

Evaluation of an Automated Executed Version of an ELISA for the Serological Diagnosis of Dengue Infections

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ABSTRACT

The incidence of dengue is increasing in many countries and at the present time, around half of the world's population is at risk of infection. Dengue is an arthropod-borne virus (arbovirus) that continues to pose an important challenge to public health authorities worldwide. A critical task in managing arboviral outbreaks and epidemics is the selection of laboratory technology for timely and accurate diagnosis. In this study, we assessed the performance of the DYNEX[®] DS2 Automated Processing System for the diagnosis of dengue vis a vis the manually executed procedure. The levels of agreement were calculated and showed 94%, 96%, and 90% positive percent agreement, negative percent agreement, and overall percent agreement, respectively. In conclusion, both tests displayed comparable performances.

Keywords

Dengue, Diagnosis, ELISA.

Abbreviations

DENV: Dengue viruses, WHO: World Health Organization, DHF: Dengue hemorrhagic fever, DSS: Dengue shock syndrome, FDA: Food and Drug Administration, ELISA: Enzyme-linked immunosorbent assay, CDC: Centers for Disease Control and Prevention, PDSS: Passive dengue surveillance system, DENRA: Virus-derived recombinant antigens, NCA: Normal cell antigen, PBS: Phosphate buffered saline, HRP: Horseradish peroxidase, TMB: Tetramethylbenzidine, OD: Optical densities, ISR: Immune status ration, CI: Confidence intervals, DPO: Days post onset, NP: Not performed.

Introduction

Puerto Rico has faced dengue, chikungunya and Zika epidemics in the past decade. These arthropod-borne viruses (arboviruses) are

transmitted mainly by the bite of an infected female *Aedes* mosquito (*Ae. aegypti* or *Ae. albopictus*) [1], which is a hematophagous arthropod highly prevalent in both urban and rural areas of the Island [2]. Among these three vector-borne diseases, dengue has the greatest significance for humans [3].

Dengue viruses (DENV 1-4) are rapidly spreading to new areas around the world resulting in public health concerns and costly disruptions affecting healthcare services. According to the World Health Organization (WHO), dengue fever ranks as the most important arthropod-borne viral disease in the world. The disease is endemic in more than 100 countries, with approximately 3.9 billion people at risk. There are estimates of 390 million annual infections, including 96 million clinical cases and 40,000 deaths every year [4].

Dengue is a febrile disease with a broad clinical spectrum. It might range from asymptomatic to a mild resolving form called dengue

fever or “break bone fever” with rash, intense headache, pain in the eyes, joints, and muscles, nausea, and vomiting [1]. It might progress to a more severe, life-threatening manifestation known as dengue hemorrhagic fever (DHF), especially in individuals who are infected for a second time [4].

DHF is an acute, potentially lethal complication, whose symptoms might come after the fever vanishes and it may be characterized by abrupt changes in temperature (from fever to hypothermia), persistent abdominal pain and vomiting, breathing difficulty and fatigue and spontaneous bleeding of the gums, nose, or blood in stool or vomits [5,6]. In sporadic occasions, the disease progresses to leakage of the blood vessels, dengue shock syndrome (DSS), and death [7].

Although there is not a specific antiviral drug to treat dengue and the focus depend on treating the symptoms, supportive care of patients can be improved with timely interventions performing early differential diagnosis from other infectious diseases and rapid testing during the acute phase of infection. In the past years, the Food and Drug Administration (FDA) cleared the DENV-1-4 Real-Time RT-PCR assay for the detection of viral RNA in human serum or plasma (CDC Dengue Branch, San Juan PR) [8] and the InBios DENV Detect IgM capture enzyme-linked immunosorbent assay (ELISA) for the qualitative detection of IgM antibodies in serum (InBios International, Inc., Seattle, WA) [9]. This ELISA test is a sensitive, specific, and a reliable manual procedure [10,11], however, as of today, it has not been validated in an automated processing system.

