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Prevalence and Detection of Dengue Serotyping by Real Time PCR at a Tertiary Care Hospital Udaipur Rajasthan

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Abstract

Background: Dengue fever is an emerging arboviral public health problem in a large endemic population in the tropical and sub-tropical areas of the world, with varying degrees of clinical presentation.All four dengue serotypes cause infection,with one of them predominantlyreported from a particular geographical region.This study was carried out to detect the predominant dengue serotype and presence of infections.

Aim and Objectives: The present study was conducted to determine theSero-Prevalence of dengue serotyping cases among dengue patients infection in a tertiary care hospital, Pacific Institute of Medical Science, UdaipurRajasthan.

Material and Methods: Acute-phase serum samples of patients suffering from dengue infection were collected.DengueNS1 antigen and IgM & IgG antibody against dengue virus detected by Rapid Dengue Duo Cassete kit and ELISA, and sample were carried out for serotyping of the dengue virus by the real time PCR.

Results:A total of 210 samples were positive by the Enzyme linked immunoassay.85 were positive only for IgM, 45 were positive only for IgG and 80 samples were positive for both IgM and IgG antibodies. A total of 15 cases were found to have infection with more than one dengue serotypes. