International Journal of Bioassays
ISSN: 2278-778X
www.ijbio.com

Research Article

A CLINICO-MICROBIOLOGICAL STUDY OF DENGUE FEVER CASES IN A TERTIARY CARE CENTRE OF NAVI MUMBAI

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Received for publication: September 11, 2013; **Revised:** September 27, 2013; **Accepted:** October 17, 2013

Abstract: Dengue is an endemic viral disease affecting tropical and subtropical regions around the world, predominantly in urban and semiurban areas. Dengue fever (DF) and its more serious forms, dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), are becoming important public health problems and were formally included within the disease portfolio of the Indian Development. During 2010, there was an increase in dengue virus infections in Mumbai as compared to 2009. This study determined the socio demographic status of suspected dengue cases from 2010 to 2012 in a tertiary care centre of Navi Mumbai. The comparative outcome of dengue NS1, IgM and IgG ICT, dengue NS1, IgM and IgG ELISA, and RT-PCR for dengue NS1 antigen to assess which of them is better for patient management and prevention of complications like DHF and DSS. Distribution in 320 positive samples were NS1 positive 51.56%, IgM positive 22.81%, IgG positive 12.2%, NS1 + IgM positive10.31%, IgM + IgG positive 2.19% and NS1 + IgM + IgG positive 0.93%.In 100 randomly selected ICT positive cases, there was a gain of 6 (3+3) positive samples by the NS1 Microlisa test as compared to the NS1 immuno chromatography test. In 66 ICT positive samples, there was a gain of 7 (2+5) positive samples by IgM Microlisa and a gain of 1 sample by IgG Microlisa. Out of 30 samples subjected to RT-PCR, all 15 positive Microlisa samples were positive, while 4 out of 15 Microlisa negative samples were positive by the RT-PCR. Overall there was a gain of 4 positive samples by the RT-PCR. As we found RT-PCR to be the gold standard, sensitivity and specificity of NS1 ICT were found to be 91.2% and 78.4%, while that of NS1 ELISA were found to be 75% and 88%. For the early diagnosis of dengue infection, Immuno chromatography test is a good screening test with good sensitivity, while dengue ELISA is more specific and can be used as confirmatory tests in routine laboratory setup. RT-PCR for dengue antigen is the most confirmatory test with highest sensitivity and specificity but is expensive. A combination of the three tests is recommended for dengue diagnosis in laboratories.

Keywords: Dengue, Dengue Haemorrhagic Fever (DHF), Dengue Shock Syndrome (DSS)

INTRODUCTION

Dengue is one of the most serious and the most common mosquito-borne viral infections of man affecting mainly the tropical and the subtropical countries in the world and caused by the bite of aedes mosquito(1). Dengue is an acute viral disease caused by a virus belonging to the broad group Arboviruses, family Flaviviridae, subfamily Flavivirinae and genus Flaviviruses. Dengue virus has a positive sense, ssRNA viral genome (2). Previously it was considered to be a disease of the urban and the semiurban areas, but now it has started affecting the affluent class as well causing a major public health concern. Since there is no immune prophylactic or specific antiviral therapy available, timely and rapid diagnosis plays a vital role in patient management and implementation of control measures (3). Outbreaks caused by the four types of dengue virus - DENV1, DENV2, DENV3 and DENV4 - have become increasingly frequent over the past 25 years (4). Individuals infected with one strain maintain lifelong homotypic immunity while remaining susceptible to infections with other heterotypic strains as no cross-immunity is provided by infection with one strain (5).

Distinct genotypes have been identified within each serotype, highlighting the extensive genetic variability of the dengue serotypes (6). About 55% of the world's population lives in areas where there is a risk of dengue fever (7). It is estimated that more than 3.6 billion people are at risk of infection and 124 countries have endemic dengue virus transmission. Out of these, nearly 70-500 million people are infected annually. These include the asymptomatic cases. Cases of dengue fever per year are 36 million. About 2.1 million cases of Dengue Haemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS), constituting 5-10% of the total cases, are reported annually although the true incidence is not really known. At least 21,000 deaths occur, mainly among children, per year (8). In dengue endemic regions, which include countries in Asia and the Americas, the burden of dengue is approximately 1,300 disability-adjusted life years (DALYs) per million

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populations, which is similar to the disease burden of other childhood and tropical diseases, including tuberculosis, in these regions (9). The "Asian" genotypes of DEN-2 and DEN-3 are frequently associated with severe disease accompanying secondary dengue infections (10). India had recorded 28,292 cases and 110 deaths in 2010; 18,860 cases and 169 deaths in 2011; but the corresponding figure till November, 2012, stood at over 35,000 cases and 216 fatalities. The Maharashtra State, as a whole, saw a 47% rise in dengue cases in 2012 with second highest number of deaths (59) after Tamil Nadu (60) and reported dengue cases at 1,464. Experts have blamed this rise in dengue cases to the fluctuating weather, the extended monsoon resulting in intermittent rain followed by great heat and stagnant pools of water (11).

Laboratory tests are essential to provide an accurate diagnosis of dengue virus infection so that appropriate treatment and patient management may be administered. An early diagnosis of the disease can prevent the further complications like DHF and DSS (12). It has been recorded that most patients seek medical care 3 to 6 days after their symptoms begin. During this time, a powerful combination of Dengue Non-Structural Antigen (NS1), Dengue IgM Antibodies and Dengue IgG Antibodies is detectable in the blood of the patients (13).

The significance of this prospective study, done over a two year period from September 2010 to August 2012 in a tertiary care centre of Navi Mumbai, is to provide a comprehensive and relevant socio demographic data for the region of Navi Mumbai and compare the results of three diagnostic methods for dengue; namely, the Immuno chromatographic (Rapid) Tests for Dengue Non-Structural Antigen 1 (NS1), IgM and IgG Antibodies; ELISA for NS1 Antigen, IgM & IgG Antibodies; and RT-PCR for Dengue Antigen detection.

MATERIAL AND METHODS

A total number of 1782 blood samples from clinically suspected cases of dengue fever, according to the WHO criteria, were obtained from both outdoor and hospitalized patients from a tertiary care center of Kamothe, Navi Mumbai. Serum was separated by centrifuging samples at 3000 rpm for 5 min and tested immediately. In case of a delay in processing, they were stored in a refrigerator at a temperature of2-8°C.

Rapid solid phase Immuno chromatographic test (ICT) for qualitative detection of Dengue NS1 Ag & IgM/ IgG antibodies was performed on the 1782 serum samples as the screening test using Dengue Day 1 Combi Card by J Mitra. The kit consisted of two devices; one device for detection of Dengue NS1 antigen and second device for the differential detection of IgM/ IgG antibodies in human serum/ plasma. The antigen device contained two lines; "C" (Control Line) & "T" (Test Line,) while the antibody device contained "C", "M" (IgM test line) & "G" lines (IgG test line) respectively. A visible pink line at "T" or "M"/ "G" was taken as a positive test.

320 samples were positive for any one of the three dengue parameters, namely NS1 antigen, IgM or IgG antibodies, bythe ICT. NS1 ELISA (enzyme-linked immunosorbent assay) test using Dengue NS1 Ag Microlisa kit by J Mitra was performed in randomly selected 100 ICT positive cases out of the 320.25 negative samples served as the controls. The PC and NC from the kit were put up with the test samples as per the kit literature provided. Two kits from the same manufacturer containing 96 microwells and 48 microwells were simultaneously used for testing a total of 125 samples. The test was a solid-phase ELISA based on "Direct Sandwich" principle. The microwells were coated with monoclonal Anti-dengue NS1 antibodies. A positive reaction was indicated by a yellow colour which was finally read at 450nm spectrophotometrically by an ELISA reader. The Cut-off Value (COF) was calculated using the formula Mean Absorbance Negative Control + 0.40.Test specimens with O.D. value greater than or equal to the COF were taken as positive, while those with a lesser O.D. were taken as negative. Randomly selected 66 ICT positive samples out of the above 100 samples were also subjected to IgM and IgG Ab ELISA test using Dengue IgM & IgG Microlisa kit byJ Mitra.25 negative samples were taken as controls and PC, NC and Calibrator were also put up. The test was based on "Capture ELISA" principle. Anti-human IgM/IgG antibodies were coated onto microtiter wells. Kits containing 96 microtiter wells were used for testing. Results were interpreted according to kit literature.