Automated clinical laboratory testing with high sensitivities and specificities can facilitate the differential diagnosis of dengue in endemic areas, such as Puerto Rico, where periodically epidemics (dengue, chikungunya and Zika) with elevated number of cases are experienced. In addition, these diagnostic tests can be valuable tools for surveillance activities, epidemiological responses, and clinical investigations. In this study, we conducted an evaluation of a commercially available manual ELISA test using computerized processing instrument.

The study

This study provides an independent assessment of the performance of the InBios DENV IgM Capture ELISA when implemented using a DYNEX® DS2® Automated Processing System to determine precision and benefits of using DS2 technology, in comparison with the manually executed diagnostic procedure. Prior to conducting the study, approval from the Institutional Review Board of the University of Puerto Rico, Medical Sciences Campus, was obtained.

In Puerto Rico, the Centers for Disease Control and Prevention (CDC) Dengue Branch in San Juan, Puerto Rico and the Puerto Rico Department of Health used to maintain a passive dengue surveillance system (PDSS) through weekly reporting (nowadays known as Arboviral Diseases Weekly Report). A well-characterized

reference panel of serum samples (n=80) was created by the CDC Dengue Branch. The panel of samples was tested through the manual procedure of the InBios DENV IgM Capture ELISA (49 positives, 27 negatives and 4 equivocal results). The samples were numerically coded for blind assay and then transferred to the Biological and Chemical Emergencies Laboratory of the Puerto Rico Department of Health. Serum samples were stored at -80°C until assayed using the DYNEX® DS2® Automated ELISA Processing System.

Briefly, the samples were run in duplicate, one for the virus-derived recombinant antigens (DENRA) and the other for the normal cell antigen (NCA). Both samples were diluted 1:100 using the DENV sample dilution buffer (Tris-HCl with Tween 20, pH 7.2-7.6). Then, 50 µL of the diluted samples were transferred to the microtiter wells pre-coated with anti-human IgM antibodies and incubated for 1 hour at 37 °C. The wells were washed 6 times with 300 µL of phosphate buffered saline (PBS) with Tween 20 (pH 6.8-7.0), and then 50 µL of the DENRA and the NCA were added to the corresponding wells, followed by an incubation for one hour at 37 °C. After an additional washing, 50 µL of DEN-specific monoclonal antibody labeled with horseradish peroxidase (HRP) was added, followed by another incubation for one hour at 37 °C. Another set of washings preceded the addition of 150 µL of EnWash solution (PBS with Tween 20, pH 7.2-7.6), followed by five minutes incubation at room temperature. After a last set of washings, the wells were incubated with 75 µL of tetramethylbenzidine substrate (TMB) at room temperature for ten minutes. Finally, 50 µL of the stopping solution (1 N Sulfuric Acid) were added, followed by incubation at room temperature for one minute. Afterwards, the wells’ optical densities (OD) were read at 450 nm. The ratio between the DENRA OD and the NCA OD determined the immune status ration (ISR) or the presence of antibodies in the samples. Samples with ISR values ≥ 2.84 were considered positive, samples with $ISR \leq 1.65$ were considered negative and samples with ISR values between 1.65 and 2.84 were considered equivocal. Samples that tested equivocal were repeated in duplicate, as per the InBios established protocol.

For the statistical analysis, the qualitative results obtained from the DYNEX® DS2® Automated ELISA Processing System were compared to the results of the manual method. Positive percent agreement, negative percent agreement, 95% confidence intervals (95% CI) and coefficient of determination (R^2 , $p < 0.0001$) were estimated [12]. All data analyses were executed using Minitab® 16 software and MedCalc software version 13.2.2 (www.medcalc.org/) [13].

Results and Discussion

Results obtained after performing the automated procedure were 46 positives (57.5%), 33 negatives (41.25%) and 1 equivocal (1.25%). Six of the 80 samples (7.5%) yielded equivocal results initially, however five of them (6.25%) turned into positives after repetition and one (1.25%) remained equivocal. These results were in accordance with those obtained through the manual procedure

of the InBios DENV IgM Capture ELISA assay (49 positives, 27 negatives and four equivocal results). A 94% Positive Percent Agreement and 96% Negative Percent Agreement were observed, as compared to the analysis completed manually. An overall 90% Agreement was observed between InBios automated method and InBios manual method (Table 1).

