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Serological Diagnostic Assays for Detection of Ns1 Antigen, IGM and IGG Antbodies to Dengue Virus

Purimitla Usha Rani, Payala Vijayalakshmi*

Department of Microbiology, GITAM Institute of Medical Sciences and Research, GITAM University, Visakhapatnam, Andhra Pradesh, India

Email address:

bavisettyvijayalakshmi2@gmail.com (P. Vijayalakshmi)

*Corresponding author

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Abstract: Complications in managing the dengue virus infections include the lack of rapid diagnostic procedures and at the same time the symptoms of dengue infection are often confused with those of other diseases. Two commercial rapid serological diagnostic kit methods (Dengue Day 1 test, J Mitra and Co. Pvt. Ltd., New Delhi, ImmunoComb II Dengue IgM/IgGBispot kit (Orgenics Pvt. Ltd., Israel)were evaluated for the detection of NS1 antigen, Immunoglobulin IgG and IgM specific to dengue virus in the serum samples of patients suffered with dengue acute primary infection and secondary infection. The total assay time was 20 min-2hrs. The results of these methods were compared with the gold standard assay methods Dengue IgM-Capture Microplate ELISA and Dengue Indirect IgG ELISA (Pan Bio, Brisbane, Australia). The total assay time was 6-7hrs. Nine serum samples were positive to NS1 antigen and negative to IgG and IgM by Dengue day 1 test. The results of Bispot assay method revealed that, the number of IgG positive samples was 11, IgM positive samples were 31 and both IgG and IgM positive samples were 8. Majority of the positive cases were noticed in the age group 35-68 years and males were more prone to dengue infection while comparing with females. By performing IgM MICROLISA, 34 samples were positive which in turn indicated that, three of them were false negative by the immune comb bispot method giving a sensitivity of 91.17%. Through indirect IgG ELISA, the number of positive samples was 15 and four of the 15 positive samples of IgG were false negative by the immuo comb bispot method giving a sensitivity of 73.33%. The gold standard ELISA methods were more efficient than rapid serological tests and gave an overall sensitivity of 99%. Thus the alternative of an assay that is to be used in the diagnosis of dengue infections depends on factors like laboratory infrastructure, preference and availability of equipment. The allied performance of the Rapid test, followed by confirmation with MAC-ELISA on those samples, ensures both speediness as well as quality of reported results.

Keywords: Dengue, NS1 Antigen, IgM, IgG, Primary Dengue, Secondary Dengue, ELISA

1. Introduction

Dengue is caused by four serologically related flaviviruses called dengue-1, dengue-2, dengue-3 and dengue-4 [1]. Infection to one serotype confers immunity only to that particular infecting serotype [2]. Subsequent infection with one of the three remaining serotypes results in immune-enhanced disease in the form of severe hemorrhagic fever or dengue shock syndrome. Of the more than 100 million cases of dengue fever, 250, 000 cases result in dengue hemorrhagic fever, resulting in approximately 25, 000 deaths annually [3]. These viruses found in most tropical parts of the world [4]. Dengue normally affects adults and older children. Dengue presents as an acute febrile illness with chills, head-ache, retro-ocular pain, body aches and anthralgia in more than 90% of apparent cases, with nausea or vomiting and a maculopapular rash resembling measles lasting for 2-7 days in about 60% of cases [5]. Illness persists for 7 days, fever remitting after 3-5 days followed by relapse (Saddleback fever) and pains in the body muscles and joints sufficiently severe to earn the breakbone fever. When the fever falls, some patients present bleeding manifestations, thrombocytopenia and hemoconcentration [6]. Hepatomegaly can also be observed. Rash occurs more

