Molecular detection and characterization of dengue isolates circulating in north India

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ABSTRACT

Background and Objectives: In recent decades, the incidence of dengue has increased dramatically. In dengue-endemic countries, changes in dengue virus serotypes, genotypes, and lineages have been reported. This study was designed to detect and characterize the dengue virus isolates circulating in North India by serological and molecular techniques.
Materials and Methods: This study was conducted at the Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India. NS1 antigen and IgM antibody against dengue were detected by ELISA methods, viral RNA was extracted and amplified by conventional PCR and one-step single-tube multiplex PCR. The purified PCR products were cycle sequenced and a database search was implemented for the confirmation of the sequence product. Phylogenetic analysis was carried out with previously reported sequences.
Results: Among 1509 samples, 205 (13.6%) were found positive for IgM antibodies with the highest number (n=67) among the 21 to 30 years age group with peak positivity during post-monsoon months. Among acute samples, NS1 antigen was positive in 62.9%. Seven patients out of 13 had dengue viral RNA in PCR. It comprised six DENV-2 serotypes and one DENV-3 serotype. On phylogenetic analysis, DENV-2 strains grouped with genotype IV and DENV-3 with genotype III.
Conclusion: Dengue infection was found frequently during post-monsoon season. The positivity rate of the dengue NS1 antigen test was greater than that of the antibody test. The dengue isolates were characterized as genotype IV and genotype III of DENV-2 and DENV-3 respectively.

Keywords: Dengue virus; Serotype; Genotype; Immunoglobulin M; Antigens

INTRODUCTION

Globally, dengue, the mosquito transmissible viral infection spreads by the bite of infected *Aedes* mosquitoes with a higher number of areas at risk (1). In recent decades, the global dengue incidence has in- creased dramatically. About 50% of the population in the world are at risk where 100-400 million infections are reported yearly (2). The majority of cases are asymptomatic or mild, self-limited, and misdiagnosed due to other febrile illnesses (3). According to Bhatt et al. (4), the *Dengue virus* (DENV) infects

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390 million population yearly with clinical manifestations in about 96 million with various degrees of severity. Thus, 129 countries are at risk where Asia contributes 70% of the disease burden (4, 5). The World Health Organization (WHO) reported an increase of eight-fold over the last two decades, over 4.2 million in 2019 from 505,430 cases in 2000. The death rate was increased to 4032 from 960 between 2000 and 2015 (2). The disability-adjusted life-years (DALY) in dengue accounted for 1.14 million cases in 2013 with 52% of the disease burden existed in the Southeast Asia region according to the Global Burden of Disease study. In 2010, out of 96 million asymptomatic dengue cases globally, 34% of the cases were contributed by India. The case-fatality rate was 2.6% (95% CI 2.0-3.4) according to the published meta-analysis from India (6).

Dengue virus exists in four genetically and antigenically distinct serotypes (DENV 1- 4) under the family *Flaviviridae*. DENV is approximately 10.7 kb containing a single open reading frame. This encodes for three structural proteins and seven non-structural proteins (7). Clinically the disease ranges from asymptomatic infection to self-limiting disease and complicated dengue, the dengue shock syndrome/ dengue hemorrhagic fever (DSS/DHF) (8). In addition to the serotypes, even the genotypes within each dengue serotype may influence the severity of DENV infection (9). In dengue-endemic countries, changes of DENV serotypes, genotypes, and lineages have been reported and associated with variations in disease severity. This emphasizes the need for improving surveillance to monitor the re-emergence of DENV strains that may cause outbreaks (10). Dengue is endemic in India and was responsible for many outbreaks in the past where DENV-2 was attributed in major outbreaks. From 2003 onwards, there has been a shift from DENV-2 to DENV-3 in the outbreaks, which circulated predominantly in North India. So far there have been few comprehensive molecular epidemiological studies describing the circulating genotypes of DENV in North India (11, 12).

Therefore, this study was designed to detect and characterize the dengue virus isolates circulating in North India by serological and molecular techniques.

