Chapter 4

Laboratory Tests Used in the Diagnostic and Research of Dengue Virus: Present and Future

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Additional information is available at the end of the chapter

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Abstract

Dengue is a significant public health problem. There are four dengue virus serotypes identified; however, its diagnosis is difficult due to the existence of many viruses, bacteria, and parasites producing the same clinical presentation, being present in the same geographical area and even producing coinfections. Therefore, determining whether a person has, had, or is infected with dengue virus is of great importance. In order to do so, direct and indirect laboratory tests have been developed to identify the virus or part of its structure that generally detects the antibody response. These techniques are used for diagnosis, epidemiological studies, monitoring, assessment and production of vaccines and antivirals, etc. They range from the use of cell cultures, animal models, inoculation by insects, and serology tests to the use of detection molecular tests and quantification of genetic material that are described in this chapter herein, a brief explanation of this methodology, its strengths and weaknesses, and its application in the dengue research.

Keywords: dengue, arbovirus, flavivirus, laboratory test, diagnostic test

1. Introduction

Dengue is a dynamic systemic infectious disease and the most important arbovirus worldwide. Its occurrence has increased in past decades, and it is estimated that 390 million of infections occurs annually of which 67–136 million manifest clinically with any severity of disease. Another study shows that 3.9 billion people are at risk of infection with dengue virus (DENV) in 128 countries [1]. Diagnostic tests providing a proper identification of DENV infection by any of its four serotypes in symptomatic or asymptomatic cases in the population, and especially in areas that have more than one arbovirus or another micro-organism (virus, bacteria or parasite) producing similar signs and symptoms are the key aspect of any dengue research

and surveillance programs [2]. Laboratory tests are based on the detection of the virus, part of its genome or structure, or specific result from an infected person or animal as immune response. In this chapter, the most used laboratory tests in an arbovirus study focused on diagnosis and research of dengue virus (DENV) will be assessed, some comparisons will be carried out with other laboratory tests, its features, advantages and disadvantages, and cautions need to be considered during the process.

2. Laboratory test

2.1. Characteristics of diagnostic tests

A diagnostic test intends to identify whether or not a patient has a disease that cannot be recognized by signs and symptoms. Ideally, such test must meet the following requirements:

- To achieve high levels of sensitivity and specificity.
- Accurate and precise results.
- To deliver rapid results.
- To be cost effective.

Different studies can be carried out in the dengue research. For example, clinical diagnosis diseases, epidemiological studies, clinical assays, viral load tests, vaccines and anti-viral assessments, etc. Thus, it is necessary to know the testing capacity to detect the presence or absence of the disease *(validation of diagnostic tests)*, so it is very useful to know the following diagnostic test indicators (**Table 1**).

Result of the laboratory test	**Disease**		
	Positive	**Negative**	**Total**
Positive	**a** (True positive)	**b** (False positive)	**a + b**
Negative	**c** (False Negative)	**d** (True negative)	**c + d**
Total	**a + c**	**b + d**	**a + b + c + d**

Sensitivity: It is the probability that a sick person delivers a positive result in the diagnostic test (a/(a + c)).

Specificity: It is the probability that a person who is not sick delivers a negative results in the diagnostic test (d/(b + d)).

Positive predictive value: It is the probability that a person is sick and gets a positive result in the diagnostic test (a/(a + b)).

Negative predictive value: It is the probability that a healthy person gets a negative result in the diagnostic test (d/(c + d)).

Efficiency: It is the probability that a person is properly diagnosed using the diagnostic test ((a + d)/(a + b + c + d)).

Positive likelihood ratio (LR^+**)**: It is obtained when dividing sensitivity by the portion of false positives (1 − specificity), and it indicates the probability of being sick if the result is positive (Sensitivity/(1 − specificity)).

Negative likelihood ratio (LR^-**)**: It is obtained when dividing false negatives (1 − sensitivity) by specificity, and it indicates the probability of negative results obtained from a sick person ((1 − Sensitivity)/Specificity).

Table 1. A double-entry table to obtain indicators of diagnostic tests from analyzing a sample showing all possible results.

The sensitivity and specificity have distinctive features of diagnostic tests, they are not compromised by the prevalence of the disease, and they are inversely proportional. If a study in which the majority of people are suffering from this disease is carried out, a high sensitivity test is needed in order to identify the highest number of true positive and the lowest number of false negative. However, it may increase the number of false positive. If you want to obtain a good disease diagnosis, a high specificity test must be used to detect the highest number of true negative. Here, the false positive will also be low. Moreover, positive and negative predictive values of the diagnostic tests are affected by the prevalence of the disease in the study population. The likelihood ratio (LR) that is independent of prevalence is used when the laboratory tests do not present dichotomous results but cut-off value. This is another way of assessing the accuracy. According to the results, the test can be classified into adequate ($LR^+ \geq 10$ to $LR^- \leq 0.1$), moderate ($LR^+ \geq 5 < 10$ to $LR^- > 0.1 \leq 0.2$), scarce ($LR^+ \geq 2 < 5$ to $LR^- > 0.2 \leq 0.5$) and insignificant ($LR^+ \geq 1 < 2$ to $LR^- > 0.5 < 1$). Not only one but also many tests can be used to diagnose dengue in the epidemiological studies. It can be done sequentially or in parallel. For example, when performing a test with two sequential tests, all positive people need to be assessed with a second test upfront, and this will cause the reduction of net sensitivity and a net specificity enhancement obtained from both tests. It will be considered as positive if their tests are positive in all tests. Likewise, the negative ones will have negative results in the confirmatory test. On the other hand, if two simultaneous tests are used, a net sensitivity is gained, while a net specificity is reduced. This is different when tests are done independently. Negative is considered people whose negative results were in all tests and positive the ones whose positive results were in at least one of the tests [3, 4].

2.2. Biological samples for dengue studies

The type of sample taken in the right moment, storage and transport to the laboratory to be processed, and the appropriate documentation plays a key role to obtain results because if there is a change of sample quality, this can reduce antibody titers, viruses or genetic material resulting in lower titers or concentrations from the real ones in quantitative tests or false negative results in quantitative or qualitative tests.

