; after blocking, dengue 3 antigen previously diluted 1/40 in PBS and undiluted were used. Positive (duplicate) and negative controls were included in each test. Samples were incubated for two hours at room temperature for IgM and IgA and at 37°C for IgE antibody detection. An antigen mixture of the four dengue serotypes was added. After overnight incubation, 50 μL of human conjugate IgG anti-dengue diluted 1/5000 was added. Orthophenylenediamine (OPD) and hydrogen peroxide were used as substrate. The reaction was stopped after 30 minutes incubation. The test was read at 492 nm. The inhibition percent was calculated as:

\[
\text{Inhibition\%} = \left(1 - \frac{\text{OD sample}}{\text{OD negative control}}\right) \times 100
\]

The antibody titer for each sample was considered as the highest dilution with a percentage of inhibition ≥50. An IgG antibody titer <20 in the acute serum (collected at days 3 or 4 of fever onset) and <1280 in the early convalescent serum (collected at days 7 or 8 of fever onset) were taken as evidence of a primary infection. By contrast, an IgG antibody titer ≥20 in the acute serum (collected at days 3 or 4 of fever onset) and ≥1280 in early convalescent serum (collected at
days 7 or 8 of fever onset) with a four-fold or higher increase of the antibody titer or titer ≥ 10,240 in any serum were taken as evidence of a secondary infection.16,19

Capture IgG ELISA (GAC-ELISA)

An IgG capture ELISA test (GAC-ELISA) was employed to detect IgG antibody in urine samples. A protocol similar to the MAC-ELISA test5,16 with minor modifications was used. The NUNC MaxiSorp plates were coated with 100 µL per well containing 10 µg/mL of goat IgG anti-human IgG (Sigma) and urine samples were used without dilution (50 µL/well). Positive and negative controls in each test were included. All samples with an OD ratio ≥ 2 were considered positive.

Results

Type of infection: primary or secondary

From the total of 22 cases, 19 were classified as dengue fever (DF); four of them were primary infections and 15 secondary infections (Table 1). The last three cases were classified as dengue hemorrhagic fever (DHF) and all of them were secondary infections (Table 1). The comparative study was performed between the different types of samples in cases of primary and secondary dengue infection.

<table>
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</tr>
<tr>
<td>2</td>
<td>&lt;20/20</td>
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<tr>
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<td>&lt;20/20</td>
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</tr>
<tr>
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</tr>
<tr>
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<td>Secondary</td>
</tr>
<tr>
<td>7</td>
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</tr>
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<td>320/10 240</td>
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a Primary case: IgG antibody titer in acute serum <20 (days 3 or 4 after onset) and in early convalescence <1280 (days 7 or 8 after onset). Secondary cases: IgG antibody titer ≥20 in acute serum (days 3 or 4 of fever onset) and early convalescence ≥1280 (days 7 or 8 of fever onset) with a four-fold or higher increase in the antibody titer or titer ≥10,240 in any serum.

Kinetics of IgM, IgA, and IgG antibodies in serum, saliva, and urine samples

Figure 1 shows the kinetics of specific dengue IgM, IgA, and IgG antibodies in serum, saliva, and urine samples according to the type of infection (primary or secondary).

Primary cases: For these cases it was only possible to obtain serum samples between three and seven days following onset of symptoms.

An increase of dengue IgM antibodies was observed in serum and saliva of patients with a primary infection (Figure 1A and C). In serum, the increase of IgM was first detected at day 4 with an OD mean ratio ± standard deviation of 2.83 ± 0.86. The highest values were observed at day 6 (15 ± 1.56). In saliva, this increase was on day 5 (2.29 ± 0.99) and the maximum value was on day 7 (4.9 ± 1.25). No IgM antibody was detected in urine samples (Figure 1E). In addition, slow increments in the OD mean ratio values of IgA antibodies in serum, saliva, and urine were observed. IgA in serum showed values from 1.38 ± 0.42 to 6.73 ± 3.81 (Figure 1A). The OD mean ratio fluctuated from 1.34 ± 0.48 to 1.91 ± 0.80 in saliva (Figure 1C) and from 1.10 ± 0.13 to 2.90 ± 0.75 in urine samples (Figure 1E). Positive IgM and IgA antibody values in serum were first detected on average at days 4.25 and 5.5, respectively, after onset of fever. Positive IgM in saliva was detected on average at day 5.75. Specific IgG was first detected in serum at day 7 (geometric mean titer = 30). No IgG antibody was detected in saliva and urine samples.

Secondary cases: In serum samples from secondary cases, the abrupt appearance of IgM antibodies was observed at day 3 (2.09 ± 1.94) being maximum at day 7 (11.36 ± 3.00). IgA and IgG antibodies increased slowly during the first days of the study. Values for IgM ranged from 1.24 ± 0.47 (day 3) to 7.07 ± 3.51 (day 7) (Figure 1B). The GMT of IgG titer fluctuated from 15 (day 3) to 15 343 (day 8).

IgM, IgA, and IgG immunoglobulins in saliva (Figure 1D) and IgA and IgG in urine samples (Figure 1F) showed similar kinetics although the values obtained were lower than those observed in sera. The IgM OD mean ratio in saliva showed values of 1.79 ± 0.48 (day 3) to 7.10 ± 4.07 (day 6) while IgA values were 1.39 ± 0.31 (day 3) and 3.15 ± 1.79 (day 7).

