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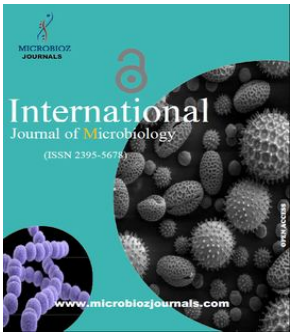

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Serological and virological profile of dengue fever in a tertiary care hospital, southern part of Hyderabad, during 2011-12

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Abstract

Dengue fever is endemic in India. Andhra Pradesh is one of the hotspots for dengue but an understanding of the incidence of the disease as well the prevailing serotypes of dengue virus is lacking. The study was conducted to determine the prevalence of dengue syndrome among patients admitted with clinical suspicion of dengue, the clinical, demographic profile, serological profile of dengue syndrome, and to identify the dengue virus serotypes prevalent in the year 2011 and 2012. Samples (n = 237) with clinical suspicion of dengue fever were tested for the presence of dengue NS1 antigen (NS1Ag) as well as DENV-specific IgM and IgG antibodies by ELISA. Serum samples were subjected to cell culture followed by RT-PCR using group-specific and type-specific primers. The seroprevalance of dengue syndrome was 35% among the study samples. IgM ELISA and NS1Ag ELISA detected 68.6% and 43.3% of the cases, respectively. NS1Ag could detect 10% of the cases that were negative by IgM ELISA in the early pyrexic stage. Primary and secondary dengue fever was more frequent in children (0-10 yr) and young adults (21-30 yr), with a frequency of 59% and 45.9%, respectively. Five samples from 2011 contained DENV1 serotype, and ten samples from 2012 indicated the co-circulation of DENV1 and DENV3 serotypes. Hemorrhagic manifestation was more common in patients having co-infections.

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Introduction

Dengue, the most important mosquito-borne viral infection, is prevalent in most parts of the tropics, and responsible for more illness and death than any other arboviral disease in humans. An estimated 2.5 billion people live in the areas at risk for epidemic dengue 1-3..

Dengue is diagnosed by either detecting virus or antibody to the virus in blood. Isolation of virus in cell culture or in infant mouse brain remains the gold standard for diagnosis, but it takes more than a week for the test to be completed making it impractical in most situations. Detection of anti-dengue IgM and IgG in the serum by ELISA is the most commonly used criteria for presumptive diagnosis of DENV infections. These serological methods are unable to detect the infection during the early phase of the disease. Thus there is a need for rapid and sensitive methods for detection of DENV early in the course of infection for better patient management.

The dengue virus genome is positive-sense RNA , has NS1 protein is an ~50-kDa (353 or 354 amino acids) glycoprotein which has a high amino acid and nucleotide homology among flavi viruses 2 . NS1 does not form part of the virion but is released from the dengue virus-infected cells, and it has been found circulating from the first day after the onset of fever up to day 9, once the clinical phase of the disease is over.

The NS1 protein could be detected even when viral RNA was negative in reverse transcriptase-PCR or in the presence of IgM antibodies. 4 The combination of NS1Ag and IgM ELISA has been reported to enhance the diagnosis without the requirement of paired sera.

It is crucial to determine which serotypes of DENV are circulating since previous infection with one of the four serotypes can be an important risk factor for developing DHF-DSS upon infection with a heterotypic serotype. The 1996 epidemic in India was mainly due to the DENV2 whereas all four serotypes were found in co-circulation in 2003 and 2005.1 In South India, few reports from Chennai 5 and Kerala 6 have identified the prevalent serotypes as DENV1, DENV2 and DENV3. In Andhra Pradesh, except for a study 7 in 2007, which reported DENV4 as the predominant serotype followed by DENV3, information on the circulation of or the changing patterns of the serotypes is not available.

Rapid serological tests such as IgM or NS1Ag ELISA with a single serum sample do not furnish information about the serotype of the virus. Molecular methods based on PCR offer a suitable alternative to conventional virus isolation.