MATERIALS AND METHODS

Study design and setting. This work was carried out in the Department of Virology, Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh, India from August to December 2013. The blood samples from dengue suspected patients received from outpatient departments, emergency, and inpatient departments of PGIMER, Chandigarh for routine dengue diagnosis were enrolled and tested. Sera from Government Medical College (GMC), Jammu and Sentinel Surveillance Hospital (SSH), Punjab under quality control programme as the Apex referral laboratory activity of National Vector Borne Disease Control Programme (NVBDCP), New Delhi were also included in the study. The samples collected from dengue suspected patients with less than five days of fever were categorized as acute samples. Both male and female patients with febrile illness were included in the study. Only acute cases were included for antigen test while the patients having beyond 5 days of symptoms were subjected to Dengue IgM tests. All the samples were transported in an icebox to the virology laboratory. The samples were recorded, sera centrifuged under refrigeration, and preserved in -70°C deep freezer until the test was completed.

Dengue IgM antibody testing. Dengue IgM antibody was detected in dengue suspected patients by dengue IgM Antibody Capture ELISA (MAC-ELISA) kit. The kit is developed by "National Institute of Virology (NIV)" (Pune, India) for the qualitative testing of dengue IgM antibodies in serum. A 50 µl of each 1:100 diluted patient serum sample, positive control, and negative control was tested by the procedures provided in the manufacturer's protocol. The absorbance of reaction termination was measured within 10 minutes at 450 nm in an ELISA reader. The samples with optical density (OD) ≤ 2 times than the OD of negative control were reported as "Negative". The samples with OD ≥ 3 times than the OD of negative control were reported as "Positive". If the OD was in between the above two values, it was considered as "Equivocal". The invalid tests were repeated (13, 14).

Dengue NS1 antigen. Acute samples collected within five days of symptoms were tested for NS1 antigen by Panbio Dengue Early ELISA (Brisbane, Australia). Panbio Dengue ELISA is an NS1 antigen test in sera of patients with clinical symptoms consistent with dengue fever. A 100 µl of each 1:2 diluted patient serum sample, calibrators, positive control, and negative control was tested according to the kit instruc-

tions. The absorbance of reaction termination was measured within 30 minutes at 450 nm in an ELISA reader. Panbio Units >11.0 were reported as "Positive" (an active primary or secondary dengue infection) and Panbio Units <9.0 were reported as "Negative" while Panbio Units between 9.0-11.0 were considered as "Equivocal" (15, 16).

Molecular detection and characterization of dengue isolates. The samples having positive NS1 antigen were subjected for viral RNA detection by conventional reverse-transcriptase polymerase reaction (RT-PCR) assay.

Viral RNA extraction. The viral RNA was extracted and purified by using a commercially available RNA extraction kit (KIAamp® Viral RNA Extraction Kit, Qiagen, Germany). The purified template was collected in 60 µl of eluent buffer and preserved at -80°C deep freezer until use (11, 17).

cDNA synthesis. Extracted RNA was subjected to cDNA synthesis by reverse transcription using a mixture of oligonucleotides of random hexamer primers. For the synthesis of cDNA, a commercially available "Thermo Scientific RevertAid First Strand cDNA Synthesis Kit" was used. The reverse transcription reaction product was stored at -70°C for the further process (11, 18).

Dengue conventional PCR for detection of C-prM gene. Conventional dengue PCR assays were performed on cDNA using dengue virus group-specific consensus primers D1 and D2 (Table 1). 50 µl Reaction mixture containing buffer (5 µl), dNTP (5 µl), $MgCl_2$ (3 µl), D1 [10 µM (2.5 µl)], D2 [10 µM (2.5 µl)], Taq polymerase [1U/µl (1.5 µl), DEPC treated water (25.5 µl), and cDNA (5 µl) was amplified. The thermal profile for the amplification of the C-prm gene was: denaturation at 94°C for 30 seconds, annealing at 55°C for 60 seconds, and extension at 72°C for 2 minutes with each step of 35 cycles. The PCR products were visualized in agarose gel electrophoresis (19, 20).