The most used samples to diagnose and to search about dengue are whole blood, serum, plasma, and human organs like spleen, liver, and heads of mosquitoes, pools of mosquitoes, brains of mice, serum samples saturated with filter paper, etc. The serum samples that will be processed for virus isolation and/or polymerase chain reaction tests and fluorescent focus assay to quantify the virus in serum are collected in tubes without anticoagulants. They must be taken within 1–5 days after the onset of symptoms, to detect the antigen (NS1 protein), within 1–6 days to detect IgM antibodies for enzyme-linked immunosorbent assay (ELISA) or a rapid test after day 5 of starting the symptoms, within 1–5 to detect IgG using matched serums to assess seroconversion for ELISA, neutralization test or hemagglutination inhibition used in acute serums, and convalescent serums after day 15. In the case of liver, spleen, kidney or nodes samples, a sample is immediately taken after the person's death or the animal in experiment. The samples must be taken to the laboratory as soon as possible and preferably dealt with in dry ice or liquid nitrogen [5, 6].

2.3. Primary and secondary dengue infections and dengue diagnostic

Although most infections are asymptomatic or subclinical, a set of symptoms starts after a dengue infection elapses the 4–10-day incubation period. A four-fold increase of the IgG antibody titers in matched serums measured by ELISA IgG test or hemagglutination inhibition indicates recent flavivirus infection. When people are infected with the virus on the first time, dengue infections are known as a primary infection in which a viral load and the relevant antibody formation (IgM, IgG and IgA) are triggered. In a primary infection, the titer of IgM is generally much higher and more specific than in a secondary infection. Some studies consider that an infection is primary if the IgM/IgG relation is higher than 1.2 with diluted samples at 1:100 or 1.4 using diluted serum at 1:20. When people are previously exposed to any serotype or flavivirus, or even after a vaccine (i.e., yellow fever vaccine), dengue infections are secondary and the IgM/IgG relation is lower than 1.2 or 1.4. In secondary infections, the IgG is detected in the highest levels and even on the acute phase. It remains higher for 10 months and even lifelong in order to consider a person being infected with dengue virus (DENV), the following laboratory test interpretations need to be followed:

- When a sample taken from the acute phase is positive for dengue due to the PCR test, viral isolation, and IgM serocoversion in matched serum samples, the IgG seroconversion in matched serum samples or the fourfold increase of IgG titer is considered confirmed cases.
- When a positive IgM occurs in a single serum sample or a positive IgG in a single sample with hemagglutination inhibition titer is the same or higher than 1:1280, it is considered a suggestive case [5–8].

2.4. Laboratory test

The laboratory tests can be interchangeably used in different researches, both basic and applied ones. We can classify them into direct methods that allow virus detection or part of its structure and indirect methods which identify a reaction produced by the presence of DENV in the organism.

2.4.1. Direct detection methods

2.4.1.1. Viral RNA extraction

The genetic material extraction has a key role for PCR tests so that the quality of a product extracted can vary depending on the type of sample being used, and the extraction method applied will directly affect the test sensitivity. The dengue RNA can be recovered from serum, blood, urine, plasma samples and other organs. However, the viral load in blood is much higher (7.9×10^2–1.9×10^5 PFU/mL) in comparison with saliva and urine samples (1×10^1–5×10^1 PFU/mL). The RNA extraction can be done by guanidine thiocyanate and trizol methods and by the use of commercial kits like Qiagen kit (QIAamp® RNA Viral mini kit), etc. RNA extraction techniques in serum/plasma samples from patients using QIAamp® UltraSens Virus Kit (Qiagen Inc., Valencia, USA) were compared to the modified Chomczynski-Sacchi extraction

technique in order to extract plasma RNA so that the original technique is used for cell or tissue samples being more cost effective than the kit commercial one. It was found that 34 samples out of 47 were positive by using the Chomczynski-Sacchi method, and the remaining 27 samples were positive by using the kit commercial method [8–10].

2.4.1.2. Reverse transcription polymerase chain reaction (RT-PCR)

The RT-PCR techniques and their different variants are converted into one of the main tools for diagnosing DENV and other arbovirosis. Less time to process the results, being able to identify the circulating serotypes of the virus, presenting the highest sensitivity and specificity levels are among its advantages. This type of test has the benefit of obtaining rapid results while identifying circulating serotypes of dengue. The RT-PCR technique is the extraction of a RNA sample followed by a reverse transcription process, the actual PCR (nested or not), and the last screening in gel obtaining a qualitative result. The technique developed by Lanciotti and his collaborators or the variant developed by Harris and his collaborators, which have been used for many years, is recommended by the Pan American Health Organization (PAHO/OPS). The technique developed by Lanciotti starts with converting RNA into DNA by using a reverse transcriptase enzyme. Then, the PCR is carried out when primers are used to amplify prM genes and C virus areas to continue with a specific primer-nested PCR for each virus serotype. Harris and his collaborators developed a multiplex RT-PCR from Lanciotti and his collaborators' method that uses five pairs of primers (four specific pairs for serotypes and one pair of the region of the capsid gene). There are many different variants in this technique having different sensitivity levels that are used in research laboratories. One of the main disadvantages is the possible existence of a false positive due to the contamination produced by amplicons during the reverse transcription of the genetic material, and it is necessary to consider that a negative result in these tests does not rule out DENV infection or other arboviruses, and the analysis must be complemented by serological findings (**Figure 1**) [9, 11].

2.4.1.3. RT-PCR multiplex to DENV and different microorganism identifications

Chikungunya (CHIKV), Zika (ZIKV), Yellow Fever (YFV) and DENV are arboviruses with the highest prevalence in the American continent. They are transmitted by the same vector *Aedes aegypti y Aedes albopictus* facilitating its cocirculation in some areas of the region. Because of these arboviruses, the affected patients develop similar symptoms but its clinical management and its possible results as the aftermath of the disease, and mortality rates are different. They can even produce coinfection with other microorganisms making a proper identification necessary in an early stage of the disease (acute phase). There are commercial and standardized testing in a laboratory allowing qualitative identification of DENV, ZIKV, and CHIKV in serum, plasma, and even some urine samples [12]. Other commercial testing like FilmArray Global fever panel has the capacity of detecting genetic material in viruses, bacteria and protozoa (nine viruses like YFV, DENV, ZIKV, WNV, CHIKV, among others, six bacteria and four protozoa) in whole blood (EDTA), with automated equipment. A study to determine a detection limit for microorganisms using FilmArray Global fever panel found the following results for DENV like DENV-1 (Hawaii) 2.7×10^1, DENV-2 (New Guinea C)

Figure 1. Reverse transcriptase semi-nested reactions, using primers targeted to the C/PrM genomic region as described by Lanciotti et al. (a photo taken on April 24, 2018 in the molecular biology laboratory with the authorization of the U.S. Naval Medical Research Unit Six (NAMRU-6).

