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DENGUE OUTBREAK IN ANANTHAPURAM DIST. RAYALA SEEMA, A. P.

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ABSTRACT: Dengue viral infection can result in range of clinical manifestations from asymptomatic infection to dengue fever and the severe dengue hemorrhagic fever/ dengue shock syndrome which can result in death if it is not managed appropriately. So efficient and accurate diagnosis of dengue is of primary importance for clinical care surveillance activities and outbreak control. The present study was conducted between July 2014 to November 2014 which was an outbreak in ananthapuram dist, Andhra Pradesh. A total number of 2410 serum samples were collected from acute suspected cases of dengue fever and were subjected to IgM anti-body captured ELISA supplied by NIV PUNE and J. Mitra in the dept. of microbiology, Govt. medical college, ananthapuram, out of which 234 samples were positive. IgM anti-body detection by IgM captured ELISA has been an important tool for routine diagnosis of dengue with 90% sensitivity and 98% specificity when used from the 3rd to 5th day of fever.

KEYWORDS: Dengue fever, IgM Antibody capture ELISA, NS1 Ag.

INTRODUCTION: Dengue is an acute potentially fatal viral infection that can culminate into Dengue hemorrhagic fever (DHF) and Dengue shock syndrome (DSS). It is caused by four serotypes of dengue virus namely DEN-1, DEN-2, DEN-3 and DEN-4 belonging to genus flavivirus and family Flaviviridae. It is spread through the bite of infected *Aedes Aegypti* and *Aedes Albopictus* Mosquitoes. Most primary infection is uneventful with non-specific illness or with dengue fever. But infection with one serotype confers lifelong immunity to that serotype cross react with other serotypes. So secondary infection with a serotype different from that causing primary infection may lead to complications like DHF & DSS due to their cross reactivity.^{1,2}

The incidence of dengue has increased due to environment risk factors like infestation with *Aedes* mosquitoes, hot and humid climates enhancing mosquito breeding and mosquito density, water storage pattern in house, presence of all 4 serotypes with secondary infection in host, urbanization, increased international travels and global warming.³ A prevalence of *Aedes Aegypti* and *Aedes Albopictus* together with the circulating Dengue virus of more than one type in any particular area tends to be associated with the outbreaks of DHF/DSS.⁴

Over the past 3 decades, there has been dramatic global increase in the frequency of DF, DHF & DSS and their epidemics in urban, semi urban and also rural areas. Some 2.5 billion people i.e. two fifth of world's population in tropical and subtropical countries are at risk of these diseases. An estimated 50 million dengue infections occur worldwide annually and 90% of them are children below 5 years.⁵ The first major dengue epidemic occurred in Madras in 1780 which later spread to all over the country. And now Dengue is almost endemic throughout India. All the four serotypes i.e. Dengue 1, 2, 3 & 4 have been isolated in India by 1990, but at present DEN-1 & DEN-2 serotypes are widespread⁶. The widespread DHF epidemic during 2003 continues to spread because of the difficulty in developing vaccine and difficulty in vector control.

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During 2011, about 18,059 cases were reported with 119 deaths. The highest number of cases was reported from Punjab followed by Tamilnadu, Gujarat, Kerala and AP.⁷

As per the WHO classification the proposed probable diagnosis of Dengue is made as an acute fibril illness with two or more of the following manifestations, headache, retro-orbital pain, arthralgia, rash, hemorrhagic manifestations, leucopenia and a positive Ig M antibody test on serum sample collected five or more days after the onset of fever.⁸ There is a need for specific, inexpensive dengue diagnostic tests that can be used for clinical management, surveillance and outbreak investigation and would permit early intervention to treat patients and prevent or control epidemics. So early diagnosis by an ideal diagnostic test plays a crucial role in forecasting an early warning of an epidemic. In order to provide timely information for the management of patients and early public health control of dengue out breaks. It is important to establish the diagnosis of acute dengue viral infection during the first few days after the manifestation of clinical symptoms. So during the out breaks, the rapidity and specificity of diagnostic tests is more important than test sensitivity. Moreover a single serological test detecting Ig M is merely indicative of a recent dengue viral infection which can be used as screening tests in epidemic periods.