Several PCR based methods for detecting DENV nucleic acid in the serum have been reported, the most widely used test is the nested RT-PCR developed by Lanciotti et al., 10.

Since 1945, outbreaks of dengue have been reported regularly from different regions of India. In recent years, the disease has been manifesting in severe form as DHF and with increasing frequency of outbreaks. The present study was conducted to determined the prevalence of dengue fever, its serological profile and serotype prevalence of DENV in a population of southern part of Hyderabad, Andhra Pradesh.

Materials and Methods

Settings

Department of Microbiology, Gandhi Medical College, Secunderabad.

Study design

Prospective cross-sectional study

Study period

6 months spread among two seasons (August -October 2011, August – October 2012)

Inclusion criteria

- Febrile patients admitted in medical and pediatric wards of Gandhi Hospital.
- Patients of all age groups, showing a temperature of >38.5oC for >24 hours, and clinically diagnosed as having dengue fever.

Exclusion criteria

- Febrile cases with definite source of infection (e.g. respiratory or urinary tract infection, meningitis).
- History of bleeding tendency since birth.
- Immuno compromised patients.

Sample Size

Number of patients included in the study was 237.

Methodology

Sample collection

Approval of the Institute's Ethics Committee was obtained to carry out the study. Informed consent was obtained from each patient. Information on demographic features and symptoms of the patients were collected by a structured questionnaire. A detailed physical examination was done. Tourniquet test was done in predicting any bleeding manifestations.

5 ml of blood was collected from each patient within 24-48 hours after admission .Other laboratory investigations included hemoglobin, total and differential leukocyte count, platelet count and liver transaminases. Chest X-ray and ultrasonography of whole abdomen were done in selected patients where clinical findings were suggestive.

Serodiagnosis

All the samples were tested for DENV NS1Ag and anti-DENV IgM and IgG antibodies by ELISA. NS1Ag was detected by sandwich ELISA (Panbio, Australia), IgM was detected by MAC ELISA (Division of Arbovirus Diagnostics, National institute of Virology, Pune), and IgG was detected by Indirect ELISA (NovaTec Immundiagnostica, GmbH, Germany). All the tests were performed according to the manufacturers' instructions. Primary dengue fever (PDF) was declared if samples were positive for NS1Ag and/or IgM antibody and negative for IgG antibody.9 Secondary dengue fever (SDF) was declared if IgG was detected in addition to NS1Ag and/or IgM.9 The WHO classification and case definitions were used to classify disease as DF and DHF, and DHF was further divided into four grades (I, II, III, IV).2

Virus isolation

Eleven samples positive for NS1Ag were subjected to virus isolation on the Aedes albopictus mosquito cells line C6/36 obtained from the National Institute of Virology, Pune. C6/36 cells were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with foetal bovine serum (FBS) and penicillin and streptomycin; cells were maintained under 5% FBS and virus was propagated using 2% FBS.

For virus isolation, each serum was diluted 1:50 in medium containing 2% FBS and filtered through a 0.2m syringe filter. The supernatant medium from a 25 cm2 tissue culture flask was removed and one mL of the diluted serum was added. The test serum was allowed to adsorb to the cells for 1 hour at 37oC at 25-30oC.

Following adsorption, the inoculum was supplemented with 4 ml of medium containing 2% FBS. The cells were observed daily for the development of cytopathic effect (CPE). The supernatants were harvested on 6th day post-inoculation and used for RT-PCR and the left-over cells were used for the detection of NS1 protein.

Immunoperoxidase test

NS1 protein expressed in infected cells was detected by immunoperoxidase test using the AEC chromogen kit (Sigma Aldrich, Missouri, USA). The cells were observed under an inverted microscope at 250X magnification. A red-brown precipitate indicating granular inclusions in the cytoplasm of infected cells indicated a positive result.

Serotype identification by RT-PCR

Cell culture supernatants from 11 samples from 2011 and 54 seropositive samples collected in 2012 were subjected to RT-PCR.