Single-tube dengue multiplex PCR for serotyping. A single-tube multiplex PCR was carried out with cDNA with forward D1 primer and four serotype-specific reverse primers (TS1-TS4) (Table 1). 50 µl reaction volume containing Taq buffer (5 µl),

dNTP (5 µl), $MgCl_2$ (3 µl), D1, TS1, TS2, TS3, and TS4 [10 µM (2.5 µl each)], Taq polymerase [1U/µl (1.5 µl)], DEPC treated water (18 µl), and cDNA (5 µl) was subjected for amplification with the thermal profiling as under denaturation at 94°C for 30 seconds, annealing at 55°C for 45 seconds, and extension at 72°C for 2 minutes with each step of 35 cycles followed by product visualization in gel electrophoresis (19, 20).

Agarose gel electrophoresis. The amplified products were visualized in 2% agarose gel stained with ethidium bromide (10 mg/ml).

Sequencing and genotyping. The purified products of 6 samples were cycle sequenced in ABI PRISM 310 genetic analyzer using the Big Dye Terminator cycle sequencing method. For confirmation of the sequence product database search using the BLAST program was carried out. For genotyping of the dengue strains, phylogenetic analysis was carried out with previously reported sequences available in the gene bank using the neighbor-joining method with a bootstrap value of 1000 replicates (11).

Ethical consideration. The ethical approval for the study was obtained from the Institutional Ethics Committee of the PGIMER, Chandigarh, India.

Statistical analysis. The data generated during the study were entered and analyzed by using Microsoft Office Excel Sheet.

RESULTS

During the 2013 dengue epidemic, a total of 1548 samples were processed which included 1499 samples from different wards of PGIMER for routine dengue diagnosis, 34 acute samples from SSH, Punjab for NS1 antigen detection, and 15 samples from Government Medical College (GMC), Jammu, out of which 10 were for routine dengue diagnosis and five were known dengue positive acute samples.

A total of 1509 samples (1499 from PGIMER and 10 from GMC, Jammu) were processed for IgM antibody detection. Out of 1509 cases, 205 (13.6%) were found positive for IgM antibodies. Among 205 IgM-positive patients, the maximum (n=67) were from the 21 to 30 years age group and the minimum (n=31) had ≥40 years of age (Fig. 1). The post-monsoon seasonal

Table 1. Oligonucleotide primers used to amplify and type dengue viruses

Primers	Sequence (5'-3')	Product size, in bp
D1	TCAATATGCTGAAACGCGCGAGAAACCG	511
D2	TTGCACCAACAGTCAATGTCTTCAGGTTC	511
TS1	CGTCTCAGTGATCCGGGGG	482
TS2	CGCCACAAGGGCCATGAACAG	119
TS3	TAACATCATCATGAGACAGAGC	290
TS4	CTCTGTTGTCTTAAACAAGAGA	392

transmission of dengue is being reflected by its peak positivity during September to November (positivity rate 12.2% to 19.7%) (Fig. 2).

Out of a total of 1548 cases, 62 samples (23 from PGIMER, 34 from SSH, Punjab, and five known cases from GMC, Jammu) were categorized as acute samples as they had a history of less than five days of symptoms. The NS1 antigen was detected in 39.1% (9/23), 73.5% (25/34), and 100% (5/5) of cases from PGIMER, SSH, Punjab and GMC, Jammu, respectively (Table 2).

Among 39 NS1 positive samples, only 13 (eight from PGIMER and five from GMC, Jammu) were subjected to the detection of viral RNA by conventional RT-PCR because of budget constraints. Dengue viral RNA was detected in 75.0% (six out of eight) from PGIMER cases and 20.0% (one out of five) from GMC, Jammu. Serotyping was done by single-tube dengue multiplex PCR on seven dengue isolates that were positive on conventional PCR. Among them, DENV-2 serotypes were detected in six positive samples (five from PGIMER and one from GMC, Jammu) while one DENV-3 serotype was isolated from PGIMER (Fig. 3). On performing the phylogenetic analysis, DENV-2 strains clustered with DENV-2: genotype IV (n=5) and DENV-3 strain clustered with DENV-3: genotype III (n=1) (Fig. 4).