3.6×10^1, DENV-3 (H87) 1.6×10^3 and DENV-4 (H241) 7.6×10^1. The main advantage of this test is time reduction to obtain results (about an hour), discriminating the amount of pathogens, and minimizing cross-contamination problems so that all reactions are carried out among a closed system. The cost of the product and a machine analyzing only a sample at a time is within its limiting [13].

2.4.1.4. Real-time PCR

It uses conventional RT-PCR principles, and it combines with fluorochromes like SYBR Green or TaqMan probes with fluorochromes capable of producing proportional fluorescence to the DNA copy samples. The strengths of this test are the same as the conventional RT-PCR, it also reduces time when releasing the results as well as the cross-contamination risk post PCR, the levels of sensitivity and specificity are higher than the conventional RT-PCR, and overall it allows quantifying the genetic material. There are different commercial kits in the market to diagnose DENV, and its sensitivity and specificity levels vary when they are compared among them [14, 15]. The CDC elaborated a real time RT-PCR in order to diagnose four serotypes in serum or human plasma samples using an ABI 7500 FAST DX thermo-cycler of Applied Biosystems and hydrolysis dual-marker TaqMan probes, and it is the first RT-PCR approved by Food and Drug Administration (FDA) to detect DENV [16].

2.4.1.5. Viral isolation in cell lines

Viral isolation in cell cultures or mosquitoes followed by the virus detection using indirect immunofluorescence is considered as gold standard [8, 17]. In order to carry out the DENV viral isolation and as a general rule to any virus, it is necessary to consider the following:

- To know the isolated virus (virus characteristics, replication, transmission mechanism, etc.)
- To know which biosafety level a virus can be performed. In the case of DENV, biosafety level 2 is needed [18].
- To determine which cell line to use and to be able to isolate the virus, it is essential to identify the most sensitive cell line from mosquitoes and mammals, and its use for the isolation and DENV propagation, being the most sensitive cell lines of mosquitoes as follows:
    - C6/36: they are easy cell propagation, highly sensitive to DENV infection, and cultivated at 28°C that are obtained from salivary glands of *Aedes albopictus*.
    - C6/36 HT (hot temperature): they can be spread at 34°C and have a bigger sensitivity to detect DENV. This cell line maintains its high sensitivity only for some weeks due to its higher temperatures, so some researchers suggest adapting at 34°C, a C6/36 cell line growing at 28°C, and using an alternative method.
    - TRAS-284-SF cell has the main benefit of not requiring the use of fetal bovine serum (FBS) as a culture medium, and it presents a higher sensitivity to the DENV isolation [6, 9, 17].
- Knowing the viral isolation technique that provides better results to isolation and virus propagation. The standard method is based on the virus propagation in a sensitive cell line for inoculating a previous diluted sample in a cell culture medium. After the infection process, the cultures are placed on incubation for the binding of the virus to the cell; subsequentially, it is placed on a means of maintenance with the essential nutrients to maintain the live cultures for a period of time that can be 13–15 days. Then, the infected cells are recovered, and the virus presence is determined by an immunofluorescence process, ELISA, molecular techniques, and others. Bottles, tubes, 6–96 well culture plates, and others are used in order to sustain the binding to cell cultures. A modified shell vial technique allows the recovery of a higher number of YFV, SLV, WNV, ILHV, GCV, OROV, MAYV and DENV isolations. This technique follows the same steps as a standard method, but after inoculating cells, the cultures are centrifuged to velocities between 1800 and 2200 rpm. This technique can also be used to isolate DENV coinfections. However, it seems not to have good results for VEE isolation [19–22].
- The sample type to be used. The sample type to be used and its proper preservation until the processing time are extremely important to isolate a virus. The most used dilutions to a viral isolation vary from 1:5 to 1:20. A very concentrated dilution of the sample could generate a toxic effect in the cells. On the other hand, a much diluted sample could cause the inability of isolating the virus because of having a low concentration virus in the inoculum [19, 21].

Figure 2. Plaque assay titration for DENV-2 using VERO-76 cells and a semi-solid method (a photo taken on March 2017, in the Virology and Molecular Biology Laboratory of the Faculty of Biological Sciences at National University of San Marcos, Peru).

2.4.1.6. Plaque assay

It is the most used test to determine viral vaccine titers so that it quantifies the virus to infect cells. It is based on the infection of a cell monolayer with different virus dilutions to evaluate. After an incubation period, the viral infection results in lytic plaques. If they are colored, they are displayed as holes in the cell monolayer. Each plaque corresponds with an infectious virus. One of the main disadvantages of this technique is that it can only use viruses being able to produce a cytopathic effect. Another one is that all native strains of DENV are not always capable of producing well-defined plaques, and the viral titers can vary depending on the cell line used. For a dengue virus case, the most used cell lines are VERO and BHK-21 (**Figure 2**) [5, 6, 23].

2.4.1.7. Fluorescent focus assay

It is a combination of plaque assay and immunofluorescence. Viruses are inoculated in different dilutions in the cell line, then a cell incubation period is fixed to plaques with any organic solvent, and an immunofluorescence is carried out. Positive cells are observed with fluorescent foci that can be counted. One of its advantages is to reduce the incubation period

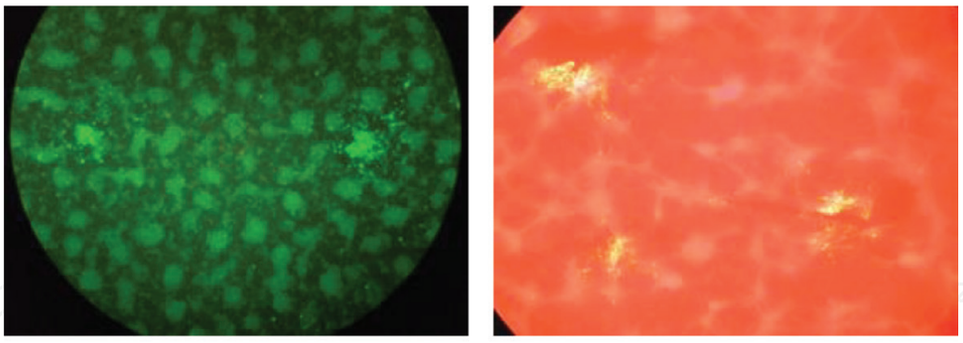

Figure 3. Fluorescent focus assay for DENV-2 using the C6/36 (A) and VERO-76 cells (B). The indirect immunofluorescence performed to visualize the foci was carried out on the fifth day of incubation, using the fluorescein isothiocyanate (FITC) conjugate (a photo taken on March 15, 2016 in the Serology laboratory with the authorization of the U.S. Naval Medical Research Unit Six (NAMRU-6)).

in order to obtain the results in comparison with plaque assay. It allows processing a bigger number of samples so that it can be adapted to use 96-well culture plates in comparison with the 24-well plates which are used in the plaque assay. Another advantage is to allow the use of C6/36 cells that are highly sensitive to detect dengue virus, and they cannot be used for the plaque assay as they do not form lytic plaques (**Figure 3**) [23].