After the onset of illness, the virus can be detected in serum, plasma and tissues for the first 4 – 5 days. After day 5, dengue virus and antigens disappear from blood and specific antibodies appear. The acquired immune response to infection into dengue virus consists of the production of Ig M & Ig G antibodies directed against viral envelop proteins. Ig M antibodies are the first immunoglobulin to appear. These antibodies are detectable in 50% of patients by day 3 – 5 after onset of illness, increasing to 80% by day 5 and 99% by day 10. According to the Pan American Health Organization (PAHO) guidelines, Ig M antibody is detectable by day 5 of illness in 80% of all dengue cases, and by day 6 – 11 of illness in 93-99% of cases and may then remain detectable for more than 90 days.⁹

Ig M levels peak about two weeks after the onset of symptoms and then decline by 2 – 3 months. Ig G antibodies are detectable at low levels at the end of the first week of illness increasing slowly thereafter and probably even for life⁸. So during a primary dengue infection, Ig M response is typically with higher titer and more specific than during secondary infection in contrast during, Ig G levels peak with high titers even in acute phase and increase rapidly over the following 2 weeks and persists for more than 10 months to life and but the Ig M levels are significantly low, so false negative results can occur in anti-dengue Ig M tests.¹⁰

Laboratory diagnostic methods for confirming dengue virus infection may involve detection of the virus, viral RNA, antigens and antibodies or a combination of these techniques. So during the early stages of dengue infection 1 – 5 days, viral isolation by inoculating the sample into mosquito cell culture like C6/36 & AP61 or human cell cultures like vero and LLC-MK2 cells or intra cerebral inoculation of suckling mice can be done, but they are expensive and time consuming (1 week). Dengue viral RNA can be detected by Nested RT-PCR and Real time RT-PCR techniques which are expensive and also give false positives due to contamination. NSI antigen detection is a non-structural protein which appears as early as day 1 after the onset of fever and declines to undetectable levels after day 5 – 6. So it is useful for early case detection and for outbreak investigation. NSI antigen can be detected by ELISA & Rapid chromatographic tests.

Ig M antibody detection by Mac ELISA – Ig M antibody capture enzyme linked immunosorbent assay has been an important tool for routine diagnosis of dengue with 90% sensitivity and 98% specificity but only when used 3 or more days after onset of fever. Mac ELISA is based on capturing

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human Ig M antibodies in a micro titer plate using anti-human Ig M antibody followed by the addition of dengue viruses specific antigen (Den 1 – 4). Ig G antibody ELISA are used for the detection of a past dengue infection and secondary infection. Samples with negative Ig G in acute phase and positive Ig G in convalescent phase of infection are primary dengue infection. Sample with a positive Ig G in acute phase and fourfold rise of Ig G titers in convalescent phase is a secondary dengue infection. So serological assays may be used to determine the extent of outbreaks but not useful for determination of dengue serotype due to cross reactivity of antibody even during primary infection.

MATERIAL AND METHODS: The study was conducted between July 2014 to November, 2014. a total of 2410 serum samples were collected from acute suspected cases of Dengue fever from Govt. General Hospital, Ananthapuramu, various private hospitals in Ananthapuramu town and field samples collected from various villages in Ananthapuramu District through DMO. Samples were subjected to Ig M antibody captured dengue ELISA in the Department of Microbiology, Govt. Medical College, Ananthapuramu. ELISA kits were supplied by NIV Pune & J. Mitra.

PRINCIPLE OF Ig M CAPTURE ELISA: Ig M antibodies in the patient's serum are captured by anti-human Ig M coated on wells. In the next step, Dengue antigen is added which binds to captured human Ig M in the sample. Unbound antigen is removed during the washing step. In the subsequent step biotinylated flavivirus anti-DEN monoclonal antibodies are added followed by Avidin – HRP. Subsequently, chromogenic substrate (TMB/H₂O₂) is added and the reaction is stopped by 1N H₂SO₄. The intensity of color/optical density is measured at 450 nm.

NIV DEN Ig M Capture Elisa Kit: The test was standardized and reported by NIV pune 1984(1).

LIST OF REAGENTS SUPPLIED PER KIT:

1. Anti-human Ig M coated wells (96): 12 strips with 8 wells.
2. Sample diluents for Dengue Ig M (60): Phosphate buffered saline with additives and antibiotics.
3. Wash buffer concentrate – 20 X (60 ml): Phosphate buffered saline with surfactant and antibiotics.
4. Dengue Antigen (6 ml).
5. Anti-DEN Monoclonal antibody (Biotin labeled) – (6 ml).
6. Avidin – HRP (6 ml).
7. Liquid TMB Substrate (12 ml): Tetramethy Benzidine Dihydrochloride.
8. Stop solution (12 ml) – 1 NH₂SO₄
9. DEN Ig M Positive Control (0.8 ml).
10. DEN Ig M Negative control (0.8 ml).