DISCUSSION

There has been a 30-fold increase in the incidence of dengue in the recent past in addition to the progression of transmission to new countries and also from urban to rural settings (4, 21). The increasing incidence of dengue infection causes significant mortality and morbidity especially in resource-poor nations with an additional burden on the economy (14). In 2012, WHO classified dengue as the most important mosquito-transmitted viral infection. Its spread into different geographic areas and the introduction of vector in virgin areas increasing the disease burden are important factors (22, 23). Due to the unavailability of specific treatment or vaccine for the prevention of dengue, diagnosis of dengue in time would help in the proper management of patients (24).

The current study reports the investigation of dengue outbreaks in 2013 in North India and the characterization of the representative strains. In our study, the highest number of cases were observed during November and the majority were young adults in the age group 21 to 30 years. The result of our study is supported by other observations in 2006 and 2007 (25). At the onset of the dengue fever and before the
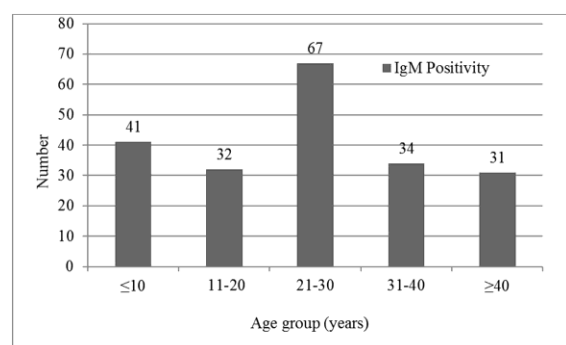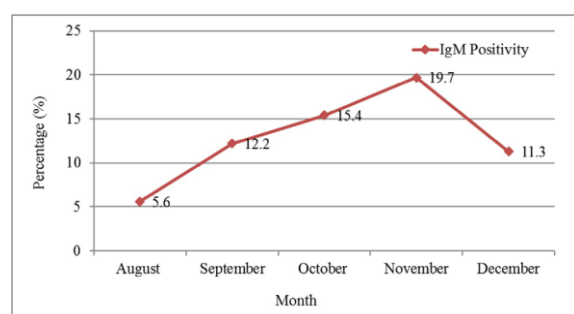

Fig. 1. Age-wise distribution of dengue IgM positive cases.

Fig. 2. Seasonal variation of dengue IgM positive cases.

Table 2. Percentage positivity of Dengue NS1 antigen and IgM antibody from different sites

Sample sites	NS1 antigen		MAC-ELISA (IgM antibody)	
	Number of samples	Positive (%)	Number of samples	Positive (%)
PGIMER	23	9 (39.1)	1499	197 (13.1)
SSH, Punjab	34	25 (73.5)	NT	NT
GMC, Jammu	5	5 (100)	10	8 (80.0)
Total	62	39 (62.9)	1509	205 (13.6)