2.4.1.8. Viral isolation in nursing mice

Suckling mice were greatly used because of their easy reproduction and handling to isolate virus as well as the antigen production. About 1–3 neonatal mice and an intracranial inoculation are carried out. Then, a 21-day daily checking to observe the occurrence of neuromotor symptoms is needed. This technique is starting to cease to use due to a great variety of cell cultures that allow good sensitivity levels in DENV detection. This is why the Institutional Animal Care Committee and IACUC Committee recommend reducing this activity. One of the most common practices to carry out euthanasia on suckling mice is using a CO_2 camera. When using this technique, it is necessary to make sure such mice are dead as they are very resilient to lacking of oxygen so it is advisable to continue with other euthanasia techniques like cervical dislocation, decapitation, etc. [6, 9, 24].

2.4.1.9. Inoculation in mosquitoes

The mosquitoes like *Aedes* genus can be used for dengue virus isolation when infection and disease transmission studies are carried out. The intracerebral and intrathoracic inoculations are used for mosquitoes which are immobilized at low temperatures. The mosquito infection technique is to feed them directly with the dengue-infected patient blood in the acute phase of the disease. The mosquitoes of the *Toxorhynchites* genus, which are not blood-feeding insects, can be used for the four-serotype dengue isolation, Japanese encephalitis and encephalitis of San Luis are more susceptible than cell culture isolation of dengue virus as well as the *Drosophila melanogaster* that can be inoculated by micro injection in the abdomen, and it could reach higher titers using less time in comparison with the *Aedes aegypti* inoculation [6, 25, 26].

2.4.1.10. Automated equipment for virus counting

There are redesigned flow cytometers to quantify a virus in solutions like The ViroCyt® Virus Counter (VC) 2100 (ViroCyt, Boulder, CO, USA) using a specific fluorescent dye for the envelope proteins and the other one for the nucleic acids that allows recognizing viral particles having both components in its structure. Then, it eliminates anything that represents one type of fluorescent [27].

2.4.1.11. Enzyme-linked immunosorbent assay (ELISA) for NS1

The NS1 nonstructural protein is produced during dengue infection and accumulated in higher concentrations in the human serum (up to 50 μg/ml) being detected during the acute phase (day 0–6) after the symptoms in primary and secondary infection start. Some studies have reported a correlation between elevated NS1 protein levels with hemorrhagic dengue cases, and even this technique seems to be effective to detect DENV in the vector. When evaluating three of these commercial tests from different manufacturers with human serum samples, it is found sensitivity between 85.5 and 95.9% and specificity between 95.0 and 100% using the viral isolation as a reference test [28].

2.4.2. Indirect methods

2.4.2.1. IgM ELISA

IgM can be detected on 50% infected people within 3–5 days and after the symptoms onset, and it reaches approximately to 80% infected people on day 5 and to 99% infected people on day 10 reaching maximum levels in humans at the 2 weeks to falter until they are not detected on 2–3 months. An indirect capture ELISA is usually used for detecting IgM, and it allows increasing sensitivity of the test in detecting antibodies. However, IgM antibodies are not specific, and they could present a cross-reaction with other flavivirus like YFV, ZIKV, etc. Besides, some particular test characteristics could alter the result of the test as the rheumatoid factor depending on IgM ELISA type causes false positives [5, 7–9].

2.4.2.2. IgG ELISA

The IgG is detected with low titers when ending the first week of the onset symptoms in humans, and they could even be detected for a lifelong. The tests to detect an IgG using the virus bind to a plate in a smooth antigen way (protein cocktail) usually present a low specificity so that there is a cross-reaction with other viruses from the same genus due to the proteins found in the antigen, and this test cannot be used to determine the infectious dengue serotype but it can present a higher sensitivity than the hemagglutination inhibition test. The ELISA is also used for studying how different IgG sub-classes react in a dengue infection [5, 7–9].

2.4.2.3. IgG inhibition ELISA tests

It can be used to differentiate a primary infection and a secondary infection from dengue replacing an hemagglutination inhibition test using a percentage of inhibition higher or the

same as 50% as a positivity criterion. In case of having only a serum sample, a primary infection is considered when the antibody IgG titer is ≤20, and a secondary infection is considered if the antibody titer is ≥1280. In both cases, the sample must be collected on 5–7 days. If there are paired samples, a primary infection is considered when the seroconversion in antibody titers from the acute and convalescent phase occurs, and a secondary infection is considered when the antibody titers increase four times or more between the acute and convalescent phase, or in both serums [6].

2.4.2.4. IgA ELISA test

It was used in some studies to identify the infected people in an early stage of dengue infection. Thus, it was found 100% of sensitivity in people with secondary dengue infection after symptom onset-day 1. However, the results were not very favorable for primary infections [29].

2.4.3. Neutralization test

2.4.3.1. Plaque reduction neutralization test (PRNT)

This test is considered the gold standard to detect neutralizing IgG antibodies because they have high sensitivity but can have a cross-reaction among members of flavivirus group. It is based on the binding of antibodies present in the sample which contains a known virus load (working dilution). The mixture of both is incubated and inoculated in a cell line until forming lytic plaques that are observed when coloring the cell monolayer. All samples neutralizing and avoiding the forming of certain number of plaques (being the most commonly used for 50–90% reduction) are with the presence of neutralizing antibodies indicating exposure to dengue. Using 1 in 30 diluted serum samples allowed discriminating between DENV-1 and DENV-2 serotypes in collected serum in Cuba, before and after the 1981 epidemic caused by DENV-2. There are many different variants of a PRNT test that could produce a variation in the results when being compared with using different used reduction rates, different PRNT methods (solid or semi-solid), and different cell lines. The most used for dengue are VERO cells that are recommended by World Health Organization (WHO), and BHK-21 that are used by other laboratories like Pedro Kouri Institute of Cuba. The use of different genotypes can alter the antibody titer results. Kochel et al. [30] evaluated Peruvian samples infected with DENV-2 American genotype. Antibody titers were found in higher levels when using the same genotype rather than using the Asian genotype. PRNT can be used to differentiate dengue infection to yellow fever infection. It has been found that the lowest serum dilution capable of distinguishing between both infections is 1 in 5. In a dengue secondary infection, PRNT can have a cross-reaction with other serotypes. In infected populations with sequentially different serotypes of dengue, the highest antibody titer pertains to the first infection (the "original antigenic" sin phenomenon) (**Figure 4**) [5–7, 30–32].

