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Molecular Characterization of Dengue Virus Circulating in Manaus, the Capital City of the State of Amazonas, Brazil

Regina Maria Pinto de Figueiredo
Foundation for Tropical Medicine Dr. Hector Vieira Dourado (FMT/HVD)
Brazil

1. Introduction

The term dengue, of Spanish origin, was used to describe joint pain from an illness that attacked the British during the epidemic that affected the Spanish West Indies from 1927-1928. Dengue was brought to the American continent to the Old World during the colonization in the late eighteenth century. However, it is not possible to say according to the historical record if the outbreaks were caused by dengue virus, as its symptoms are similar to those of several other infections, especially yellow fever (Holmes et al., 1998). The etiology of dengue has been credited to the miasma theory, bacterial or protozoan infection and finally to an ultramicroscopic agent. Similarly, transmission has been considered by respiratory airway and finally, by mosquitoes.

The isolation of dengue virus (DENV) occurred in the 1940s during the epidemics of Nagasaki (1943) and Osaka (1944). The first known strain is coined DENV Mochizuki (Kimura & Hotta, 1944). In 1945, the Hawaii strain was isolated, and that same year other DENVs showing antigenic characteristics of different serotypes were isolated in New Guinea. The first two strains were designated serotype 1 and serotype 2. In 1956, other strains designated as serotype 3 and 4 were isolated. Thus, four dengue serotypes are known to date: DENV-1, DENV-2, DENV-3 and DENV-4 (Martinez Torres, 1990).

Genetic variation within each serotype was first demonstrated by serological techniques. Subsequently analysis of the viral genome showed that DENV-1and DENV-2 can be classified as having five genotypes or subtypes each while DENV-3 and DENV-4having four and two respectively (Rico-Hesse, 1990, Lanciotti et al., 1994). Recently, Rico-Hesse et al (2003) reviewed the classification of DENV genotypes by the analysis and comparison of the nucleotide sequence of the complete E gene of various strains. As a result it was defined thatDENV-2 and DENV-3 have four genotypes while DENV-1 and DENV-4 have five and three genotypes respectively (Cunha & Nogueira, 2005).

1.1 Etiology

Dengue Fever, a vector-borne disease, is the most important arboviral disease worldwide. Dengue viruses (DENVs) belong to the genus *Flavivirus*, family *Flaviviridae*. These are single-stranded positive-sense RNA viruses. DENV are grouped into four antigenically related but distinct serotypes named DENV-1, 2, 3 and 4.The four serotypes of DENV are diverse and

not phylogenetically related but strongly related to flavivirus transmitted by mosquitoes. It is reported that these viruses have emerged about 1000 years ago from a monkey virus and its transmission to humans occurred in the last 320 years. Some studies indicated its origin in Africa and others in Asia (Weaver et al., 2004).

1.2 Clinical characteristics

Dengue has two main clinical forms: classic dengue fever (DF) and dengue hemorrhagic fever (DHF) with or without shock (PAN AMERICAN HEALTH ORGANIZATION, 1994). The symptoms of DF are headache, retro-orbital pain, breaking bones sensation, muscles or joints pain, rash and leucopenia. Dengue hemorrhagic fever is characterized by high fever, hemorrhagic phenomena often with hepatomegaly and, in severe cases, signs of circulatory failure. Patients with DHF may develop hypovolemic shock resulting from plasma leakage. This clinical manifestation is called dengue shock syndrome (DSS) and can be fatal (WORLD HEALTH ORGANIZATION, 2001).

1.3 Transmission

The dengue virus is transmitted to humans through the bite of hematophagous Diptera, the mosquito *Aedes aegypti*. In the Americas, *A. aegypti* is one of the most efficient vectors of Arboviruses and is highly anthropophilic thriving in close proximity to humans and adapts very well indoors, generally in humid environment (WORLD HEALTH ORGANIZATION, 2001).

Once contracted the virus, the mosquito remains infected during its entire life and may transmit the virus to individuals during blood meals. The infected *A.aegypti* females may also transmit the virus to the next generation of mosquitoes by transovarial. Although very seldom, this means of transmission is of great epidemiological significance demonstrating that the vector play an important role in the persistence of the virus in the environment and act as reservoirs (WORLD HEALTH ORGANIZATION, 2001; Castro et al., 2004; Joshi et al., 2006).