In urine samples, no IgM antibody was detected, IgA values ranged from 1.13 ± 0.13 (day 3) to 5.29 ± 2.45 (day 8), and IgG values were from 0.93 ± 0.1 (day 3) to 6.07 ± 4.03 (day 7).

Positive IgM and IgA antibody values in serum were first detected on average at days 4.1 and 4.8, respectively; in saliva these were at 4.4 and 5.7 days after onset. In urine samples, positivity to IgA was at 6.3 days after onset. In secondary infection, the IgM response in serum and saliva samples was always higher than the IgA response. The positivity to IgG in saliva was at 5.3 days after onset.

In undiluted urine samples, only 11/18 (61.1%) of the secondary cases were positive by EIM (average 6.81 days). Urine samples were also assayed by GAC-ELISA test and of most of them were IgG positive between 6 and 7 days (average 5.78) after onset with IgG values of 3.16 ± 2.18 and 6.07 ± 4.03, respectively (primary y-axis in Figure 1F).

The IgG antibody was detected in saliva and urine samples when IgG titers in serum were 1280 or higher. The OD mean ratio values of all immunoglobulins studied decreased after day 20 of fever onset in secondary cases.
Comparison of percentages of positives to IgM, IgA, and IgG antibodies in serum, saliva, and urine samples from patients with primary and secondary dengue infection

Figure 2 shows the percentage of cases (by day of illness) with positive IgM (A primary and B secondary cases), IgA (C primary and D secondary cases), and IgG (E primary and F secondary cases) in serum, saliva, and urine samples.

By day 5, 100% of both primary and secondary cases had a positive IgM response in serum. In saliva, 100% of primary and secondary cases showed a positive IgM at days 6 and 7, respectively, however a large number of patients were already positive by day 5 (75 and 83.3%). A 100% positive IgA response in serum in primary and secondary cases was observed at day 7 (Figure 2C and D). All secondary cases were positive to IgG in saliva and urine samples at day 7 (Figure 2F).

Kinetics of specific and total IgE antibodies in serum samples of patients with a primary or secondary dengue infection

Values of specific and total IgE in serum samples are shown in Figure 3 (A primary cases and B secondary cases). An increase in the OD mean ratio values of specific IgE was observed at day 7 (1.7 ± 0.20) in primary cases and at day 5 (1.45 ± 0.507) in secondary cases being highest at day 8 (2.28 ± 0.823). Most secondary cases showed a positive anti-dengue specific IgE response around day 6 (average, day 5.9). The comparative analysis between primary and secondary specific IgE using the t-test showed no significant difference (p > 0.05).

In primary cases the IgE total mean values were between 1686 IU/mL (day 3) and 770 IU/mL (day 7). In secondary cases the total IgE showed a range of values between 687 UI/mL (day 3) and 588 IU/mL (day 8) after outbreak.

Discussion

The presence of antibodies in saliva and urine has been studied in rubella, hepatitis A, hepatitis C among others; but there are few reports on dengue IgM, IgA, and IgG detection in saliva samples and we found none employing urine samples.

The kinetics of IgM, IgA, and IgG antibodies showed a similar pattern in saliva and serum samples in both primary and secondary cases. Despite the similarity in the kinetic profile of the antibody response, the IgM and IgA OD values and the geometric mean titer of IgG antibodies were lower in saliva than in serum samples. The IgA values were lower than IgM both in serum and saliva. Similar results in serum have been found by other authors.

Urine is a body fluid with low but measurable concentrations of immunoglobulins derived from plasma either through the kidney or by transudation into the lower renal tract. It has been postulated that large macromolecules such as IgM...
antibodies cannot pass through the glomerular filter under normal conditions; however, the IgM protein in its monomeric form has been detected in some viral infections. In our study, specific IgM was not detected in urine samples. In contrast, IgA antibodies were detectable in both primary and secondary cases showing a similar pattern as in serum and saliva samples. The IgA OD mean values were slightly higher in urine than in saliva in both types of infection. These results could be due to the presence of non-specific IgA secretions that could be competing with the anti-dengue IgA of saliva in the capture ELISA.

EIM is an ELISA test standardized to detect dengue IgG antibodies in serum samples with high sensitivity and specificity, and it has been used to classify into primary or sec-

Figure 2  Percentage of positives in serum, saliva, and urine samples to IgM antibody (A primary and B secondary cases), to IgA antibody (C primary and D secondary cases), and to IgG antibody (E primary and F secondary cases) at different days after outbreak.

Figure 3  Kinetics of specific and total IgE antibodies in serum samples (A primary and B secondary cases). The principal y-axis shows the IgE specific OD (mean ratio + SD). The secondary y-axis shows a plot of the total IgE expressed in IU/mL (mean + SD); n = the total of the serum samples tested by day.
In this study the kinetics of three serological markers (IgM, IgA, and IgG) in serum, saliva, and urine samples from adult patients with primary or secondary dengue 3 infection were studied for the first time showing their behavior and usefulness in dengue virus diagnosis. The specific IgE could also play a role as a serological marker in secondary infections.

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Conflict of interest: No conflict of interest to declare.

References


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