2.4.3.2. Focus reduction neutralization test (FRNT)

It combines the PRNT test with immunofluorescence or ELISA to count fluorescent foci or spots and calculate their reduction rate in the samples. Among its advantages, this allows reducing the days to obtain the results and undertaking studies with native strains so that

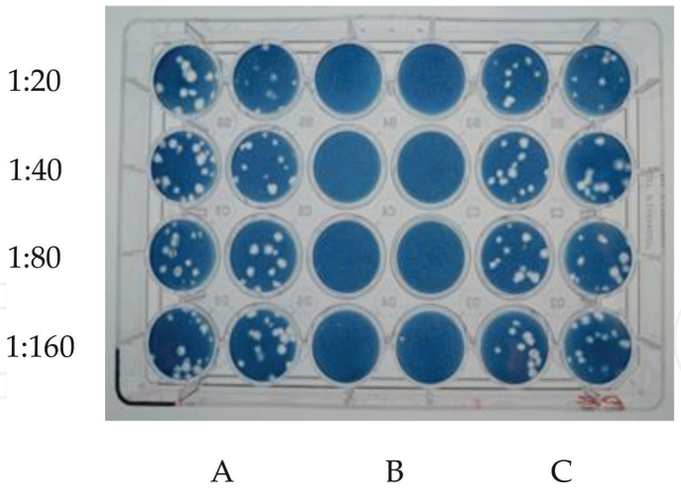

Figure 4. Plaque reduction neutralization test for DENV-2 using BHK-21 clone 15 cells. Dilutions from 1:20 to 1: 160 were used. Sample A and C do not present antibodies against the DENV2, and sample B is positive up to a dilution greater than 1: 160 (a photo taken on March 23, 2012 in the PRNT laboratory with the authorization of the U.S. Naval Medical Research Unit Six (NAMRU-6)).

some of them produce tenuous plates that are difficult to count but they make easier to detect fluorescent foci. Moreover, more sensitive lines can be used to detect dengue, not forming C6/36 plates, and they can be adapted to plates with a higher number of wells enabling to process a higher number of samples and saving materials. And, fluorescent foci can be counted with an ultraviolet light microscope or using computerized readers capable of reading fluorescent foci and reducing time to obtain results [33, 34].

2.4.3.3. Microneutralization-ELISA

It is a variable in the PRNT test and uses the same immunological basis with the advantage of being worked on 96-well plates different from the PRNT that generally uses 24-well plates. This allows saving materials and handling a higher number of samples. This technique is used to detect the presence of neutralizing antibodies in the sample which is mixing the patient's sample that is previously inactivated in different dilutions with the dengue virus serotype to be evaluated, once the antigen-antibody binding is inoculated in the specified cell line as VERO-76 cells. If there were neutralizing antibodies in the sample, these ones would block the entry from the virus to the cell and would not produce the infection. The presence of the virus in the cell is revealed by an ELISA procedure. Samples exposing the color change in a substrate will be considered as negatives to an IgG antibody against the dengue serotype under study, and the samples that do not produce color change in the substrate will be positive for the presence of IgG antibodies (**Figure 5**). When this technique was evaluated with serum samples from patients with primary infection, the results were very similar to the ones in PRNT. However, the result correlation was very poor in comparison with this technique using samples from patients with secondary infections [35].

2.4.3.4. Immunofluorescence test

This test can be direct or indirect, and it is the most commonly used to identify the infected cells deriving from cell lines, salivary glands of mosquitoes, etc. It is based on the binding of

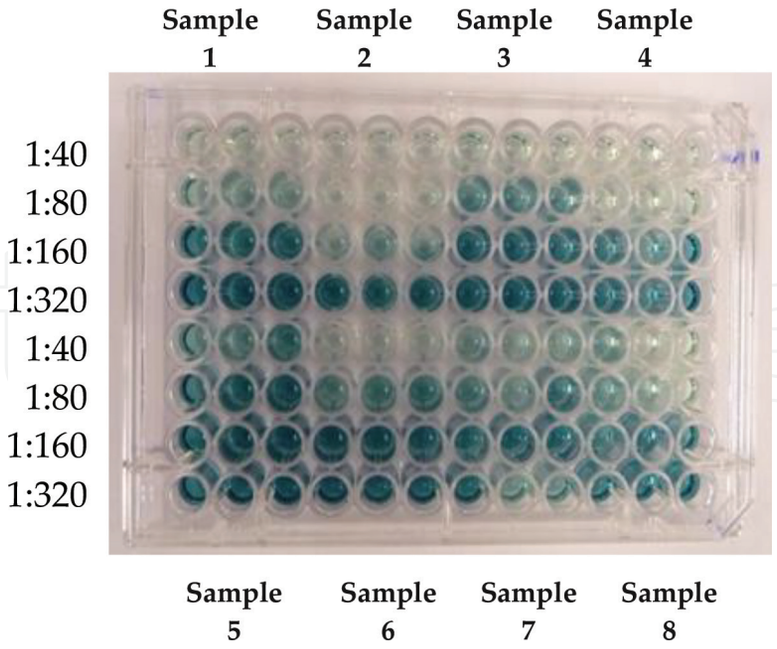

Figure 5. Enzyme-linked immunosorbent assay-format microneutralization test for DENV-2. The samples were inoculated in triplicate in VERO-76 cells using dilutions from 1:40 to 1: 320, and the ELISA test was performed on day 5 post inoculation (a photo taken on April 24, 2018 in the serology laboratory with the authorization of the U.S. Naval Medical Research Unit Six (NAMRU-6)).

the actual virus to a sample that can be infected cells with a dengue virus antibody joined to a fluorescent marker named conjugated (direct immunofluorescence). One of the most used fluorochromes for this technique is the fluorescein isothiocyanate (FITC). First, a specific antibody is to bind to a specific virus in the indirect immunofluorescence (the used antibody can be monoclonal or polyclonal, and it is bind to a conjugated). Observing the samples under ultraviolet light of the microscope, the fluorescent cells indicate the presence of the virus in the cell. This test is quite cost effective, and its sensibility and specificity can vary depending on the antibody quality used for virus identification. The polyclone antibodies are produced by sensitizing mice with the specific virus to detect. If an inactivated and structurally complete virus is used, the mice will produce antibodies against virus proteins resulting in a high cross-reaction within the virus of the same genome as it is the case of DENV, YFV, ZIKV, SLV, WNV, etc. Monoclonal antibodies produced in hybridomas can eliminate or reduce cross-reactions (cell lines are able to produce antibodies against one or various virus epitopes) with bigger specificity. There are antibodies produced in hybridomas capable of identifying and differentiating flavivirus group, a general dengue virus (dengue complex), and to each dengue virus serotype (**Figure 6**) [5, 6].