Total RNA was extracted using the Qia Amp RNA Extraction Kit (Qiagen, Gmbh, Germany) and RT-PCR was performed using the Enhanced RT-PCR kit (Sigma Aldrich, Missouri, USA). The primers10 used are shown in Table 1. Complementary DNA (c DNA) was synthesized in a 20µl reaction by using RNA template, dNTPS (10mM each), and random nonamers (50µM). Incubated at 25oC for 15minutes. 10x buffer for AMV-RT, enhanced avian RT and dengue virus group specific consensus downstream primer(D2) (10mM) was added to the reaction and incubated at a temperature between 42-500C for 50minutes.

The amplification of c DNA was carried out in a 50 µl final reaction using 10 mM

d NTPs, PCR primers D1 and D2 (10 mM each), and Jumpstart AccuTaq LA DNA polymerase. The thermal cycling profile of the PCR reaction was (1) RT inactivation and initial denaturation at 95oC for 5 mins followed by (2) 35 cycles of (a) denaturation at 95oC for 30 sec, (b) annealing at 55oC for 30 sec, (c) extension at 72oC for 60 sec, and (d) final extension at 72oC for 10 min. Amplification of the 511 bp DENV-specific product was assessed by 1% agarose gel electrophoresis and UV transillumination.

The primary PCR product obtained from RT-PCR was then further typed by nested PCR employing internal serotype specific primers (TS1, TS2, TS3 and TS4) as downstream primers in a single tube along with D1 as the upstream primer. The rest of the procedure was the same, and the products were analysed by 2% agarose gel electrophoresis and UV transillumination.

Primer	Sequence (5' to 3')	Genome Position	Size(bp)of PCR Product
D1	TCAATATGCTGAAACGCGCGAGAACCG	134-161	511
D2	TTGCACCAACAGTCAATGTCTTCAGGTTC	616-644	511
TS1	CGTCTCAGTGATCCGGGGG	568-586	482 (D1 and TS1)
TS2	CGCCACAAGGGCCATGAACAG	232-252	119 (D1 and TS2)
TS3	TAACATCATCATGAGACAGAGC	400-421	290 (D1 and TS3)
TS4	CTCTGTTGTCTTAAACAAGAGA	506-527	392 (D1 and TS4)

Table 1. Sequences of primers used in the study

Statistical analysis

Statistical analysis of the data was done by chi-square ($\chi 2$) test using EPIINFO 2000 software. The P values were interpreted as not significant if >0.05, significant if <0.05, and highly significant when <0.001.

Results

Out of 237 samples tested by NS1Ag and/or IgM ELISA, 83 (35%) were positive with a male-to-female ratio of 1.59:1. Most of the cases of dengue fever (44.3%) were from urban slum areas, and the highest prevalence was in the age group 21-30 years. The majority of the seropositive cases (56.6%) had fever of 5-7 days' duration. Headache, arthralgia, vomiting, bleeding manifestations, abdominal pain, hypotension, rash and positive tourniquet test were all significantly associated with positive cases as compared to negative cases (P = <0.05) . Except for serum bilirubin, all the other laboratory tests showed abnormal profile in dengue syndrome (not shown).

Out of 83 positive samples, 36 (43.3%) were positive for NS1Ag and 57 (68.6%) were positive for IgM (Table 2); 26.5% and 73.5% were primary and secondary dengue fever cases, respectively. Primary dengue fever was more common (59%) in younger age group (0-10 yr) whereas secondary dengue fever was more frequent (45.9%) in young adults (21-30 yr).

The most common symptoms in primary dengue fever were vomiting, arthralgia and rash, whereas headache (91.8%) and bleeding manifestations (49.1%) predominated in secondary dengue fever.