Human beings are the main host and the virus replicates in the blood stream. Uninfected mosquitoes can contract the virus during blood meals from an infected individual. The virus multiplies in the cells of the mosquito during a period of 8 to 10 days. After this period, the vector is able to transmit the virus to humans again. In humans, the incubation period of dengue fever ranges from 2 to 7 days. Laboratory experiments showed that the mosquito *A. aegypti* may be infected simultaneously by different arboviruses and is also capable of transmitting them simultaneously (Araújo et al., 2006). According Wenming et al. (2005) it is possible that mosquitoes infected with DENV-2 and DENV-3 can transmit both in areas where two or more serotypes circulate. The poor environmental conditions of urban centers, the humidity and temperature as in Brazil associated with resistance of eggs of *A. aegypti* for long periods of desiccation favor the proliferation of mosquitoes and contribute to the spread of DENV. Dengue is currently considered the most important arbovirus and is a public health problem in tropical and subtropical countries (Guzman et al. 2006; WORLD HEALTH ORGANIZATION, 2001).

1.4 Dengue in Manaus

Infection by dengue virus in Brazil has increased significantly over the last decade, particularly after 1994, as a consequence of the spread of *A.aegypti*. The following serotypes,

DENV-1, DENV-2 and DENV-3 were common in most Brazilian cities. The DENV-4, co-circulating with DENV-1,was detected during the first epidemic reported in Brazil in Boa Vista - Roraima in 1981-1982 (Osanai, 1984).

In early 1998, the Laboratory of Arbovirology at the Foundation for Tropical Medicine Dr. Hector Vieira Gold (FMT/HVD) in Manaus in the state of Amazonas, started a program of monitoring and diagnosing of viral diseases transmitted by arthropods (arboviruses) to determine their etiological agents. The diagnosis was made by serologic studies using the MAC-ELISA test for detection of IgM antibodies. Sera samples from 8557 patients suspected of dengue were analyzed and 40% of the sera were ELISA positive for dengue virus. The DENV-1 was considered responsible for this epidemic (Figueiredo et al., 2004). In 2001, DENV-1 and DENV-2 were isolated in the State of Amazons and dengue hemorrhagic fever cases were registered (Figueiredo et al., 2002). In 2003, DENV-3 was isolated for the first time in the state of Amazonas from a patient coming from the state of Bahia (Figueiredo et al., 2003).

A variety of acute febrile diseases with and without hemorrhagic manifestations were diagnosed as dengue in Manaus in March 1998 by detection of specific IgM antibodies. Mayaro (MAYV) and *Oropouche* (OROV) viruses were also diagnosed. Infections with rubella and parvovirus were also observed (Figueiredo et al., 2004).

In 2008, DENV-4 was first identified in Manaus (Figueiredo et al., 2008). The Amazon is situated in the Northern Region of Brazil, bordered to the north with the State of Roraima, Venezuela and Colombia. Serotype DENV-4 was endemic to Venezuela and Colombia and this may have influenced the detection of this serotype in the Amazon due to its proximity to these countries (Figueiredo, 2008). The four serotypes of dengue and cases of DENV-3/DENV-4 co-infection are shown in Figure 1.

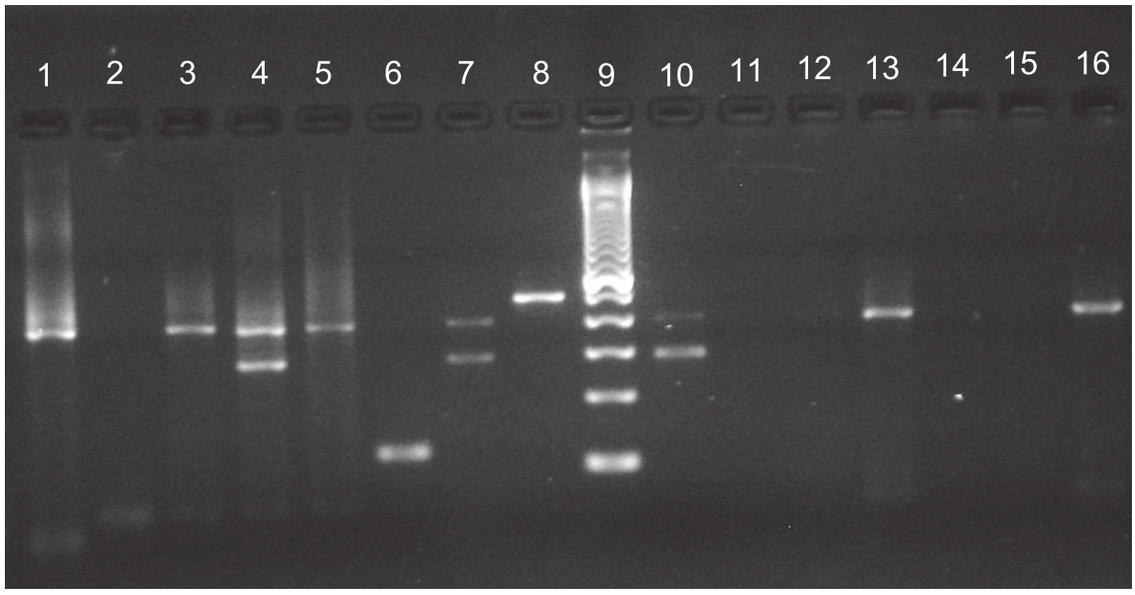


Fig. 1. Agarose gel electrophoresis. Lanes 1,3, 13, 16: DENV-4; Lane 4,7 and 10: DENV-3/DENV-4; Lane 6: DENV-2, Lane 8: DENV-1; Lane 9: DNA size standards 100Kb;

Nucleotide sequence analysis of DENV-4 present in the State of Amazonas showed to be of genotype I which is only present in the Asian continent and never described in the

Americas (De Melo et al., 2009). The introduction of this virus in Manaus is probably due to the fact that this is a city with eco-tourism development, and possess an Industrial Center (Free Zone of Manaus), with over 450 factories of large, medium and small size (PORTAL AMAZÔNIA) and many of them are of Asian origin (Figueiredo, 2008). It is possible that the virus has been introduced from an Asian-infected visitor or vector *A.aegypti*-infected.

1.5 Co-infection

Natural co-infection with dengue virus can occur in highly endemic areas where several serotypes have been transmitted for many years. Cases of simultaneous infection by more than one species of arboviruses in mosquito or human host were reported (Meyers& Carey, 1967; Gubler et al., 1985). In Brazil, one case of co-infection by DENV-1 and DENV-2 was reported in the patient with classic dengue fever (DF) from Southeastern region through immunofluorescence and RT-PCR (Santos et al., 2003). Another case of co-infection by DENV-2 and DENV-3, was observed in 2005 in the Northeastern region from a patient with DF (Araújo et al., 2006). Simultaneous infection by different strains of dengue virus in mosquitoes and humans underscores the potential for recombination (Santos et al., 2003). Although recombination is rarely recorded in positive-stranded RNA viruses (Lai, 1992), recombination occurrence in picornavirus, coronavirus, and alphavirus have been suggested. The latter viruses are also transmitted by mosquitoes (Hahn et al., 1998.Variations in dengue virus and the occurrence of co-infections with different DENV serotype may lead to genetic exchange between strains increasing the likelihood of recombination (Kuno, 1997).

During an outbreak of dengue in São José do Rio Preto, State of São Paulo, 365 samples were positives to DENV-3, 5 samples were to DENV-2, and 8 to Saint Louis encephalitis flavivirus (SLEV). Among the positive samples, one co-infection was detected for DENV-2 and DENV-3. Co-infection of each distint DENV serotype or other flavivirus during dengue outbreaks seems to be common (Terzian et al., 2010).

2. Laboratory diagnosis

2.1 Isolation of virus

The two basic methods for establishing a laboratory diagnosis of dengue fever are: the detection of viruses (egg, culture), or detection of IgM antibodies anti-dengue (serology). Blood samples for viral isolation should be collected within five days from the onset of symptoms. Serum is obtained by centrifugation and stored at -70°C.The inoculation of clinical specimens in adult mosquitoes or larvae in the culture technique is more sensitive for the detection of DENV. In laboratories where colonized mosquitoes are not available, samples can be inoculated in any mosquito cell lines available such as C6/36 (*Aedes albopictus* clone) that has a high sensitivity to DENV and other arboviruses.