Table 1: Comparison of results between the automated and manual methods for the InBios DENV Detect IgM Capture ELISA.

| Automated InBios DS2 | Manual InBios | | | Total |
|----------------------------|---------------|----------|-----------|-------|
| | Positive | Negative | Equivocal | |
| Positive | 46 | 0 | 0 | 46 |
| Negative | 3 | 26 | 4 | 33 |
| Equivocal | 0 | 1 | 0 | 1 |
| Total | 49 | 27 | 4 | 80 |
| Positive percent agreement | 46/49 | | | 94% |
| Negative percent agreement | 26/27 | | | 96% |
| Overall percent agreement | 72/80 | | | 90% |

Eight (10%) of the 80 samples exhibited discordant results between methods (Table 2). Three of the 49 samples that tested positive on the manual method (samples 3, 57, 59), tested negative on the automated method. Similarly, one out of the 27 negative samples on the manual method (sample 42) tested equivocal on the automated method and the four equivocal samples on the manual method (samples 1, 6, 7, 8) tested negative on the automated method. In overall, a significant correlation in the performance between the manual InBios method and the automated method was observed (Figure 1A). Linear regression analysis revealed a correlation coefficient (R^2) of 0.7959 ($P < 0.0001$).

Table 2: Samples with discordant results between the automated and manual methods for the InBios DENV Detect IgM Capture ELISA.

| Sample code | DPO* | Manual | | | Qualitative result | Automated DS2 | | | | Qualitative result |
|-------------|------|------------|-----------|---------------|--------------------|---------------|-------------|-------------|---------------|--------------------|
| | | Run 1 ISR† | Run 2 ISR | Run 2 ISR AVR | | Run 1 ISR | Run 2 ISR 1 | Run 2 ISR 2 | Run 2 ISR AVR | |
| 42 | 5 | 1.41 | NP | Negative | 1.85 | 2.34 | 2.52 | 2.43 | Equivocal | |
| 1 | 10 | 1.81 | 1.65 | Equivocal | 1.45 | NP‡ | NP | NP | Negative | |
| 6 | 10 | 1.79 | 2.31 | Equivocal | 1.11 | NP | NP | NP | Negative | |
| 7 | 16 | 1.83 | 2.60 | Equivocal | 1.08 | NP | NP | NP | Negative | |
| 8 | 19 | 1.80 | 1.80 | Equivocal | 1.29 | NP | NP | NP | Negative | |
| 3 | 6 | 2.23 | 2.98 | Positive | 1.62 | NP | NP | NP | Negative | |
| 57 | 6 | 1.81 | 4.68 | Positive | 1.34 | NP | NP | NP | Negative | |
| 59 | 5 | 2.84 | 3.85 | Positive | 1.62 | NP | NP | NP | Negative | |

*DPO, Days post onset, refers to how many days elapsed between the appearance of symptoms and the collection of samples. †ISR, Immune Status Ratio. Samples with $ISR < 1.65$ were considered negative, ISR between 1.65 - 2.84 equivocal; $ISR > 2.84$ positive. Samples tested by the manual method with equivocal results were repeated singly and the ISR from the second run determined the qualitative result. Samples tested by the automated method with equivocal results were repeated in duplicate and the ISR average from the second run determined the qualitative result. ‡NP, not performed.

Although the manufacturer recommends repeating equivocal

results in duplicate, the 15 samples that tested equivocal originally on the manual method were retested singly and yielded nine positive results, two negative results and four remained equivocal. Seven of the eight samples with discordant results in our study belong to this group of samples. This might represent a limitation since there was a slight difference in execution procedures. When results were reevaluated, without taking into consideration these 15 samples, overall agreement increased from 90% to 98%; positive percent agreement increased from 94% to 100%, and the correlation coefficient (R^2) increased (Figure 1B) to 0.98 ($P < 0.0001$).