*NT; not tested
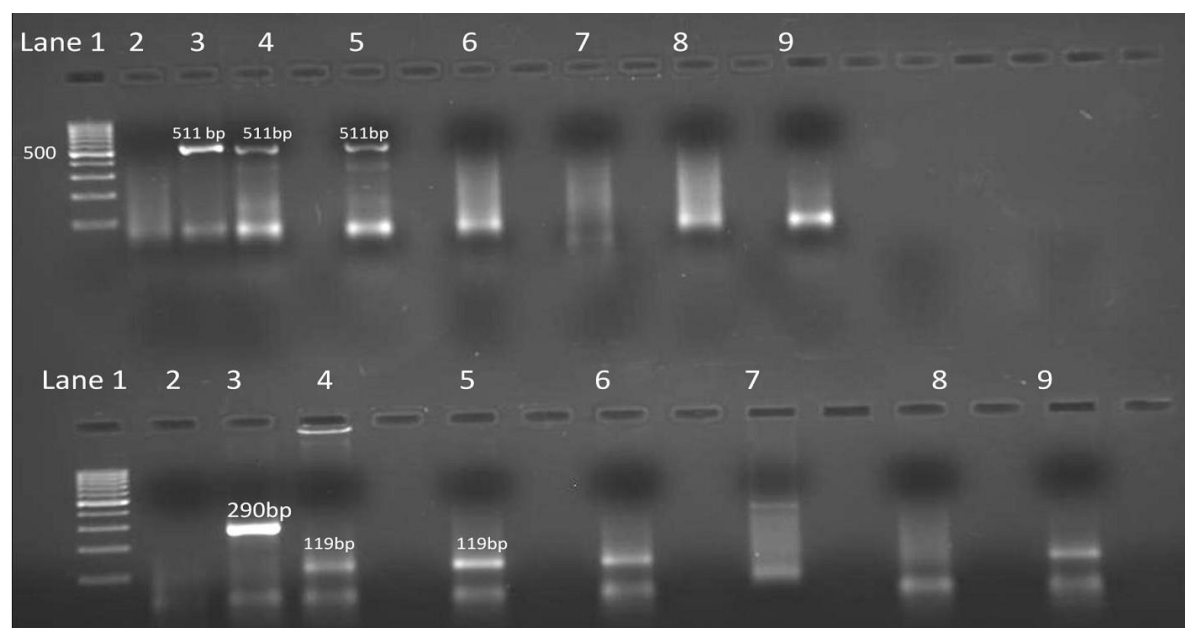
Fig. 3. PCR product analysis on 2% agarose gel. Upper; Conventional PCR positive: Lane 5 Sample1. Lane 1 MM, Lane 2 NC, Lane 3 PC1, Lane 4 PC2, Lane 5-9 Samples. Lower; Single-tube multiplex PCR positive: DENV 2 (Lane 5 Sample1). Lane1 MM, Lane 2 NC, Lane 3 PC1, Lane 4 PC2, Lane 5-9 Samples.

dengue IgM appears, detection of NS1 antigen of DENV can be chosen as a suitable option for dengue diagnosis. For the timely detection of dengue infection, WHO has recommended the use of an NS1 antigen detection test. In this study, only 13.6% of cases were detected by dengue IgM antibody test while the NS1 antigen test was positive in 62.9% of tested cases. The discrepancy in this result could be due to the fact that Dengue NS1 antigen detection was performed only on sera from patients with a history of less than five days of fever and clinical symptoms consistent with dengue fever resulting higher positivity rate (62.9%). However, Dengue IgM antibody detection was performed on all suspected patients, giving a low positivity rate (13.6%). The samples from acute patients are more likely to give a positive result in the NS1 antigen test, whereas suspected cases, among which antibody test was performed, likely to have other febrile illnesses and most of them tested negative in the antibody test. Kumar et al. (14) from Bundelkhand region, India also reported almost similar positivity rate of dengue infection by IgM antibody test (12.4%) but the lesser rate of NS1 antigen test (24.8%) and in the year 2015, Prakash et al. (26) also reported 22% dengue cases from Lucknow, Uttar Pradesh, India.