2.2 Serological diagnosis

Sera should be collected from patients from the sixth day of illness and stored at -20°C. The MAC-ELISA is an antibody-capture assay of IgM from sera of patients suspected of DF. Briefy, the plate is sensitized with an anti-human IgM, and after various steps of the assay (blocking, dilution, washing, incubation overnight with the pool of antigen (DENV-1,

DENV-2 and DENV-3), the presence of specific IgM antibodies to dengue in the patient serum is shown by color change of the substrate that undergo enzymatic action of the conjugate. The color intensity is directly proportional to the amount of IgM antibodies contained in serum (Kuno et al., 1987).

2.3 Polymerase chain reaction (PCR) coupled with reverse transcription(RT-PCR)

The technique of PCR can be used to detect the presence of the DENV. Viral RNA is extracted using commercial kits. The RNA is reverse transcribed to cDNA in a first step. Then specific primers for DENV nucleotide sequence are used for the amplification of targeted sequence so as to detect a small amount of RNA molecules of DENV. This method is fast and rapid compared to cell culture. Reverse transcription and PCR technique was shown for the first time as a powerful technique for the detection of the DENV during convalescence, when the antibodies would limit its detection (WORLD HEALTH ORGANIZATION, 2001).

However, this method should still be considered an experimental approach. Is implementation on a large scale awaits further experiments. Moreover, a consensus on the adequate preparation of samples and for determining the sequences of bases in oligonucleotides capable of detecting all or most circulating genotypes of dengue need to be reached (WORLD HEALTH ORGANIZATION, 2001).

2.4 Virological diagnosis

The technique used to isolate DENV is continuous cell lines of mosquito cells C6/36 (*Aedes albopictus* clone) (Igarashi, 1978), grown in 25 cm flask with growth medium L-15 plus 5% of fetal bovine serum. To 1.5 mL of theL-15 plus 5% of fetal bovine serum containing the C6/36 cells in disposable falcon tubes (15 mL), 70 µl of serum from patients suspected of DF in acute phase was added and incubated at 28° C for two week. Culture media is changed two times per week and incubated. After 10 days of incubation, immunofluorescence (IF) technique is used for the identification of serotypes of dengue.

3. Discussion

In Brazil, first cases of DHF occurred after the introduction of DENV-2 in the State of Rio de Janeiro. The cases of DHF by DENV-2 occurred after an epidemic by DENV-1 in Rio de Janeiro four years ago (Dias et al. 1991; Zagne et al., 1994). The same pattern was seen in Cuba during the 1981 epidemic with sequential infection by two serotypes (DENV-1 and DENV-2), and an interval of six months to five years or so (Kouri et al., 1986).The dynamics of epidemic of dengue in the Amazon is similar to that of other regions of Brazil and America. In Manaus – Amazonas, the first cases of dengue were registered in March 1998 by serological studies during the first epidemic of dengue (Figueiredo et al., 2004). In 2001, it was possible to identify the DENV-1 as the causative agent of that epidemic (Figueiredo et al., 2002). That same year, cases of dengue hemorrhagic fever were registered in the State of Amazonas, with the viral isolation of DENV-2 (Figueiredo et al., 2002). In 2002, DENV3 was first isolated from a patient coming from Bahia. Since then, DENV-3 was diagnosed by viral isolation from several patients with DF (Figueiredo et al., 2003).The virus DENV-4 was detected during the first epidemic reported in Brazil in Boa Vista-Roraima in 1981-1982. At

that time the DENV-1 was also found (Osanai, 1984). Since then, no isolate of DENV-4 was recorded anywhere else in the country until later in the year 2008 DENV-4 was isolated in Manaus from patients with DF (Figueiredo et al., 2008).

The DENV-4 present in the State of Amazonas and analyzed by nucleotide sequence was shown to belong to genotype I, only present in the Asian continent and never described in the Americas (De Melo et al., 2009).