A total of 30 samples were further tested to RT-PCR (Real-time polymerase chain reaction) of which 15 were ELISA positive and 15 was ELISA negative samples. RT-PCR was done using Geno-Sen's Dengue 1-4 RT-PCR kit for Rotor gene 2000/3000/6000 based on the Taqman principle. During PCR, forward & reverse primers hybridized to a specific sequence product. A Taqman probe hybridized to a target sequence within the PCR product. Taq polymerase cleaved the probe. Reporter & quencher dye were separated, resulting in increase in fluorescence for the reporter, which was directly proportional to target amplification of PCR. Roche Light Cycler^{480} RT-PCR machine was used for the same. RT- PCR was taken as the gold standard against which the other two tests against which the other two tests i.e. ICT and ELISA were compared for dengue antigen.

RESULTS

In the 1782 clinically suspected dengue patients who presented to the tertiary care centre over a period of two years, socio demographically the percentage of males was 67.96% and the females was 32.04%. The male: female ratio was calculated and reported 2.1:1 (Figure 1). The age- group distribution waschildren 0-10 years age group6.4%, 11-20 yrs age group12.8%, 21-30 yrs age group 56.84%, 31-40 yrs age group 14.36%, and above 40 yrs age 9.6% in overall clinically suspected dengue fever cases (Figure 2).

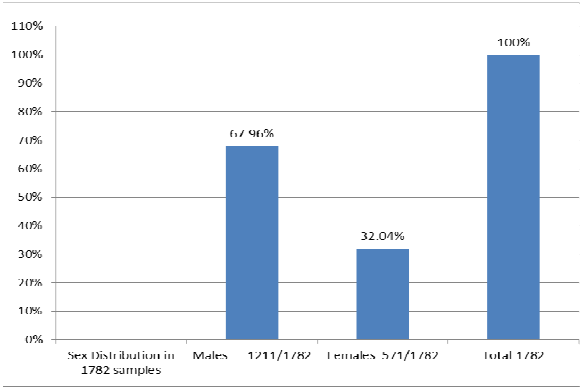

Figure.1: Sex distribution in clinically suspected dengue patients

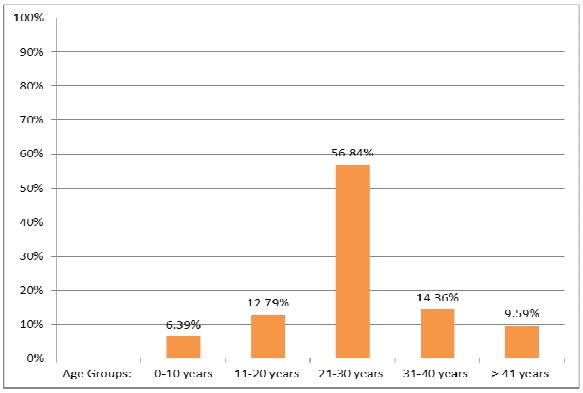

Figure.2: Age Group distribution in clinically suspected cases

Clinical and Laboratory findings were out of the 1782 serum samples of clinically suspected dengue cases, 320 were positive by the immune chromatographic test for any one of the three dengue parameters, namely NS1 antigen, IgM or IgG antibodies. Thus the incidence of dengue was found to be 17.95% in Kamothe, Navi Mumbai (Figure 3). Out of 320 ICT positive samples, only NS1 positive were 51.56%, only IgM positive 22.81%, only IgG positive12.20%, NS1 + IgM positive 10.31%, IgM + IgG positive 2.19%and NS1+ IgM + IgG positive 0.93% (Figure4).There was a peaking of serologically positive cases from July to October (62.50% and 67.40%) in each of the years of study, while showing a major decline in the other seasonal months i.e. from November to February and from March to June.