2.4.3.5. Rapid tests

There are rapid tests based on lateral flow chromatographic immune assays produced by different laboratories. Proteins such as NS1, IgM, IgG in serum, blood or plasma samples can be detected through these tests, and they can simultaneously identify antibodies or proteins produced by ZIKV, DENV, and CHIKV. Assessing four rapid tests for NS1, its sensibility was in 71.9–79.1% range and its specificity in 95–100% antibodies compared to the viral isolation. These tests have the benefit of being cost effective, not requiring qualified personnel to be

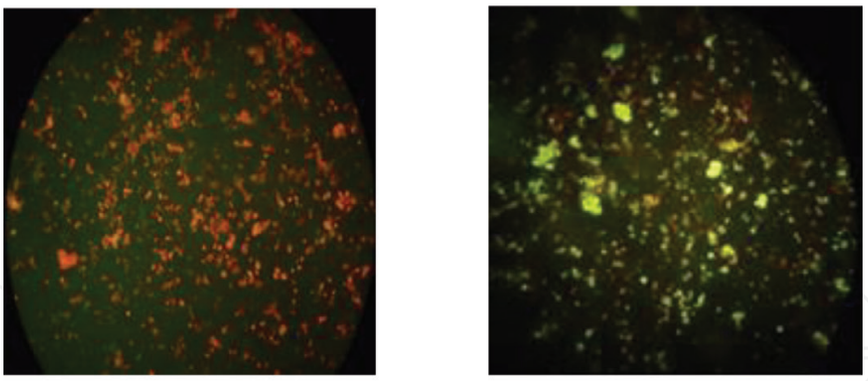

Figure 6. Indirect immunofluorescence test performed on C6/36 cells. In (A), uninfected red cells are observed and in (B), infected green cells are observed (a photo taken on April 24, 2018 in the serology laboratory with the authorization of the U.S. Naval Medical Research Unit Six authorization (NAMRU-6)).

done nor using sophisticated equipment. Within its limitations, it is not possible to identify the circulating serotype in a virus, the band intensity is not related to the antibody titers nor the actual NS1 antigen in the sample, cross-reactions with other flavivirus are common, a negative result in these tests does not exclude that the patient has been exposed to the virus, and some samples present high titers of rheumatoid factor affecting the results. The positive samples for these results should be verified with other alternative methods [28].

2.5. The animal models for dengue virus

Studies to evaluate antivirals against dengue, vaccines, plant extracts, etc. mainly use the mouse model and nonhuman primates (NHPs). However, each of them presents a series of constraints that impair research developments. The infections of the mouse model are used for pre-clinical development of vaccines. In this model, a neurotropic disease can occur; however, this does not usually happen in humans. Some mice like C57BL/6, BALB/c and A/J enable viral replication, but A/J and BALB/c mice develop paralysis. Some studies show that C57BL/6 mice can have hemorrhages when being infected with a DENV2 strain 16681 using a virus with a titer of 3×10^9 PFU/mL. Moreover, an infection and endothelial cell damage as well as hemorrhages in tissues can occur without showing signs of disease. The DENV replication in mice is low, but suckling mice inoculated by an intracranial via at high DENV doses can induce neurological illnesses and paralysis that is used to measure the effectiveness of the vaccines. They can also be used to test a virus neurovirulence or an attenuation to produce vaccines. NHPs are used to research an immune reaction against DENV, an evaluation of candidates' vaccines, a replication kinetic, etc. This model has a major constraint because it does not produce signs of clinical disease. Nonetheless, DENV can infect some cells in the body. An inoculation with 10^5 PFU DENV concentrations enables a lower viral replication in humans, and it is restricted to lymphoid-rich tissues producing lymphadenopathy, lymphocytosis, and leukopenia. Rhesus macaques can produce antibodies against DENV and a similar viral load to the humans. The inoculation of high titer DENV via intravenous in rhesus macaques can produce hemorrhage. The physiology of pigs is similar to the human one. Yucatan miniature swine models present

an immunological and physiological result similar to the human ones. It is known that it is susceptible to a flavivirus such as the Japanese encephalitis, YFV, and Murray valley encephalitis. Studies about infected porcine models administered subcutaneously with DENV-1 (10^7 PFU) resulting to developed viremia, and IgM and IgG antibodies without symptoms are carried out. The secondary infection with the same serotype produced extensive macular and papular rashes similar to the ones affecting humans. When infecting the swine model with a DENV-1 intravenous line, a rash skin and dermal edemas appeared on the animal [36, 37].

2.6. The future of laboratory tests and its connection with dengue studies

Throughout a dengue research, the use of laboratory tests play a fundamental role in identifying the virus (serotype, genotype and lineage), its genetic material, viral proteins, the presence of antibodies against the virus or assessing the patient's condition, and for instance, hematocrit, platelet count, white blood cell count, blood count, etc. together with patient's signs and symptoms and some epidemiological criteria, it allows categorizing the disease for dengue with or without warning signs or severe dengue. This classification assists in deciding what therapy to choose and preventing from a severe dengue development. Besides the above mentioned techniques in this chapter, there are laboratory techniques which are not commonly used at present because they are expensive and/or they require sophisticated equipment but they could be used for DENV studies. Among them, there is the transmission electron microscopy (TEM) which is a gold standard technique for the absolute quantification of particles. However, it does not allow differentiating an infectious virus from a non-infectious one. The high performance liquid chromatography (HPLC) where the virus load is quantified through the UV analysis of fractions produced during HPLC. Flow cytometry (FC) can be used to quantify viral proteins being present on the surface of the infected cells. The next-generation whole genome sequencing is the most advanced sequencing technology which allows learning the complete and detailed genome sequence of an organism in a short period of time (days), though its cost is still quite higher to some researches [27, 38].

Dengue researches in the future will try to respond to different problems, needs, and gaps in knowledge like:

The production and improvement of the vaccine against DENV like Dengvaxia® (CYD-TDV), produced by Sanofi Pasteur, is a prophylactic, tetravalent, live attenuated recombinant viral vaccine which uses a 3-dose vaccination schedule, and it is recommended for people ranged from 9 to 45, or 9 to 60 year old (depending on the license) [39]. Nonetheless, it is necessary to continue with searches to develop a vaccine which can be used from the first year of life enabling to achieve the maximum immunity with a single dose and can be used in different endemic areas obtaining the same immunity level regardless the disease prevalence.

