EXPERIMENTAL TECHNIQUE OF LATEX AGGLUTINATION IN THE DETECTION OF DENGUE VIRUS

ABSTRACT

Dengue is the arthropod-borne viral disease that causes the most mortality and morbidity worldwide. Its proportions are pandemic infecting an estimated 1.6 million individuals annually. The clinical presentation associated with epidemiological factors, such as the prevalence of dengue in the patient's home region, and has been the only means of early diagnosis. Definitive laboratory diagnoses take several days until viral isolation is achieved. Serological methods depend on high levels of specific antibodies, and molecular methods are not available in the majority of routine diagnostic laboratories. The purpose of this study was to develop an agglutination method using latex to detect dengue virus using biological samples from mice infected intracerebrally with type-1 dengue, the Moshizuki strain, and specific anti-dengue-1 antibodies from immunized mice. According to the results, this method is capable of producing a diagnosis of dengue virus within ninety minutes in positive samples from experimental animals. Thus, the method provides new prospects for the rapid detection of dengue in a susceptible population during the first days of the symptoms.

INTRODUCTION

The Chinese Encyclopedia of Disease Symptoms and Remedies written in 265 AD and reedited in 450 AD by Tang & Sung, describes a 'water fever' associated with flying insects and water (Nobuchi 1979). The disease has periodically emerged in many countries: in India in 1635, in Panama in 1699, in Asia, Africa and in North America (Hirsch 1983, Howe 1977) from 1779 to 1980, when it eventually became known as "dengue fever". With the destruction of forests, an increase in the transmission rate was seen. In Southeast Asia "dengue hemorrhagic fever" (DHF) and the "Dengue Shock Syndrome" (DSS) were identified for the first time (Gubler 1988, Gubler & Trent 1994). The first epidemic of DHF was in Manila in the Philippines between 1953 and 1970 (WHO 1986, Gubler 1999, Gubler 1997). Since 1827 the disease has been described in the Americas in clinical terms (Ehrenkranz et al. 1971) but only with the advent of virologic techniques and serological diagnosis, could the clinical suspicion be confirmed. Four serotypes have been identified (Rosen 1958, Downs 1959). The different strains of the viruses were identified in 1977 (DEN-1), in 1981 (DEN-2), in 1994 (DEN-3) and in 1981 (DEN-4). In the 1970s,
there was a drop in the numbers of people affected, but at this time the mosquito, *Aedes aegypti*, was identified as the main transmitting vector followed by *Aedes albopictus* and *Aedes polynesiensis* (Pinheiro 1989, Westaway et al. 1985, Halstead 1980). DHF and DSS were confirmed by laboratory tests in 27 countries during the period from 1981 to 1997. An estimated one million six hundred thousand people become sick with dengue annually (Gubler 1987, Gubler & Clark 1994, Monath 1994, Brandt et al. 1967).

In Brazil, *Flaviviruses* are the cause of outbreaks (Pinheiro 1982, Figueiredo 1994, Vasconcelos et al. 1992). These viruses are perpetuated in nature in vertebrate hosts and hematophagous arthropods and are transovarially or venereally transmitted in arthropods. Around the world, 500 different types of *Arboviruses* are known but only 200 are considered important for man, including dengue viruses (Rehle 1989, Calisher et al. 1980, Karabatsos 1980). Brazil is the South American country that has isolated these viruses the most (Degallier et al. 1987, Pinheiro 1982). Dissemination of the virus is linked to demographic growth and uncontrolled urbanization. The use of insecticides is still ineffective (Reiter & Gubler 1997) and gives a false sense of protection. In addition to the symptoms characteristic of viral infection, individuals with dengue present with retro-orbital pain, myalgia, anorexia, vomiting, prostration, joint and bone pain, and respiratory complications. In severe cases, petechiae and bleeding of the mucous membranes are seen and the tourniquet test is positive (Simmons et al. 1931). DHF and DSS rapidly evolve to prostration, irritability, high blood pressure, tachypnea, cyanosis, tachycardia, shock, pleural effusions, hepaticomegaly, an elevation of the hematocrit levels, reduction in the complement level and lipoproteinemia. There may be an improvement in the state of shock after hydration or electrolyte replacement (Fields et al. 1990, Sangkhawibha et al. 1984). On the Island of Tonga in the Pacific Ocean, a great variation in the onset and duration of viremia was demonstrated in cases of primary infection (Burke et al. 1988, Singer & Plotz 1956). These variations were due to different genotypes that express different behavior for each viral sample.