Dengue fever in uncomplicated cases of co-infection has also been observed by other authors, contradicting the hypothesis that simultaneous infection with dengue virus permits the emergence of a more severe disease (Santos et al., 2003; Araújo et al.,2006). In areas where more than one serotype are transmitted at the same time, clinical cases caused by more than one serotype of dengue fever can be common (Lorono et al., 1999). The high rate of cases occurring during epidemics can result in many infections with multiple serotypes in humans (both clinical and subclinical), and also provide opportunity for mosquitoes to become infected with two or more serotypes (Gubler et al., 1985; Burke et al., 1988). This suggests that co-infection by multiple dengue serotypes may influence the clinical expression of disease and it was initially considered as an explanation for the emergence of DHF (Hammon, 1973).The Amazon is situated in the Northern Region of Brazil, bordered to the north with the State of Roraima, Venezuela and Colombia to the east with the State of Pará, the southeast by the State of Mato Grosso, to the south with the State of Rondônia and southwest with the State of Acre and Peru (Viverde Tourism). The proximity to other countries where endemic DENV-4 is observed as in Venezuela and Colombia may have influenced the detection of this serotype first in the Amazon. Besides the geographical location, being today a city with eco-tourism development, and the Industrial Pole of Manaus, with over 450 factories of large, medium and small size (PORTAL AMAZON), which attracts investors from around the world. Manaus receives many people from these and other states and countries.

It is important to remember that the DENV-3 was first detected in Manaus from a patient coming from Salvador-Bahia (Figueiredo et al., 2003).The analysis of the region the C/prM of the DENV-3 in this study belonged to genotype III strains. All DENV-3 isolated in the Amazon were very close to the Indian strain GWL-60 (N°acessoAY770512) and the Brazilian strain BR-74 886 (N access AY679147). Subtype III is related to outbreaks of DHF in India (Dash et al., 2006). In Brazil, the prevalence of DENV-3 two years after its introduction in 2000 was associated with major epidemics in terms of more severe clinical manifestations, and the number of deaths (Nogueira et al., 2005). Twenty-two isolates were classified as DENV-3 subtype III (Miagostovich et al., 2002). The similarity of these strains to other represented by the same genotype III ranged from 96% to 98% and 98-99% for sequences nucleotides and amino acids, respectively. These data demonstrate that this virus is circulating around the world, again indicating high potential for distribution, adaptation in in various geographic areas of the world. This subtype has been implicated in outbreaks of DHF in Asia, Africa and the Americas, and has high potential to cause a pandemic of dengue (Messer et al., 2003).

4. Conclusions

The emergence of dengue virus in Manaus follows the same pattern as to what occurs in other Brazilian cities. Every two to three years there are new outbreaks of dengue and the

emergence of new serotypes followed by cases of dengue hemorrhagic. The emergence of DENV-4 in 2008 did not just caused a major epidemic with severe and fatal cases. Other serotypes were also observed. It is possible due to competition between with other serotypes, DENV-4 took some time to become established and the population might have been protected by antibodies obtained in heterologous infections by heterologous serotypes. The viral strain should be studied in detail since it is an Asian genotype that has been associated with DHF in the Asian continent.

- In relation to the DENV-2 which appears in 2011 with very high frequency, Manaus already has the DENV-2 genotype II for some time and often associated with cases of hemorrhagic dengue fever in other regions.
- Another important point is the co-infections with DENV-3 and DENV-4 in patients with DF. Most of them are associated with mild DF.
- Considering the large number of samples negative for dengue fever in our study, and to take into account the occurrence of other viruses, clinically similar to dengue fever, and the possibilities available to new viruses emerging or reemerging as Manaus is surrounded by the Amazon rainforest and also a large urban center that welcomes visitors from around the world, other viruses need to be investigated.

5. Recognition

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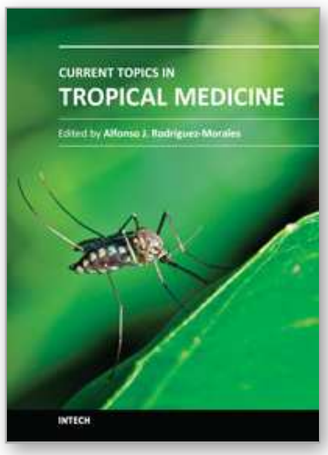
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Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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