commonly in patients aged less than 14 years. The incubation period is 5-11 days. Patients usually recover after fluid and electrolyte therapy [7]. Shock is followed by death in 5-10% cases if rehydration is insufficient or delayed plasma leakage is the main characterization of DHF/DSS [8] [9]. The pathogenesis of DHF/DSS is not very well understood nor do the host conditions that favor the severity of disease; however children, females, individuals with chronic diseases such as asthma and diabetes are appear to be at high risk. Recently it was proved that, the risk of DHF/DSS is higher if the interval is longer between primary and secondary infection [10]. The febrile clinical symptoms associated with dengue are similar to those of other arboviruses from other families; this has resulted in confusion in the diagnosis of dengue [1]. In particular the high incidence of dengue has resulted in the misdiagnosis of some arbovirus. Serological studies are needed to differentiate these. DENV are thought to have originated in Asia as sylvatic cycle involving arboreal mosquitoes and monkeys. However, endemic viruses have evolved and are now maintained in nature by a cycle involving man as both reservoir and amplification host, and domestic mosquitoes, principally *Aedes aegypti* as vectors [11]. It is the most prevalent arbovirus in the world; more than 100 million people are infected annually. It is the leading cause of illness and death in the tropics and subtropics. Humans are the main reservoir for this virus, and person-to-person transmission occurs through a mosquito vector [12]. The use of good dengue diagnostic tools is critical for laboratory confirmation of DHF/DSS, including the number of case fatalities, determining which strains are involved, and to derive estimates of total incidence following epidemics. IgM antibody is the first immunoglobulin isotype identified in the serum of dengue patients [13]. Anti-dengue IgG appears in a low titer at the end of the first week of disease onset, and increases slowly. By contrast, during a secondary infection, antibody titers rise extremely, rapidly and antibody reacts broadly with many flaviviruses. High levels of IgG are detectable even in the acute phase and they rise dramatically over the following two weeks [14]. However by day 5 of illness, 80% of cases have detectable IgM antibody, and by day 6 to 10, 93-99% of cases have detectable IgM that may persists for over 90 days. Laboratory diagnosis is based on the presence of virus-specific IgM antibody, a fourfold rise in specific IgG antibody, or a positive RT-PCR amplification for dengue genomic sequences [15]. Though IgM capture ELISA, virus isolation in mosquito cell lines and live mosquitoes, dengue specific monoclonal antibodies and PCR have all represented major advances in dengue diagnosis [16]. However, an appropriate rapid, early and accessible diagnostic method useful both for epidemiological surveillance and clinical diagnosis is still needed [17]. Also, tools that suggest a prognosis allowing for better management are also needed. In terms of morbidity and mortality, Dengue hemorrhagic fever and Dengue shock syndrome are one of the important arthropod-borne viral diseases. The emergence and reemergence of dengue can be attributed to a number of underlying causes. These include demographic and societal changes such as population growth and unplanned urbanization; this can result in large crowded human populations living in urban centers with substandard housing and inadequate water, sewage and waste management systems [18]. When these factors are combined with increased movement of individuals from endemic areas, the deterioration of effective mosquito control measures and the limited financial and human resources dedicated to the public health infrastructure, dengue can gain a foothold within the population. Anti-dengue IgM detection using Enzyme linked immuno sorbent assay (ELISA) represents one of the most important advances and has become an invaluable tool for routine dengue diagnosis. Specifically IgM antibody capture ELISA (MAC-ELISA) diagnosis is based on detecting dengue specific IgM antibodies in the test serum by capturing them using anti-human IgM antibody previously bound on a solid phase [19]. In areas where dengue is not endemic, IgM based immunoassays can be used in clinical surveillance for viral illness or for random population based serological surveys, with the likelihood that any positive results detected indicate recent infections. In general 10% false negative and 1.7% false positive reactions have been observed. ELISA for anti-dengue IgG detection is currently widely used for classifying cases based on the kind of infection, primary or secondary. MAC-ELISA provides more information, is more efficient than other serological tests and is especially valuable for laboratories that perform a high volume of testing [20]. An early diagnosis helps in prompt patient management and immediate implementation of appropriate vector control measures which in turn helps to prevent the spread of infection. Additionally, diagnostics provides the key data on the epidemiology and health burden of dengue, which is very useful for accurate public health surveillance. Commercial serological assays are commonly used in diagnostic laboratories for dengue confirmation. Serological assays are comparatively simple to perform and the specimens required for the assay, such as serum or plasma, are stable in the tropical climate.

2. Materials and Methods

Blood samples were collected from the suspected patients of dengue infection in sterile dry vials. Serum was separated from the blood by centrifugation at room temperature. The laboratory criteria for dengue infection was characterized by thrombocytopenia (< 1 lakh/mm3), hematocrit increased by > 20%. Fever, hemorrhagic manifestations, thrombocytopenia with rising hematocrit was sufficient for clinical diagnosis of dengue hemorrhagic fever (DHF). Pleural effusion and hypo albuminaemia are supportive evidences.