Thirty six patients (43.3%) fulfilled WHO criteria for DHF, presenting with fever, petechiae, gingival bleeding, hematemesis, melaena, hypotension and shock. Timely supportive treatment in the form of fresh plasma (25.3% of the cases) or platelet (30.1%) or whole blood (13.2%) transfusions, or ionophores in the case of shock (9.6%) helped 75.9% of the patients recover. Eight patients (9.6%) showed severe dengue fever of grade 3 and 4, and two of these, both infants of age <1 yr, and in grade 4, had profound shock, and succumbed (mortality rate of 2.4%).

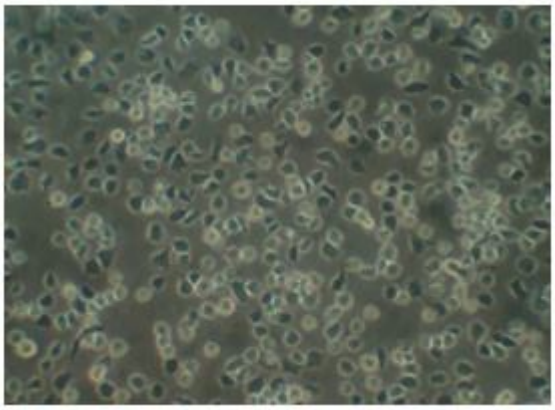
NS1Ag	IgM	IgG	Positive Cases
+	-	-	9 (10.8%)
+	+	-	6 (7.2%)
+	+	+	4 (4.8%)
+	-	+	17 (20.4%)
-	+	-	7 (8.4%
-	+	+	40 (48.1%)

Table 2. Antigen and serological profile of dengue cases (n = 83)

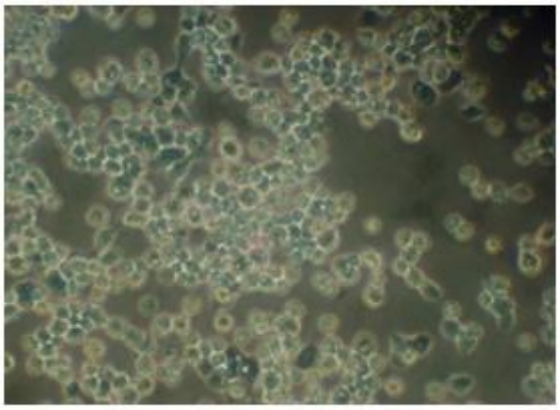
Eleven samples collected in 2011 were processed for virus isolation in C6/36 cells. Five showed cytopathic effects upon blind passaging the infected cell supernatants three times. First the cells became rounded and swollen, then small aggregates appeared. After 2-3 days multinucleated giant cells and cell debris were observed (Fig. 1).

Infected cells at initial stage of infection were subjected to immunoperoxidase test to detect intracellular NS1 (Fig. 2) to confirm DENV infection. Infected cell culture supernatants were then subjected to RT-PCR (Fig. 3), where five samples were found to be positive for DENV1. The 54 positive serum samples (NS1Ag and /or IgM and/or IgG) from 2012 were directly subjected to RT-PCR. Whereas two and six samples were positive for DENV1 and DENV3, respectively, ten patients had co-infection with DENV1 and DENV3. Detection by RT-PCR was better with samples received during the acute stage of the disease i.e., on 4th day of fever. Virus could be detected up to 9th day of fever. Hemorrhagic complications were more associated with co-infections (DENV 1 and DENV3) than with monotypic infection.

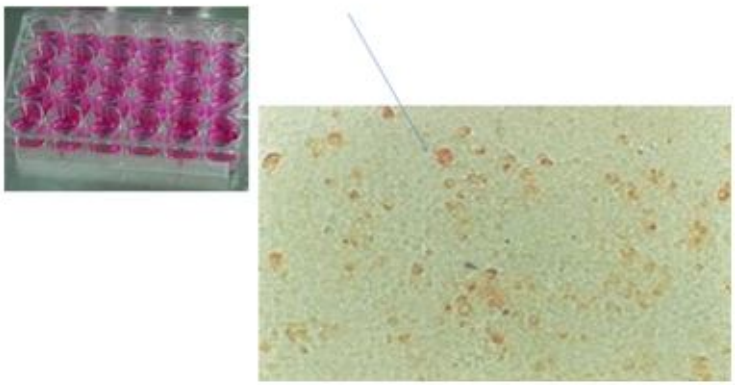
Figure 1. Cytopathic effect and antigen detection in cultured cells. Positive serum samples were diluted 1:50, inoculated onto C6/36 cells. Uninoculated cells (A) and cells infected 5 days earlier (B) are shown. In C, cells infected for 3 days in a multiwell plate were fixed, permeabilized and reacted with anti-NS1 antibodies and secondary antibodies conjugated to horse radish peroxidase before adding the chromogenic substrate.