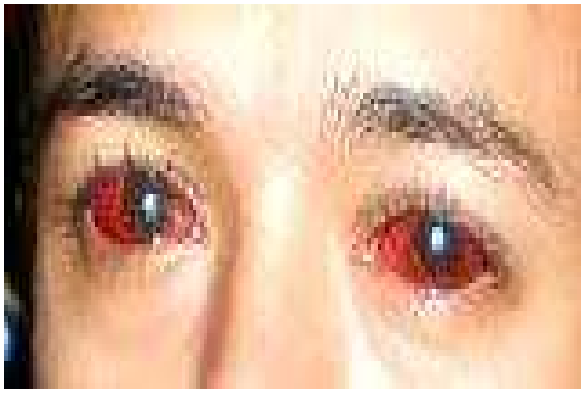

Figure.3: Subconjunctival haemorrhage in dengue haemorrhagic fever

100 ICT positive samples for any one of the three dengue parameters out of the total 320 positive samples were randomly selected for further testing by NS1 Microlisa.25 negative ICT samples were also tested which served as controls. The history of presenting complaints was noted for each of the100 ICT positive patients from the lab requisition forms filled up by the clinicians. They were confirmed from either the patient themselves in case of hospitalized patients or tallied with the registers in the case of OPD patients. It was noted that 71% of the patients presented with fever with chills only, followed by fever with chills and rashes 13%, fever with chills and joint pains 5% and fever with myalgia 3%. Sub-conjunctival haemorrhage, a presumptive sign of dengue haemorrhagic syndrome was seen only in 2% patients in the present study (Figure 5). The rest showed other insignificant associated symptoms and signs. There was no mortality reported throughout the period of study.

The platelet counts were recorded from the haematology laboratory for the patients studied. It was found that 52.8% patients had platelet counts <50,000/cu.mm, followed by 30.40% with > 80,000/cu.mm and the rest between 50,000-80,000/cu.mm of blood (Figure 6). It was found that 75 out of the 100 samples were positive for NS1 Microlisa, while 3 of the 25 negative controls were positive for NS1 Microlisa. The NS1 ICT test positives in the same samples were found to be 72.Thus there was a gain of 6 (3+3) positive samples by the NS1 Microlisa test as compared to the NS1 immunochromatography test.