Dengue Day 1 test: The test was performed according to manufacturer instructions (J Mitra and Co. Pvt. Ltd., New Delhi, India) and it is a rapid solid phase immune-chromatographic test for the qualitative detection of Dengue

NS1 antigen and differential detection of IgM and IgG antibodies to dengue virus in human serum or plasma. This test is used for an early diagnosis of dengue infection and presumptive diagnosis between primary and secondary dengue infection. However this test is not used as the sole criteria for detection of dengue infection. Initially two drops of nearly 70µl serum sample was added to sample well of antigen device in Dengue day 1 test card and left for 20 min. After 20 min coloured lines on control and test lines were identified. If the sample contains NS1 antigen it will bind to the anti-dengue NS1 gold colloid conjugate and forms antigen-antibody complex. This complex migrates along the membrane to the test region and forms the visible pink line at T as antibody-antigen-antibody gold conjugate complex. For the detection of IgM and IgG, specimen at a concentration of 10µl was added to the sample well S of antibody device and left for 20 min. If three red coloured lines were identified on the C, M, G lines indicates secondary dengue infection. If two lines on C and M indicates primary dengue infection and on C, G indicates secondary dengue infection.

ImmunoComb II Dengue IgM/IgG Bispot kit (Orgenics Pvt. Ltd., Israel): This test is intended for the qualitative simultaneous detection of IgM and IgG antibodies to dengue virus in human serum or plasma and for the differentiation between primary and secondary infection. It is a solid-phase enzyme immuno assay (EIA), based on an immune capture principle. The solid phase is a card with 12 teeth. Each teeth is sensitized at three positions upper spot (Biotinylated BSA as internal control), middle spot (rabbit antibodies to human IgG), lower spot (rabbit antibodies to human IgM). The kit contains 3 developing plates covered with aluminium foil. Each developing plate contains all reagents needed for the test. The developing plate consists of 6 rows (A-F) of 12 wells each. Row A contains specimen diluent, Row B dengue antigen, Row C biotinylated MAb to dengue proteins, Row D streptavidin conjugated to alkaline phosphatase, Row E washing solution, Row F chromogenic substrate solution containing BCLP and NBT chemicals. The kit is also provided with positive control, negative control, comb scale and perforator. The developing plate was incubated at 37°C for 30 min. The initial step of the procedure was antibody capture performed by adding specimens and controls to row A at a concentration of 10µl. Now card was inserted into the wells of Row A, mixed and incubated for 10 min. The antibody capture step was followed by antigen-antibody reaction. In this Row B was perforated and at the same time card was removed from the Row A. The adhering liquid was absorbed from teeth on a clean absorbent paper. The Card was inserted in Row B, mixed and incubated for 40 min. After 40 minutes the adhering liquid was absorbed from teeth. The third step of the procedure was binding of biotinylated anti-dengue to Row C which was perforated and inserted with card from Row B. Later it was mixed, incubated at room temperature for 40 min and adhering liquid was absorbed. The same procedure was done with Row D which includes the binding of modified avidin/alkaline phosphatase, Row E includes washing and Row F involves chromogen addition but for all these steps the incubation time was 10 min. After 10 min, the card was withdrawn from Row F and again inserted into Row E called stop reaction. Later than 1min, the card was removed from Row E and allowed to air dry. The positive control always produces 3 spots on the card teeth. The negative control produces an upper spot. The presence of specific anti-dengue IgM antibodies in each specimen may be assessed by comparing the color intensity of the lower spot on each tooth to that of lower spot of the positive control tooth whereas the presence of anti-dengue IgG in the serum sample can be estimated by comparing the colour intensity of the middle spot on each tooth with the colour scale on the comb scale.

Dengue IgM-Capture Microplate Enzyme-Linked Immunosorbent assay or Dengue IgM MICROLISA (Pan Bio, Brisbane, Australia): It is an Invitro qualitative detection of dengue IgM antibodies in plasma or serum sample and is used as a screening test for testing of collected blood samples. Dengue IgM microlisa test is an enzyme immunoassay based on MAC capture ELISA. This test is used for all the four subtypes of dengue virus. The serum samples to be tested were diluted with sample diluent 1:100 in separate tubes. Initially the strip holder was fitted with required number of anti-human IgM coated strips. Now 100µl negative control was added to A well, 100µl calibrator in B, C and D wells, 100µl positive control in E well. Then 100µl of each sample diluted in sample diluent was added in each well starting from F well. Incubated at 37°C for 1hr. Working washing solution and working conjugate solution were prepared. After 1hr of incubation the plate was removed and the wells were washed for five times with washing solution. 100µl of working conjugate solution was added in each well and incubated at 37°C for 1hr. Again after washing for five times 100µl of working substrate solution was added to each well and incubated at 30°C for 30 min in dark. Later 50µl of stop solution was added to each well. Optical density values for the samples were read at 450nm in ELISA reader. The cut off value and sample O. D ratio were determined for each sample. Cut off value can be calculated by multiplying mean O. D of calibrator and calibrator factor. The sample O. D ratio can be calculated by dividing the sample O. D with cut off value.

Dengue IgG Indirect ELISA Rapid (Pan Bio, Brisbane, Australia): Dengue specific IgG based assays can be used for the detection of past dengue infections and current infections if paired sera are collected within the correct time frame to allow the demonstration of seroconversion between acute and convalescent serum samples. Assays are usually carried out using multiple dilutions of each serum tested to determine an end point time. The serum samples, calibrators and controls were initially diluted at a ratio of 1:100 twice starting with 10µl samples. Then 100µl of diluted serum samples were added to the wells and incubated at 37°C for 30 min. After washing for 6 times with wash solution 100µl of HRP conjugate-antihuman IgG was added to each well and left for 30 min at 37°C. Again after washing 100µl of TMB was added to each well and incubated for 10 min at room

temperature. Finally 100µl of stop solution was added all wells and read the O. D at 450 nm in ELISA reader.

3. Results and Discussion

Of the 100 serum a sample tested by the rapid serological kit methods and assays nearly nine serum samples were positive to NS1 antigen and negative to IgG and IgM by Dengue day 1 test. When these nine serum samples were again tested with Immuno comb IgM/IgGBispot kit method, the samples showed negative results for both IgG and IgM indicates the acute primary dengue infection. Among these nine samples two samples were reported from pediatric department of age group 2-3 years and the remaining samples were reported were from age group 20-39 years. The results were interpreted by observing the pink colour lines on test and control samples of dengue day 1 test card. The remaining 91 serum samples were screened for IgM, IgG or both by using immune comb II IgM/IgG Bispot kit method. The results showed that, the number of IgG positive samples was 11, IgM positive samples were 31 and both IgG and IgMpositive samples were 8. The rate of IgM detection was 34.06% and IgG was 12.08%. Total of 50 serum samples (54.94%) were positive for dengue acute primary infection and dengue secondary infection and 41 serum samples were reported be negative with Bispot kit method. The results were interpreted by observing the colour intensities of the lower spot and middle spot of the tooth with positive control sample and with combscale calibrator. In acute primary dengue infection cases where the serum sample was positive for IgM, the colour intensity of the lower spot showed >C+ and <CO+ on comb scale. In the dengue secondary infection cases where the serum sample was positive for IgG, the colour intensity of the middle spot was higher than C+ on comb scale. The serum samples which were positive for both IgM and IgG, the colour intensities of both lower and middle spots of tooth were higher than positive control and C+ on comb scale. Majority of the positive cases were noticed in the age group 35-68 years and males were more prone to dengue infection while comparing with females (Figure 1). The positive samples for IgM and IgG were retested and compared with gold standard ELISA techniques.

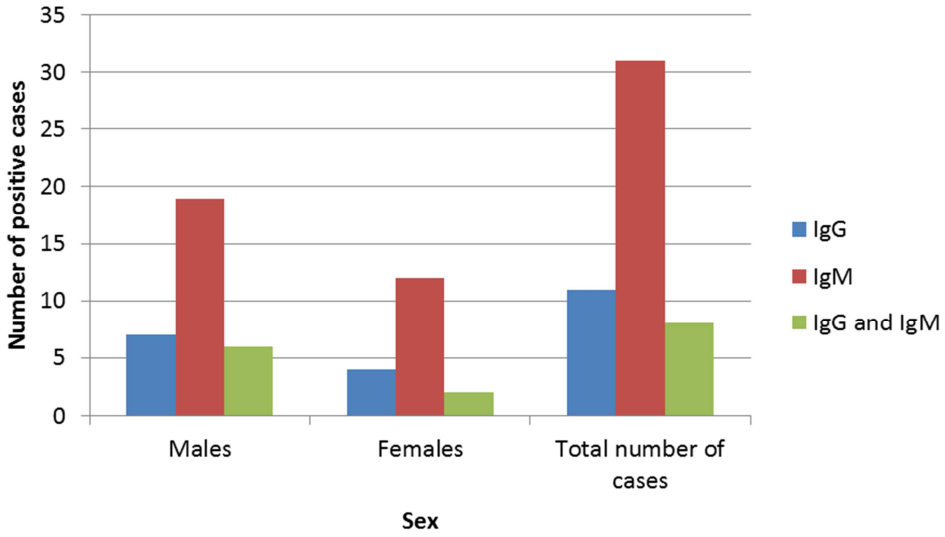

Figure 1. *Gender wise distribution of Dengue positive cases with Immuno comb Bispot method.*