PROCEDURE:

1. Serum samples were diluted 1: 100 using sample diluents for dengue Ig M.
2. Anti Ig M coated strips were taken and numbered as 1,2,3 after considering 1st and 2nd wells at negative control and positive control.
3. Wells were washed with wash buffer with 1 X (after diluting the wash buffer from 20 x into 1 x) for 3 times.

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4. 50 ul of diluted serum samples were placed into respective wells using multichannel pipette.
5. 50 ul of negative control and positive control were also added to 1st and 2nd wells and all the wells were covered with aluminum foil to prevent evaporation and wells were kept at 37 C in an incubator for 1 hour.
6. After incubation, wells were washed for five times with wash buffer and the plate was tapped after the last wash on the tissue paper to remove traces of wash buffer content.
7. 50 ul of DEN antigen was added to each well without taking them to room temperature and was incubated at 37 C for 1 hour and washed 5 times 50 ul of DEN Monoclonal antibody (biotin labeled) was added and incubated. After one hour of incubation, the wells were washed with wash buffer.
8. 50 ul of Avidin HRP was added to each well and incubated at the 37 C for 30 minutes and washed with wash buffer. Plate was tapped after every washing to remove traces of wash buffer content.
9. 100 ul of liquid TMB substrate was added to each well and incubated at room temperature in dark for 10 minutes and the reaction was stopped by adding 100 ul of stop solution.
10. The plate was measured by ELISA reader at the absorbance 450 nm within 10 minutes after termination of reaction.

QUALITY CONTROL:

1. If OD of Negative control is more than 0.18.
OR
2. If OD of the positive control is less than 6 times the OD of Negative Control.
3. In the both situations, the test should be considered as invalid.

INTERPRETATION OF RESULTS:

1. If OD value of sample tested is less than OD of Negative control by a factor 2.0 (Sample OD < Negative control OD X 2.0). The sample should be considered as "Negative".
2. If OD value of sample tested exceeds OD of Negative control by a factor 3.0 (Sample OD > more than Negative Control OD X 3.0). The sample should be considered as "Positive".
3. If OD value of sample tested exceeds OD of Negative control by a factor of 2.0 (Sample OD > Negative control OD X 2.0), but is less than OD of Negative of control by a factor 3.0 (Sample OD < Negative control OD X 3.0). The sample should be considered as "Equivocal".

DENGUE Ig M MICROLISA BY J. MITRA: Dengue Ig M Microlisa is a in-vitro qualitative detection of Dengue Ig M Antibodies in human serum and is used as a screening test for suspected cases.

PROCEDURE:

1. Serum samples were diluted to 1:100 using sample diluents.
2. Anti Ig M coated strips were taken and numbered as 1, 2, 3 after considering 1st and 2nd wells as negative and positive controls and 3,4,5 as calibrators as C1, D1, E1.
3. 100 ul of diluted serum samples were placed into the respective wells using multichannel pipette.

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4. 100 ul of negative control and positive control were also added to the 1st and 2nd wells and calibrators to 3rd, 4th, 5th wells, and all the wells were covered with aluminum foil to prevent evaporation and wells were kept at 37°C in an incubator for one hour.
5. After incubation, wells were washed for 5 times with wash buffer (after diluting the wash buffer from 25 X into 1X) and plate was tapped after the last wash on the tissue paper to remove traces of wash buffer content.
6. 100 ul of working conjugate (concentrate conjugate (0.1 ml) + conjugate diluents (0.9 ml) = 1 ml (1:10) was added to each well without taking them to room temperature and incubated at 37°C for one hour and washed 5 times and tapped.
7. 100 ul of working substrate solution (TMB substrate (1 ml) + TMB diluents (1 ml) = 2 ml (1:1) was added to each well and incubated at room temperature in dark for 30 minutes and reaction was stopped by adding 50 ul of stop solution.
8. The plate was measured by ELISA reader at the absorbance of 450 nm within 30 minutes after termination of reaction.