Today, it is acknowledged that the immunity against dengue after a natural infection can be for a lifetime [5]; however, some studies indicate that a dengue reinfection may occur under certain conditions [40].

The relation between chronic diseases and dengue has been studied as risk factors in order to determine the severity of dengue disease in individuals. In recent decades, the increase of

chronic diseases and being one of the main causes of death in today's world require to continue and deepen the study of these diseases as well as its association with the dengue disease [5].

In endemic areas, dengue studies in blood banking are essential especially in epidemic outbreaks so that when there is a large amount of asymptomatic DENV, the virus may be transmitted through blood transfusions [41].

Individuals with inapparent dengue virus infection are considered dead-end hosts for transmission because they do not present high enough levels of viremia to infect mosquitoes; nonetheless, some studies show that asymptomatic people for dengue can transmit the virus to mosquitoes when bitten, although having a lower average of viremia, increasing the risk of disease spread in different areas [42]. Therefore, it is important to carry out studies with individuals presenting inapparent dengue virus infections aimed at breaking the transmission cycle and avoiding disease spread.

Wildlife mammals with genetic material and antibody against DENV like bats have been found, but their participation in the transmission cycle have not been approved yet [43].

Because of not having found an animal model able to replicate the dengue disease, it is necessary to continue searching for different animal models which can support the study of this disease so that the use of humanized mice becomes an attractive area of research [36, 37].

The development of new drugs and the vegetal compound assessment with antiviral activity against DENV are needed because there is no specific treatment for this disease yet.

The use of mathematical models to predict the pace of dengue spread based on clinical data and laboratory results may support the prognosis of this disease.

DENV has a type of RNA genome, with a dependent RNA polymerase without a corrective activity being able to accumulate genetic material changes leading to a false positive increase in PCR tests [11]. Thus, it is required to evaluate the necessity of primer redesigning which allows detecting a larger amount of DENV circulating strains worldwide as well as its standardization and assessment.

DENV coinfections along with other microorganisms circulating in the same endemic areas like DENV/*plasmodium* Sp. may be frequent depending on the disease prevalence in the area, especially if they are transmitted by the same vector in cases like DENV, CHIKV and ZIKV. Some studies have shown infections of *Aedes aegypti* with MAYV causing a concern about a possible natural transmission of such disease in urban areas [22, 44].

3. Conclusion(s)

Dengue is still a leading public health problem. The diagnostic tests must improve and be standardized in all research and diagnostic laboratories with the use of technology. This will allow having comparable results in different studies and reference centers that contribute to the knowledge development needed to understand transmission mechanisms and DENV propagation and setting preventive and appropriate control measures as well as developing new vaccines and antivirals helping to control this disease.

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Conflict of interest

The authors declare no conflict of interest.

Disclaimer

The views expressed in this laboratory test used in the diagnostic and research of dengue virus: present and future are those of the author and do not necessarily reflect the official policy or position of the Department of the Navy, Department of Defense, nor the U.S. Government.

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References

[1] World Health Organization. Dengue and Severe Dengue [Internet]. 2018. Available from: http://www.who.int/news-room/fact-sheets/detail/dengue-and-severe-dengue [Accessed: 17-05-2018]

[2] Forshey BM, Guevara C, Laguna-Torres VA, Cespedes M, Vargas J, et al. Arboviral etiologies of acute febrile illnesses in Western South America, 2000-2007. PLoS Neglected Tropical Diseases. 2010;**4**(8):e787. DOI: 10.1371/journal.pntd.0000787

[3] Gordis L. Epidemiology. 5th ed. Elsevier Saunders: Barcelona; 2014. p. 391

[4] Donis JH. Evaluación de la validez y confiabilidad de una prueba diagnóstica. Avances en Biomedicina. 2012;**1**(2):73-81. Available from: http://www.redalyc.org/articulo.oa?id=331328015005

[5] WHO. Dengue guias para el diagnóstico, tratamiento, prevención y control [Internet]. Ginebra: World Health Organization; 2009 [cited 17 May 2018]. Available from: http://www.who.int/iris/handle/10665/44504

[6] Instituto de Medicina Tropical Pedro Kourí. Técnicas de laboratorio para el diagnóstico y la caracterización del virus dengue. La Habana: Instituto de Medicina Tropical Pedro Kourí; 2013. p. 128

[7] Peeling RW, Artsob H, Pelegrino JL, Buchy P, Cardosa MJ, Devi S, et al. Evaluation of diagnostic tests: Dengue. Nature Reviews Microbiology. 2010;**8**:30-38. DOI: 10.1038/nrmicro2459

[8] Tang KF, Ooi EE. Diagnosis of dengue: An update. Expert Review of Anti-Infective Therapy. 2012;**10**(8):895-907. DOI: 10.1586/eri.12.76

[9] Gutiérrez L, Diana Q, Martínez M. Actualización en diagnóstico del dengue: evolución de las técnicas y su aplicación real en la clínica. Medicina & Laboratorio. 2012;**18**(9-10):411-441

[10] Dettogni R, Louro I. Dengue virus RNA purification from human plasma: A comparison of two techniques. Molecular Biology Reports. 2010;**38**(8):4979-4983

[11] Usme J, Gómez A, Gallego J. Detección molecular y tipificación del virus dengue por RT-PCR y PCR anidada usando oligonucleótidos mejorados. Salud Uninorte Barranquilla (Col). 2012;**28**(1):1-15

[12] Vargas D. Dengue, Chikungunya y Zika: diagnóstico molecular mediante RT-PCR múltiple. infoLABIN. 2017;**44**:1-2

[13] Helm J, Toxopeus C, Batty N, Border L, Davidson O, Kelley A, et al. Analytical Performance of the FilmArray® Global fever panel. Presented at the American Society of Tropical Medicine and Hygiene (ASTMH) Annual Meeting; November 2017. Poster 1445

[14] Saengsawang J, Nathalang O, Kamonsil M, Watanaveeradej V. Comparison of two commercial real-time PCR assays for detection of dengue virus in patient serum samples. Journal of Clinical Microbiology. 2014;**52**(10):3781-3783

[15] Waggoner JJ, Abeynayake J, Sahoo MK, Gresh L, Tellez Y, Gonzalez K, et al. Comparison of the FDA-approved CDC DENV-1-4 real-time reverse transcription-PCR with a laboratory-developed assay for dengue virus detection and serotyping. Journal of Clinical Microbiology. 2013;**51**(10):3418-3420