By performing IgM MICROLISA, 34 samples were positive which in turn indicated that, three of them were false negative by the immune comb bispot method giving a sensitivity of 91.17%. The rate of IgM detection was 37.3%. The dengue IgM unit varies from 14 to 20 units for the 34 serum samples indicate positive for Dengue IgM antibodies (Table 1). Through indirect IgG ELISA, the number of positive samples was 15 and four of the 15 positive samples of IgG were false negative by the immuo comb bispot method giving a sensitivity of 73.33%. The rate of IgG detection was 16.4%. The IgG standard units vary from 15-49 units. The gold standard ELISA methods were more efficient than rapid serological tests and gave an overall sensitivity of 99.9%. Revitz et al. [21] also reported the overall sensitivity of ELISA was 99% for IgM and IgG detection. The previous studies of Wu et al. [22] showed that, the rate of IgM positive cases were 57.3%, Vazquez et al. [23] reported 85% of positive IgM cases which were comparatively higher than the present study 37.3% whereas Lam et al. [24] reported higher rate of IgG positive cases 78.7% and the present study showed only 16.4% positive cases. The result of the current study was similar to the results of Sathish et al. [25] reported 38.9% positive cases of IgM. The choice of an assay for the detection of dengue infections that is to be used in any laboratory is dependent on several factors including the facilities available and the anticipated workload. The ELISA methods were more extremely useful in the diagnosis of dengue infections through identification of NS1Ag, IgM or IgG [26], [27]. The ELISA assays have the advantage of being able to diagnose a large numbers of samples. The only drawback is that specialized, automated equipment is required to carry out the assay. Although serological tests are widely used in the diagnosis of dengue, cannot efficiently detect the presence of

antibodies in acute phase sera in primary dengue infections [28]. In addition, these methods are unable to distinguish the serotype of dengue causing the infection. However PCR is fast becoming the method of choice for the rapid detection of dengue virus [29]. Hence the concomitant performance of the Rapid test, followed by confirmation with MAC-ELISA on those samples, ensures both rapidity as well as quality of reported results.

Table 1. *Dengue IgM units for 34 serum positive samples of IgM antibody using MAC-ELISA.*

Serum samples	Mean O. D of calibrator	Sample O. D	Sample O. D ratio	Cut-off value	Dengue IgM units
S1	0.7	0.925	1.88	0.49	18.8
S2	0.75	0.91	1.73	0.525	17.3
S3	0.8	0.942	1.44	0.65	14.4
S4	0.62	0.89	2.06	0.43	20.6
S5	0.78	0.91	1.68	0.54	16.8
S6	0.65	0.96	2.13	0.45	21.3
S7	0.72	0.952	1.904	0.50	19
S8	0.71	0.893	1.81	0.49	18.1
S9	0.73	0.963	1.88	0.51	18.8
S10	0.81	0.92	1.43	0.64	14.3
S11	0.73	0.9	1.76	0.51	17.6
S12	0.77	0.9	1.69	0.53	16.9
S13	0.7	0.934	1.89	0.49	18.9
S14	0.82	0.87	1.52	0.57	15.2
S15	0.65	0.92	2.04	0.45	20.4
S16	0.7	0.91	1.85	0.49	18.5
S17	0.7	0.89	1.81	0.49	18.1
S18	0.7	0.982	2.00	0.49	20
S19	0.63	0.93	2.11	0.44	21.1
S20	0.72	0.9	1.8	0.50	18
S21	0.73	0.94	1.84	0.51	18.4
S22	0.75	0.86	1.63	0.525	16.3
S23	0.75	0.97	1.84	0.525	18.4
S24	0.67	0.971	2.11	0.46	21.1
S25	0.83	0.965	1.66	0.58	16.6
S26	0.79	0.934	1.69	0.55	16.9
S27	0.7	0.962	1.96	0.49	19.6
S28	0.74	0.856	1.65	0.518	16.5
S29	0.72	0.957	1.84	0.52	18.4
S30	0.85	0.935	1.58	0.59	15.8
S31	0.77	0.96	1.81	0.53	18.1
S32	0.75	0.91	1.73	0.525	17.3
S33	0.70	0.91	1.85	0.49	18.5
S34	0.72	0.983	1.89	0.52	18.9

4. Conclusion

The results obtained with rapid kit protocols available for laboratory diagnosis of suspected cases of dengue analyzed in our laboratory allocated us to notice some differences in the sensitivity of the tests. The samples were further screened by gold standard ELISA techniques. Hence dengue viral infection requires more than one methodology for confirmation and provides appropriate, rapid treatment of the patients and to prevent dengue outbreak. ELISA has become an important tool for routine dengue diagnosis of NS1Ag, IgG and IgM for detection of acute primary or secondary infection and has a sensitivity of more than 90%. At the same time it is necessary to take early prophylactic measures to control vector population as dengue is one of the life-threating illnesses.

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