TEST VALIDITY: The test is valid when the following is within specified acceptance criteria:

1. NC O.D must < 0.3.
2. Ratio of PC O.D / cut off must be > 1.1.
3. Cut off value must be > 1.5 X NC O.D.

CALCULATION OF RESULTS:

- Calibration factor is varied with different lots.
- Cut off value = Mean O.D of calibrator X calibration factor.
- Sample O.D ratio = Sample O.D.
- Cut of Value.

Calculation of Dengue Ig M units:

- Sample O.D ratio X 10.

INTERPRETATION:

1. If the Dengue Ig M units is < 9 then interpret the sample as NEGATIVE for Dengue Ig M antibodies.
2. If the Dengue Ig M units is between 9 – 11 then interpret the sample as Equivocal for Dengue Ig M antibodies.
3. If the Dengue IgM units is > 11 then interpret the sample as POSITIVE for Dengue Ig M antibodies.

RESULTS: A total number of 2410 sera samples suspected of acute dengue viral fever were processed for Ig M Dengue antibody by IgM captured ELISA during the period July 2014 to Nov. 2014.

Total number of samples tested	Total number of positives	Total number of equivocal	Total number of negatives
2410	234 (9.7%)	21	2155

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	0-10	11-20	21-30	31-90	41-50	51-60	Above 60	Total
Total No. of samples tested	815	563	383	218	186	120	125	2410
Total No. of positives	108 (13.2%)	47 (8.3%)	15 (3.91%)	23 (10.55%)	20 (10.75%)	10 (8.33%)	11 (8.8%)	234
Total No. of equivocal	07	04	3	2	3	1	1	21
Total No. of Negatives	700	512	365	193	163	109	113	2155

Table 1: Age wise distribution of dengue positives and negatives

Out of 2410 serum samples 283 were NSI Positive sera samples collected from various paediatric private Hospitals in Ananthapuramu town. (By Immuno Chromatographic Test).

Total number of NS1 Positive samples	1 st day NS1 positives	2 nd day NS1 positives	3 rd day NS1 positives	4 th day NS1 positives	5 th day NS1 positives
283	37	65	61	59	61
IgM positives(42)	nil	nil	8	11	23

TABLE 2: NS1 POSITIVE CASES (283). NS₁ POSITIVES – IgM POSITIVES

DISCUSSION: A total 2410 sera samples were subjected to Dengue IgM – ELISA, out of which 234 samples were positive, 2155 samples were negative and 21 cases were equivocal. The Overall positive percentage was 9.7%.

Highest prevalence was noticed in 1 to 10 years age group with 13.25%. A total 815 samples were processed with ELISA & 108 cases were positive. This is revealed by various authors.^{16,17}

Among 2410 samples, NS1 Positive samples were 283 from day 1 to day 5. 283 NS1 positive serum samples were received from various pediatric private hospitals from ananthapuramu town admitted with signs and symptoms of acute dengue fever (by ICT – Immuno Chromatographic Tests). 37 children were admitted with NS1 positive in 1st day, 2nd day 65, 3rd day 61, 4th day 59, 5th day 61. All the samples were processed for IgM ELISA.

Out of 283 NS1 positive Samples 42 were given positive for IgM antibodies 8 samples were IgM positive on 3rd day, 11 on 4th day, 23 on 5th day. There were no positives on 1st and 2nd day for IgM antibodies. Significant levels of IgM antibodies start appeared on 3rd day onwards and rises steadily thereafter.^{12,13,14,16,17} Out of 283 NS1 positives 7 samples showed equivocal results.

In large number of (21) cases equivocal results were observed in this outbreak. This is because of samples were collected during outbreak, and low titers of IgM were observed in secondary infection.¹⁶

As the kinetics of the IgM response are more variable as IgM significantly lower in secondary Dengue infection. False negative test results for dengue specific IgM have been reported during secondary infection.¹⁶

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CONCLUSION: Dengue is now a global threat and is endemic or epidemic in almost every country located in the tropics. As dengue is increasing in incidence, improved diagnosis, early detection of severe cases and efficient medical management are of primary importance in all areas where dengue is endemic. The currently available best diagnostic method is IgM captured ELISA which can detect IgM anti-bodies from 3rd to 5th day of fever onwards. In recent years there are several foundations and partnerships have been working with WHO and National governments to develop new tools and strategies to improve diagnostics and clinical treatments to achieve a successful vaccine.

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