The virus replicates in macrophages (Risen 1984, Sabin 1952). A patient infected by one type of dengue virus, initially reacts immunologically only to this type but, after a short period, the individual can react to the other types. This phenomenon is also due to a difference among epitopes in the viral samples. The definitive diagnosis is made through viral isolation, from detection of the antigens and the RNA in blood samples or from tissues and of specific antibodies (Eckels et al. 1985).

Isolation of dengue viruses is made in hamster (BHK21) and monkey (LLC–MK2) renal cells or by inoculation in brains of 1 to 3-day-old mice (Vaughn 2000). Dengue viruses are not very pathogenic for mice. The cytopathic effect on cells in the first passage is low (Karabatsos 1985) but more evident in later passages. Inoculation is effective in 100% of cases in which positive inoculants are used (Rosen & Gubler 1974, Kubeski & Roden 1977) in cultures of clone C6/36 *Aedes albopictus* cells (Sinarachataanant & Olson 1973, Kuno 1982, Figueiredo 1990, Igarashi 1978). Viral identification may be achieved using direct or indirect immunofluorescence assays (Henchal et al. 1983). Molecular biology techniques have moderate sensitivity but good specificity. Also techniques such as *Southern blot* and *Northern blot* are utilized using RNA fragments or synthetically marked oligonucleotides (Chandler et al. 1993). The polymerase chain reaction is also employed (Lanciotti et al. 1992, Morita et al. 1994) with the amplified product being observed using electrophoresis in agarose gel with characteristic “DNA bands” for each serotype, as all primers form bands in different positions giving a 482 bp band for dengue-1. The serological diagnosis can be made by hemagglutination (Clarke & Casals 1958), by the complement fixation reaction (Russel & Nisalak 1967) and by the neutralization test (Calisher et al. 1989). Immunoenzymatic assays are used in the capture of the IgM anti-dengue antibodies. The MAC–ELISA technique can also be employed, using serum of patients with dengue (Kuno et al. 1987).

The latex fixation technique developed by Singer and Plotz (1956), which uses spherical particles of ‘marked’ polystyrene latex in the ‘solid phase’ with specific immunoglobulins against determined antigens of virus proteins, has been successfully utilized. This is a low-cost technique that is easy to use.

The current work presents an adaptation of the agglutination technique that aims at making an early diagnosis of dengue feasible. It employs polystyrene particles marked with specific antibodies of the dengue-1 virus in 2- to 3-day-old mouse brain samples infected with the *Mosquito* strain.
MATERIALS AND METHODS

A sample of the Moshizuki strain of dengue-1 virus was propagated on clone C6/36 Aedes albopictus mosquito cells, cultivated in 75-cm² cell culture flasks containing Leibowitz L15 medium added to 10% heat-inactivated bovine fetal serum, 10% tryptose phosphate broth, 100 U/mL penicillin and 100 mg/mL streptomycin, at a temperature of 28°C. The mixture was observed daily using an inverted microscope and only on the seventh day separation of cells by gentle agitation was visible. Small 1.5-mL samples were aliquoted in tubes, which were stored in a freezer at minus 70°C.

Immediately before the aliquots were prepared, 40-mL samples of non-infected and infected cell suspensions were applied side-by-side as two spots on a microscope slide, dried under warmed air and immersed in iced acetone (4°C) during 20 minutes for fixation. Subsequently, the slides were dried again under warm air and 40 µL of ascitic fluid (AF) diluted at 1/20 in PBS at pH 7.5 was added, then they were incubated for 30 minutes at 37°C in a humid chamber for tissue culture. Next the slides were washed in PBS by immersion in a shaker for 10 minutes and dried under warmed air. Subsequently, 10 µL of conjugated anti-mouse IgG marked with fluorescein isothiocyanate was added at a titer of 1/100 in PBS at pH 7.5. Following incubation at 37°C in a humid chamber for 30 minutes, the slides were immersed in PBS at pH 7.5 and swirled for 10 minutes, rinsed with deionized distilled water and dried under a forced airflow.