[16] Centers for Disease Control and Prevention. Ensayo de RT-PCR en tiempo real para DENV-1-4 de los CDC. Puerto Rico: Centers for Disease Control and Prevention; 2013. p. 56

[17] Special Programme For Research and Training in Tropical Diseases. Dengue Diagnostic: Proceedings of an International Workshop. ENICEF/UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR); 4-6 October 2004. Geneva: WHO/TDR; 2005. (TDR/IRM/DIAG/DEN/05.1)

[18] Chosewood LC, Wilson DE. Biosafety in Microbiological and Biomedical Laboratories. U.S. Department of Health and Human Services. 5th ed. HHS Publication; 2009. p. 415

[19] Caceda ER, Kochel TJ. Application of modified shell vial culture procedure for arbovirus detection. PLoS One. 2007;**2**:e1034

[20] Rodríguez R, Álvarez M, Guzmán MG, Morier L, Kourí G. Comparison of rapid centrifugation assay with conventional tissue culture method for isolation of dengue 2 virus in C6/36-HT cells. Journal of Clinical Microbiology. 2000;**38**:3508-3510

[21] Palomino M, Gutierrez V, Salas R. Estandarización del método de centrifugación en placa para el aislamiento del virus Dengue. Revista Peruana de Medicina Experimental y Salud Pública. 2010;**27**(1):51-58

[22] Sulca J, Guevara C, Halsey E, Ampuero J. Aislamiento de los serotipos 1 y 3 del virus dengue por shell vial modificado en un paciente coinfectado. Revista Peruana de Medicina Experimental y Salud Pública. 2013;**30**(2):354-355

[23] Payne AF, Binduga-Gajewska I, Kauffman EB, Kramer LD. Quantitation of flaviviruses by fluorescent focus assay. Journal of Virological Methods. 2006;**134**:183-189

[24] Institute for Laboratory Animal Research NRC. Guide for the Care and Use of Laboratory Animals. 8th ed. Washington, D.C.: National Academies Press; 2011. p. 220

[25] Padmanabhan R, Vasudevan SG, editors. Dengue: Methods and Protocols. Vol. 1138. New York USA: Humana Press; 2014. p. 403

[26] Paingankar MS, Gokhale MD, Deobagkar DN. Drosophila Melanogaster Supports Multiplication of Dengue-2 Virus. Presented at VII- Sir Dorabji Tata Symposium Arthropod Borne Viral Infections; March 2007

[27] Rossi CA et al. Evaluation of viroCyt(R) virus counter for rapid filovirus quantitation. Viruses. 2015;**7**(3):857-872. DOI: 10.3390/v7030857

[28] Pal S, Dauner AL, Mitra I, Forshey BM, Garcia P, Morrison AC, et al. Evaluation of dengue NS1 antigen rapid tests and ELISA kits using clinical samples. PLoS One. 2014;**9**(11):e113411. DOI: 10.1371/ journal.pone.0113411

[29] Yap G, Sil BK, Ng L-C. Use of saliva for early dengue diagnosis. Lopes da Fonseca BA, editor. PLoS Neglected Tropical Diseases. 2011;**5**:e1046

[30] Kochel TJ, Watts DM, Halstead SB, Hayes CG, Espinoza A, et al. Effect of dengue-1 antibodies on American dengue-2 viral infection and dengue hemorrhagic fever. Lancet. 2002;**360**(9329):310-312

[31] Rainwater-Lovett K, Rodriguez-Barraquer I, Cummings DA, Lessler J. Variation in dengue virus plaque reduction neutralization testing: Systematic review and pooled analysis. BMC Infectious Diseases. 2012;**12**:233

[32] Roehrig JT, Hombach J, Barrett AD. Guidelines for plaque-reduction neutralization testing of human antibodies to dengue viruses. Viral Immunology. 2008;**21**:123-132

[33] Ngwe Tun MM, Muta Y, Inoue S, Morita K. Persistence of neutralizing antibody against dengue virus 2 after 70 years from infection in Nagasaki. BioResources. 2016;**5**:188-191

[34] Fournier C, Duverlie G, Francois C, Schnuriger A, Dedeurwaerder S, Brochot E, Capron D, Wychowski C, Thibault V, Castelain S. A focus reduction neutralization

assay for hepatitis C virus neutralizing antibodies. Virology Journal. 2007;**4**:35. DOI: 10.1186/1743-422X-4-35

[35] Vorndam V, Beltran M. Enzyme-linked immunosorbent assay-format microneutralization test for dengue viruses. The American Journal of Tropical Medicine and Hygiene. 2002;**66**:208-212

[36] Zompi S, Harris E. Animal models of dengue virus infection. Viruses. 2012;**4**:62-82

[37] Na W, Yeom M, Choi I, Yook H, Song D. Animal models for dengue vaccine development and testing. Clinical and Experimental Vaccine Research. 2017;**6**(2):104

[38] White Paper Provided by Virocyt: An Overview of Virus Quantification Techniques [Internet]. Vaccine Nation. 2018 [cited 19 June 2018]. Available from: http://www.vaccinenation.org/2013/05/07/white-paper-virocyt-overview-virus-quantification-techniques/

[39] World Health Organization. Dengue vaccine: WHO position paper—July 2016. Weekly Epidemiological Record. 2016;**91**:349-364. Available from: http://www.who.int/wer/2016/wer9130.pdf?ua=1

[40] Forshey BM, Reiner RC, Olkowski S, et al. Incomplete protection against dengue virus type 2 re-infection in Peru. PLoS Neglected Tropical Diseases. 2016;**10**(2):e0004398. DOI: 10.1371/journal.pntd.0004398

[41] Ashshi AM. The prevalence of dengue virus serotypes in asymptomatic blood donors reveals the emergence of serotype 4 in Saudi Arabia. Virology Journal. 2017;**14**:107

[42] Duong V, Lambrechts L, Paul R, Ly S, Lay R, Long K, et al. Asymptomatic humans transmit dengue virus to mosquitoes. Proceedings of the National Academy of Sciences. 2015;**112**(47):14688-14693

[43] Vicente-Santos A, Moreira-Soto A, Soto-Garita C, Chaverri L, Chaves A, Drexler J, et al. Neotropical bats that co-habit with humans function as dead-end hosts for dengue virus. PLoS Neglected Tropical Diseases. 2017;**11**(5):e0005537

[44] Long K, Tesh R, Higgs S, Hausser N, Thangamani S, Kochel T, et al. Experimental transmission of mayaro virus by Aedes aegypti. The American Journal of Tropical Medicine and Hygiene. 2011;**85**(4):750-757