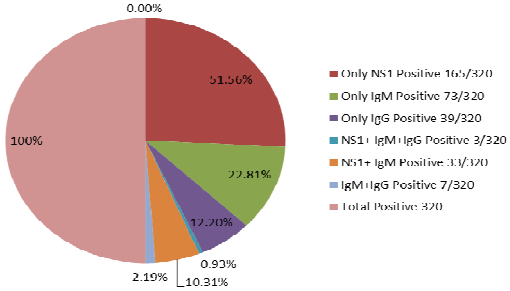

Figure.4: Distribution of dengue parameters in 320 ICT positive cases

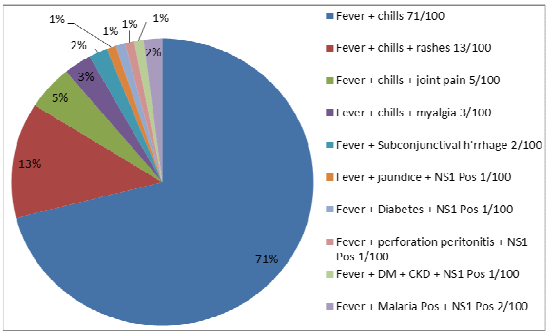

Figure.5: Presenting complaints in ICT positive patients

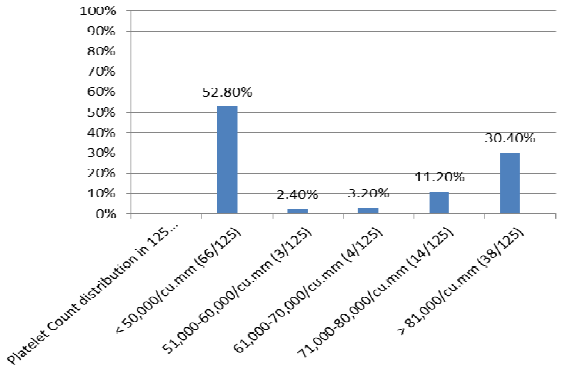

Figure.6: Platelet count distribution in 125 tested samples

66 ICT positive samples, which were also tested for NS1 Microlisa, were tested for IgM and IgG Microlisa.25 ICT negative samples, which were also tested for NS1 Microlisa, served as controls. We found that 25 out of 66 ICT positive samples and 5 out of 25 ICT negative samples were positive for IgM Ab Microlisa. 23 IgM positive were seen in corresponding 66 ICT positive samples. There was a gain of 7 (2+5) positive samples by IgM Microlisa.

For IgG Ab Microlisa, 3 out of 66 ICT positive samples and 1 out of 25 negative ICT samples were positive. IgG ICT positivity was also 3 out of the 66 positive samples. Hence the gain was of 1 sample by IgG Microlisa.

Association between the ICT and ELISA was statistically calculated using Chi-square and Fisher tests. Since the exact p-value for the Chi-square test and Fisher was 0.000 which was less than that of 0.05, it indicated that there was association between the ICT and Microlisa tests. Correlation values for the tests were calculated using contingency coefficient value and Phi.

RT-PCR was done on 30 randomly selected samples tested for Microlisa, which included 15 positive and 15 negative Microlisa samples. They were tested using Geno-Sen's Dengue 1-4 RT-PCR kit for Rotor gene 2000/3000/6000. We found that all 15 of the Microlisa positive samples were also positive by the RT-PCR, while 4 out of 15 Microlisa negative samples were positive by the RT-PCR. Overall there was a gain of 4 positive samples by the RT-PCR. As we found RT-PCR to be the gold standard, sensitivity and specificity of NS1 ICT were found to be 91.2% and 78.4%, while that of NS1 ELISA were found to be 75% and 88%.

DISCUSSION

Key contributing factors to the worldwide resurgence of dengue in the last few decades include the rise in number and size of densely populated urban cities that are conducive for the spread of the disease and the adaptation and proliferation of dengue vectors, particularly the primary carrier of dengue virus, *Aedes aegypti*. Dengue's threat to travellers has also been systematically demonstrated by various studies across the globe. In the last three decades, the number of people living in cities around the world has doubled from 1.7 billion to 3.6 billion. The number is expected to rise to 4.9 billion by 2030, and most of this is projected to occur in Asia (14).

There was a peaking of serologically positive case from July to October in each of the years of the present study. A. Chakravarti *et al.,* (2012), have reported that dengue outbreak coincided mainly with the post monsoon period of subnormal rainfall. The difference between serologically positive cases as compared to serologically negative ones in post monsoon period was significantly higher ($p<0.001$).Their study highlighted rain, temperature and relative humidity as the major and important climatic factors, which could alone or collectively be responsible for an outbreak. The peak incidence of dengue cases in Ekta Gupta *et al.,* (2006) study was in the 2nd and 3rd week of October.

The present study has shown a male preponderance (67.96%) as compared to the females (32.04%) with the male: female ratio being 2.1:1 in the clinically suspected dengue patients. Similar results were observed by Atul Garg *et al.,* (2011) who gave the male: female ratio as being 2:1 and Tank Arun G, Jain Mannu R (2012) who have given a sex ratio for dengue sero-positive patients formale to female as 2.54:1.

We found that the mean age group affected was 21-30 years. This was consistent with other studies on dengue in India. R. N. Makroo *et al.,* (2007) in their study have reported the mean age of dengue patients as 27 years and that most belonged to the 21-30 year age group (32.44%). Ekta Gupta *et al.,*(2006) have also givenan age group predominance of 21-30 years in all the 3 years of their study.