The slides were observed under a fluorescence microscope. There were intense dots of intracellular fluorescence lining the entire nuclear surface and a great part of the cellular cytoplasm. Some small cellular agglutinations revealed intense fluorescence making the artifact specific to the technique. No fluorescence was seen in the samples without infected cells. Ascitic fluids with antibodies were obtained utilizing young virgin mice (Mus musculus – Swiss albino) which had been intraperitoneally inoculated on four occasions using a 25 x 5 needle at seven-day intervals with 50 µL of a suspension of macerated new-born mouse brains infected with a suspension of C6/36 cells infected with the Moshizuki strain of the dengue-1 virus, diluted at 1/20 in a 0.85% sodium chloride solution previously centrifuged at 1200 x g for 5 minutes with an inoculation of 20 µL. The inoculated mice were kept with their mothers and after 4 days they presented with signs of movement disabilities and 30% died. The remaining animals were sacrificed and frozen at minus 70°C. A suspension of macerated brain obtained by the puncture of the encephalic mass of the frozen brains was diluted at 1/20 in a solution of 0.85% sodium chloride at pH 8.5 and subsequently centrifuged at 1600 x g for 10 minutes in a cooled centrifuge. The ascitic fluid was also intraperitoneally inoculated using 200 µL of the centrifuged suspension in adult mice that evolved with large-volume ascites after 8 days. The ascitic fluid was extracted by paracentesis performed on a single occasion for the mice that had evolved ascites.

This fluid, which proved to contain anti-dengue-1 antibodies, was centrifuged at 1600 g for 15 minutes and the supernatant was stored at minus 20°C. Before use, it was thawed and added to an equal amount of 40% ammonium sulphate solution, drop by drop and left for 12 hours after which the final volume was centrifuged at 1600 g for 30 minutes. The precipitate was resuspended and rinsed to remove the ammonium sulphate. After centrifuging again, it was resuspended in PBS and dialysis performed for 24 hours in running water on a PM30 Amicon membrane and for a similar time using 0.85% sodium chloride at 4°C. Following this, electrophoresis was performed on the proteins which revealed a concentration of 0.6 g/dL of gammaglobulin and 0.9g/dL of albumin. The presence of anti-dengue-1 antibodies was confirmed in the ascitic fluid by indirect immunofluorescence.

The agglutination test was then performed with a 10% suspension of 800-µm polystyrene spheres diluted at 1/50 in PBS at a pH of 7.5 and kept at 4°C; 40 µL of the antibody solution extracted from the ascitic fluid diluted at 1/20 in buffered borate and 0.85% sodium chloride producing solutions with pHs of from 5.5 to 9.5 at intervals of 0.5. The resulting solutions were added to 20 µL of polystyrene stock solution bound to immunoglobulin and incubated in a humid chamber at 37°C for 40 minutes. As a negative control, the same procedure as above was performed with uninfected solutions. The solutions were incubated for 360 minutes with assessments made at 60-minute intervals.

Animals similar to those employed in this procedure were subjected to intracerebral inoculation to determine the 50% lethal dose (LD50).
Calculation of the LD50 was achieved using newborn mice (1 to 2 days old) divided into groups of 5 but kept with their mothers. Each group was subjected to intracranial inoculation of 20 μL of different concentrations of the macerated brain with 10^2, 10^3, 10^4, 10^5, 10^6, 10^7 cells of the dengue virus. A control group was similarly set up. The animals were observed up to the 14th post-inoculation day, registering the numbers of deaths and survivals. The calculation of the LD50 was made according to the method described by Reed & Muench (1938).

RESULTS AND DISCUSSION

The latex fixation technique using polystyrene particles bound to antibodies against specific antigens has been the gold standard since 1958 when it was first described by Singer & Plotz (1961) for the laboratory diagnosis of cases that require fast and easy diagnosis at a low cost and using little equipment. In this work we are proposing a new quick technique to diagnose the presence of the dengue virus in samples.

A concentration of 0.05 g/mL of the antibodies extracted from the ascitic fluid in a PBS solution with a pH of 7.5 proved to be ideal to ‘mark’ the polystyrene spheres, as concentrations higher than 0.08 g/mL cause spontaneous agglutination immediately after the addition of the antibody solution to the polystyrene suspension (Singer & Plotz 1961). Lower concentrations of from 0.04 g/mL to 0.01 g/mL did not cause visible agglutination in the presence of the antigen. As the polystyrene agglutination reaction is pH dependent (Clarke & Casals 1958) and as hemagglutination reactions vary, there is an ideal range for each different arbovirus. In keeping the conditions of the immunoglobulin and polystyrene concentrations unaltered, including the temperature and humidity, reactions at a pH of 8.5 were seen to be more intense and more reproducible. Reactions at low pHs gave slight agglutination and small microscopically visible aggregates. Reactions at pHs higher than 8.5 and up to 9.5 did not present agglutination. Reactions with pHs between 5.5 and 7.5 gave spontaneous agglutination even before the addition of the antigen. The ideal concentration of the polystyrene spheres to view the agglutination under the microscope was 0.02%.