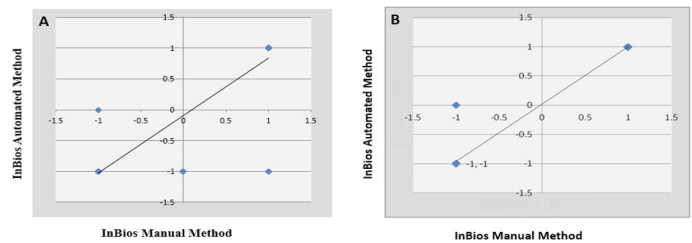


Figure 1: Linear regression analysis.

Linear regression analysis for the comparison of qualitative results between the InBios IgM capture ELISA manual method and the automated method. A) $n = 80$, $R^2 = 0.7959$ ($P < 0.0001$). B) $n = 55$, $R^2 = 0.98$ ($P < 0.0001$).

Other studies were conducted previously to evaluate commercially available diagnostic tests. Hunsperger et al. [14] performed an evaluation of nine commercially available anti-dengue IgM tests, including five ELISA kits. She reported sensitivities ranging from 21-99%, whereas specificities varied from 77-98%. Only three of the evaluated ELISA kits (Panbio, Focus, and Standard Diagnostics) showed strong agreements with the reference standards used by CDC. Blacksell et al. [15] evaluated seven commercial dengue diagnostic assays, including two IgM capture ELISA (Panbio and Standard Diagnostics). The sensitivities of the tests ranged from 85-89% and the specificities varied from 88-100%. Hunsperger et al. [16] performed another evaluation of 12 commercially available diagnostic tests from seven different companies, including one IgM anti DENV ELISA. The test displayed sensitivity and specificity of 96-98% and 78-91%, respectively.

The InBios DENV Detect IgM capture ELISA was previously evaluated using manual procedures. Namekar et al. [10] reported 92% sensitivity and 94% specificity and concluded that the test was rapid and reliable for the diagnosis of acute dengue infection. Welch et al. [11] also performed another evaluation of the test. This study revealed high sensitivity (88.7%) and specificity (93.1%), comparable to other commercially available and similar assays. The results presented in the current study revealed agreement between the InBios automated method and InBios manual method and therefore, the automated method can also be effectively used to diagnose dengue infection.

Delays in diagnosis can lead to inadequate treatment and affect the recovery rate of patients and at the same time, increase the

risk of infection for healthcare workers. These are some of the challenges we are still facing regarding dengue control, treatment, and prevention. Currently, there is not specific treatment and there is only one licensed vaccine (DENGVAIXIA) available. Despite its approval, the vaccine is limited to children and adolescents living in endemic areas. Moreover, a previous dengue infection evidenced by laboratory-confirmed results through serological or molecular testing is required to be eligible for the vaccine. On the other hand, the diagnosis of acute dengue infection guided by clinical signs and symptoms can be challenging due to the similarity with other infectious diseases. Therefore, in an environment with limited resources, it is imperative to develop new affordable tools for clinical laboratories in poor countries or otherwise, improve the tools available to reduce misdiagnosis of dengue, increase early detection, assess surveillance actions, and conduct better clinical care.

In conclusion, our study showed that the InBios DENV IgM Capture ELISA performance, when executed through the DYNEX® DS2 Automated ELISA Processing System, is comparable to the performance of the test when manually executed. The automated version of an ELISA System for the detection of IgM Dengue virus antibodies was validated and implemented in the Biological and Chemical Emergencies Laboratory. Although the DS2 is expensive and requires extensive training, it offers high throughput for larger trials, simplifies workflow, minimizes the interaction of the medical technologist with the samples, and reduces staff workload while maintaining qualitative detection of IgM antibodies. Therefore, the automated platform can facilitate the differential diagnosis of dengue, from other febrile diseases like chikungunya and Zika, thereby increasing the output of results in public health laboratories during outbreaks and epidemics. The implementation of this diagnostic platform can greatly improve the diagnosis of dengue and accelerate the availability of results, which might be an important factor for case management, surveillance, and control of the disease in the local population and visitor travelers.

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