Fever with chills (71%) was the most common presenting complaint in this study amongst the serologically positive and tested cases. Dengue haemorrhagic fever was reported only in 2% cases.

Abdul Haque Khan *et al.,* (2010) observed that in 20 serologically confirmed dengue patients, the typical clinical features included chills and rigors in 16 (80%), myalgia in 14 (70%), vomiting in 12 (60%), headache in 10 (50%), rash in 5 (25%). Out of dengue proven, 18 patients had dengue fever and 2 had dengue hemorrhagic fever. According to WHO2009guidelines, the classical dengue fever presents as sudden onset of fever with chills and high fever which was consistent with the current findings. We did not encounter any mortality in our study, the reason being our patients mostly had classical dengue fever, which is a self-limiting illness if diagnosed and treated early, with a very low case fatality rate of 0.4% in this region (22).

One of the conclusions drawn out of our study highlighted that out of 1782 clinically suspected dengue patient's acute phase samples received over a period of 2 years, 320 were tested positive by the Dengue Rapid ICT. Thus the incidence of dengue in the tertiary care center set up came to be 17.95%. In a study by Jenny G. H. Low *et al.,* (2011), 11.7% had confirmed dengue infection. D Turbadkar *et al.,* (2012) tested 3677 samples over a 3 year period in Mumbai, out of which 503 were serologically positive, making the incidence as 13.67%.

Rapid test (ICT) was performed on the 1782 blood samples by a proven standard kit with a good sensitivity and specificity. Out of the 1782 clinically suspected samples, 320 were positive for one or more dengue parameters.165 (51.56%) were positive for NS1 only, 73 (22.81%) were positive for only IgM, and 39 (12.2%) were positive for IgG only.3 (0.93%) were positive for NS1 + IgM + IgG, 33 (10.31%) for NS1 + IgM, and 7 (2.19%) for IgM + IgG.

The results obtained are similar to another study. R D Kulkarni *et al.,*(2011) used the Rapid Dengue kit from the same manufacturer to test 2104 samples. Out of 2104 samples, 320 were positive for one or more dengue parameters. Of the 320, 95 (29.69%) were positive for NS1 only, 161 (50.31%) showed IgM only, while 9 (2.81%) showed IgG only. More than one marker was detected in the remaining 55 (17.19%) samples.

In the present study, 30 samples were processed by RT-PCR which was taken as the gold standard as its sensitivity and specificity came out to be 100%. The sensitivity and specificity of NS1 ICT were found to be 91.2% and 78.4%, while that of NS1 ELISA were found to be 75% and 88%.

Results recorded in another comparative study done by Chua KB *et al.,* (2011) were out of 558 samples, a total of 190 serum samples were tested positive for dengue by either one or a combination of the four methods whereas, only 59 serum samples were tested positive by all four methods. Thus, based on single-acute serum samples, 190 of the 558 patients (34.1%) were laboratory-confirmed acute dengue. The overall test sensitivity was 91.6%, 40.5%, 48.4% and 58.9% for dengue NS1 antigen-capture ELISA, virus isolation, conventional RT-PCR and real-time RT-PCR respectively. Statistically, dengue NS1 antigen-capture ELISA was the most sensitive and virus isolation was the least sensitive test for the laboratory confirmation of acute dengue based on single-acute serum specimens. Real-time RT-PCR was significantly more sensitive than the conventional RT-PCR.

CONCLUSION

Dengue secondary infection can lead to complications like dengue haemorrhagic fever and dengue shock syndrome and subsequent mortality. As there is no prevention in the form of any vaccine for dengue, early diagnosis and treatment is recommended for preventing such complications. Immuno chromatographic tests for dengue NS1 antigen and IgM antibody are good for screening purpose, while confirmation with ELISA and RT-PCR is required as they have better sensitivity and specificity. The study concluded that qualitative RT-PCR is the standard method for early detection of Dengue antigen as it is most sensitive and specific. This can be followed by serotyping and quantitative RT-PCR for epidemiological studies.

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Source of support: Nil
Conflict of interest: None Declared