The following table demonstrates the result of the LD50 test with the virus sample employed for the inoculation of mice.

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Deaths</th>
<th>Survival</th>
<th>Mortality</th>
<th>Cumulative deaths</th>
<th>Cumulative survival</th>
<th>Mortality rate</th>
<th>Percentage mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^-2</td>
<td>5</td>
<td>0</td>
<td>5/5</td>
<td>21</td>
<td>0</td>
<td>21/21</td>
<td>100</td>
</tr>
<tr>
<td>10^-3</td>
<td>5</td>
<td>0</td>
<td>5/5</td>
<td>16</td>
<td>0</td>
<td>16/16</td>
<td>100</td>
</tr>
<tr>
<td>10^-4</td>
<td>4</td>
<td>1</td>
<td>4/5</td>
<td>11</td>
<td>1</td>
<td>11/12</td>
<td>92</td>
</tr>
<tr>
<td>10^-5</td>
<td>3</td>
<td>2</td>
<td>3/5</td>
<td>7</td>
<td>3</td>
<td>07/10</td>
<td>70</td>
</tr>
<tr>
<td>10^-6</td>
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<td>3/5</td>
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<td>5</td>
<td>04/09</td>
<td>44</td>
</tr>
<tr>
<td>10^-7</td>
<td>1</td>
<td>4</td>
<td>1/5</td>
<td>1</td>
<td>9</td>
<td>01/10</td>
<td>10</td>
</tr>
</tbody>
</table>

70- 50 = 0.8 (interpolated value)

Log 10 of the 50% lethal dose was obtained by summing the interpolated value, corrected by the log, to the dilution for more than 50% = -5.0 + (-0.8) = -5.8. The dilution of the lethal viral sample for 50% of the animals inoculated was 10^-5.8. The number of estimated viral particles was 800 x 10^3/mL or 16 x 10^3 particles/inoculated volume. Microscopically visible reactions occurred with concen-
trations varying from $10^0$, with a great intensity and slight spontaneous agglutination, to $10^8$, with strong intensity and no spontaneous agglutination. Reactions with $10^3$ showed slight agglutination seen under the microscope and with $10^4$ or less, visible agglutination occurred. It can be seen from these results there is a great 'distance' between the 50% lethal dose ($10^{5.8}$) and the dilution capable of agglutinating polystyrene spheres marked with anti-dengue antibodies ($10^8$ and $10^9$). This discrepancy must be due to methodological differences, interfering in the levels of sensitivity, as it is impossible to compare the two methods.

The total time for the new test is approximately 90 minutes. Although the scope of this work was to develop a “fast test”, some parameters could not be reduced in respect to the incubation periods and temperature. The stabilization time of the reaction was 6 hours, but when this was reduced to one hour, the reaction was totally inhibited. The temperature in the humid chamber was constant at 37°C. Adequate control of the reaction depends on the evaluation, identification and correction of possible inhibitors that interfere in the formation of antigen-antibody complexes and of the agglutination of the polystyrene spheres bound to this complex. If it is possible to eliminate possible interferants and the sensitivity of the technique has attained dilutions to the order of between $3.0 \log 10$ and $4.0 \log 10$, as intermediate dilutions were not tested, there is a possibility to apply the method to blood samples from patients suspected of having dengue fever. The dengue-1 serotype is detectable in samples up to six days after the initiation of the symptoms (Gubler et al. 1981) by the method of intrathoracic inoculation of blood in Aedes aegypti or albopictus with titers of $3.8 \log 10$ to $8.0 \log 10$. The level of viremia in blood samples in these cases will be detectable by the proposed method, demonstrating the real possibility of its utilization in the detection and diagnosis of the dengue virus.

CONCLUSIONS

The polystyrene sphere agglutination test proposed for the diagnosis of dengue-1 virus in biological samples, when it is submitted to a rigid control in respect to the reaction conditions, including the buffer solution pH determined on a decimal scale, the concentration and the homogeneity of the polystyrene particles has proved to be a good diagnostic alternative to the traditional methods, giving results in a matter of about 90 minutes.

REFERENCES