Virus isolation and viral RNA detection by RT-PCR are other methods of viral diagnosis. In the early part of dengue infection, isolation of the virus is the gold standard but is associated with low sensitivity and is time-consuming. In this study, a part of NS1 positive samples was subjected for detection of viral RNA by
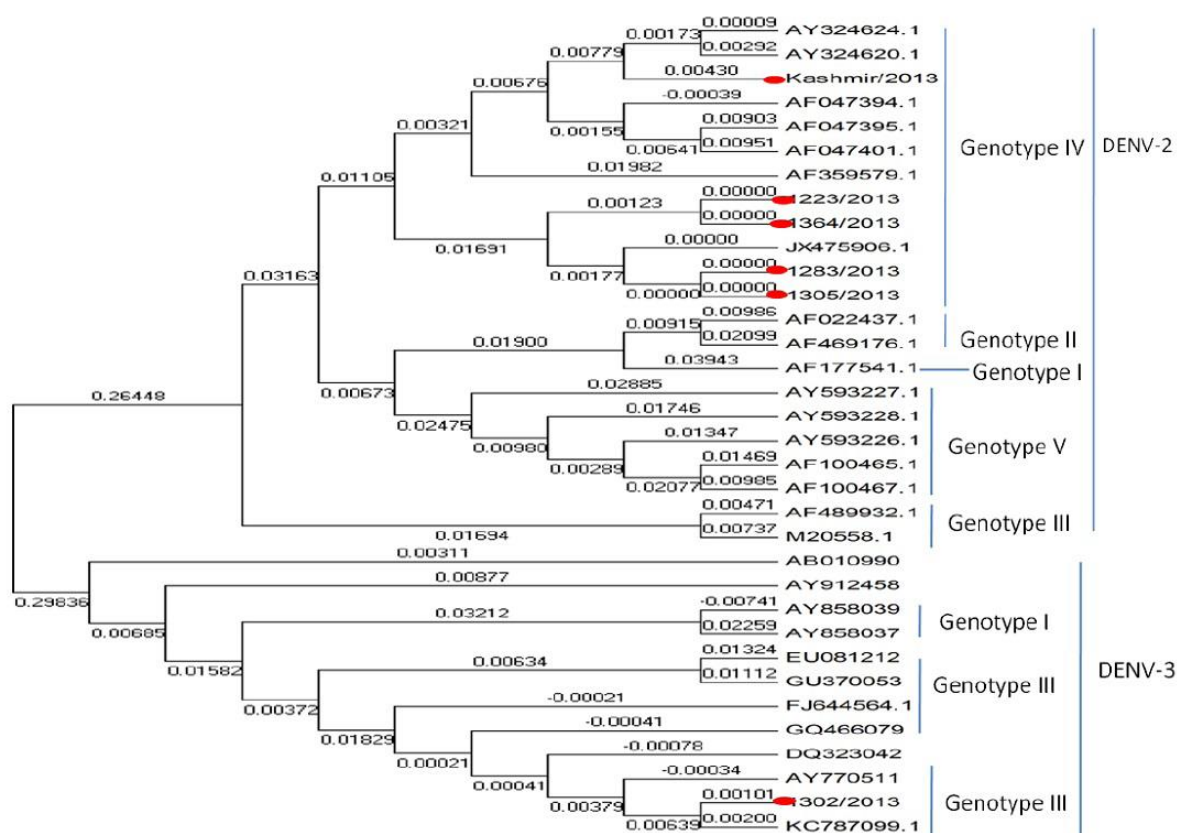
Fig. 4. Phylogenetic analysis of DENV isolates circulating in North India in 2013. On performing the phylogenetic analysis, DENV-2 strains clustered with DENV-2: genotype IV and DENV-3 strain clustered with DENV-3: genotype III.

conventional RT-PCR and the dengue serotype was identified by single-tube dengue multiplex PCR that was positive on conventional PCR. Dengue viral RNA was detected in 75.0% of NS1 positive cases. The dengue serotype identified in the current study was DENV-2 and DENV-3 whereas the outbreak reported in 2007 was due to the DENV-1 serotype (25) and DENV-2 was responsible agent in 2002 (27). The study from Delhi had reported concurrent infection by four serotypes during the 2006 outbreak where DENV-3 was predominated (28). Various studies from Delhi in the last 10 years identified the co-circulation of more than one serotype (29). However, we had not observed any co-infection.

On performing the phylogenetic analysis, DENV-2: genotype IV (n=5) and DENV-3: genotype III (n=1) were identified in our study. Various studies indicated the circulation of four dengue serotypes in India with DENV-2 and DENV-3 being commonly reported serotypes. Circulation of more than one serotype was reported by two-thirds of the studies (29). Among five genotypes of DENV-3, genotype III is predominant in India (11). The genotype V of DENV-2 strains might have been replaced by genotype IV and continues to circulate silently in North India with the potential to re-emerge as a major epidemic of dengue (30).

CONCLUSION

In this study, dengue infection was found frequently during post-monsoon season i.e. September to November. The positivity rate of the dengue NS1 antigen test was greater than that of the antibody test. The dengue isolates were characterized as DENV-2: genotype IV and DENV-3: genotype III. This type of molecular epidemiological study is of great significance in geographical areas where multiple dengue serotypes co-circulates simultaneously. Hence, active epidemiological surveillance is needed for molecular characterization of the dengue isolates to initiate effective control and management strategies in time.

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