(A)



(B)



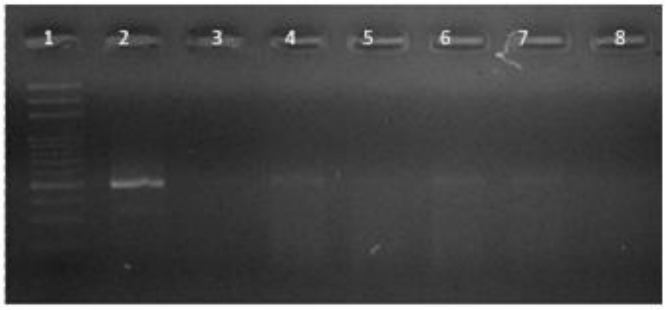
(C)

Figure 2. Detection of dengue virus genome and serotyping by RT-PCR. RT-PCR was standardized using RNA extracted from reference serotype viruses. The group-specific 511 bp segment was amplified with all the four serotypes (A) and the different serotypes could be differentiated by multiplex PCR (B).

A: Lane 1 – 100 bp ladder, Lane 2– DENV1positive control; Lane 4 –7-sample positive for dengue virus; Lane 3 – negative sample;

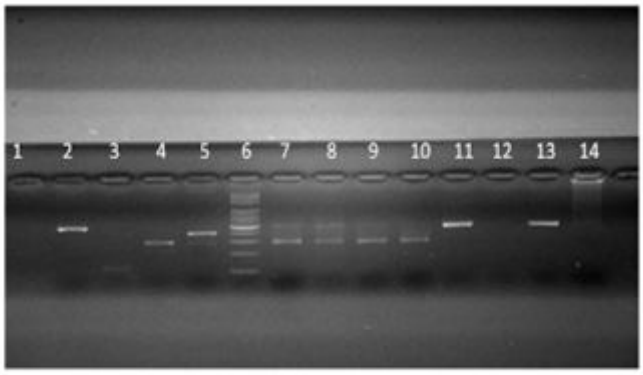
B: Nested PCR analysis of dengue virus isolates

Lane 1-negative control, lane 2-5 –positive controls for each serotype(DENV1-4),lane 6-100bp ladder,lane11 and 13-dengue virus serotype 1,lane 9 and 10- dengue virus serotype 3 ,lane 7and 8-dengue virus serotypes 1 and 3



(A)

Gel Documentation of RTPCR Analysis showing Dengue Suspected patient serum samples (lane 4-7) showing the presence of Dengue Virus Specific 511bp amplicon, Lane 1-100 bp DNA ladder, lane 3-Samples negative for 511bp amplicon, Lane 2 DENV 1Positive Control, Lane 8-negative Control



(B)

Nested PCR Analysis of Dengue Virus isolates showing the presence of Dengue Virus Serotypes 1(lane 11 & 13), Dengue Virus Serotype 3 (lane 9&10) both DENV 1 & DENV 3 (lane 7, 8), lane 2-5, showing +ve control for each serotype (DENV 1-4), Lane 1-negative Control lane 6 to 100bp DNA ladder.

Discussion

Dengue is an endemic disease in India and is associated with high morbidity and mortality. In our study, the prevalence of dengue was 35%, similar to those previously reported for North India11 and Vietnam13 but lower than that in Pakistan.14 Most of the cases (44.3%) were from urban slum areas with high population density and rapid development activities, factors which are favourable for dengue transmission.7 The male-to-female ratio of 1.59:1 was also similar to those reported previously in India1,11 and Malaysia,16 possibly reflecting outdoor occupational and recreational activities. In addition, most patients in our study belonged to the age group 21-30 years, with a mean of 22.4 years, supporting the results of Jdeveries et al.13 With household mosquito elimination programmes on an overdrive, DENV carrying Aedes mosquitoes seemingly infect young employed population which is mostly outdoors.

Rash (33.7%) and positive tourniquet test (19.2%) were infrequent findings among our patients. Other abnormal findings included hepatomegaly (31.3%) and splenomegaly (6%), and abnormal liver transaminases (57.8%), similar to observations in Bangladesh19 and Lucknow.26 The most significant laboratory abnormality was thrombocytopenia (83.1%), as observed in other studies,18,20 a finding attributable to depression of bone marrow during acute stage of DENV infection.

The IgM ELISA detected 57 out of the 83 samples whereas Ayub et al18 have reported positive serology for IgM in 48.75% of the patients in Saudi Arabia. Since we could not collect paired samples, presence of only dengue IgG antibodies without the presence of NS1Ag or IgM antibody were not considered as as dengue cases due to the confounding issue of high cross-reactivity of IgG antibodies.

On the other hand, presence of IgG with or without IgM is suggestive of secondary dengue infection, and by this interpretation, such cases in our study amounted to 65%. Using this single criteria, a previous study in our institute calculated the prevalence of secondary dengue to be 70% (unpublished data).

In our study, NS1Ag test detected nine cases which were negative by IgM ELISA, supporting the notion that a good number of cases (10% in our case) would be missed if NS1Ag ELISA is not included in the test panel.

The age demographics of primary and secondary dengue fever, i.e., 0-10 years and 21-30 years, respectively, observed in our study was similar to those reported from other parts of the country1,22.

Two infants succumbed to DSS in our study, suggesting that patients aged 6-12 months may be at high risk because the maternal antibodies may have dipped below protective levels21. Similar mortality rates were reported in Bangkok24 (2.2%) and Mumbai20 (2.9%).

Isolation of virus in cell culture or detection of viral genome by RT-PCR is considered as confirmatory diagnosis for dengue fever.2,24 In our study, 27.7% of the cases positive for NS1Ag and/or IgM and with fever lasting for nine days or less were positive for viral RNA, with the highest detection rate at 4th day of fever. Importantly, viral RNA was detected in all the nine samples positive for NS1Ag alone. On the other hand, viral RNA was detected only in 40%, 15% and 20% of the IgM, IgG and IgM and IgG positive samples, corroborating the fact that viremia is decreasing as humoral immunity develops.

In this study, DENV1 and DENV3 infections were observed but not DENV4 in 2011 and 2012. In addition, DENV1 and DENV3 co-infections were detected in 18.5% cases in the year 2012, with higher tendency for hemorrhagic manifestations. Previous studies have shown that DENV2 and DENV3 serotypes were associated with haemorrhagic manifestations whereas Chandrakanta et al26 reported that 60% cases of DHF were associated with DENV1. Concurrent infections may present the possibility of recombination between viruses leading to the emergence of more virulent strains, underscoring the need for continued surveillance and serotyping.

Conclusion

During the anticipated season, dengue represented 35% of the pyrexic cases. Application of IgM or NS1Ag ELISA detected only 43.3% when compared to declaring positivity with either one, although NS1Ag ELISA was better than IgM ELISA. Primary and secondary dengue fever were more frequent in the younger (0-10 yr) and young adult (21-30 yr) age group, respectively, and the disease proved fatal to infants under one year of age.

DENV1 was the major circulating serotype in 2011 whereas DENV1 and DENV3 were found co-circulating in ten of 18 cases in 2012. Hemorrhagic manifestations were more common in patients having co-infections.

Acknowledgements

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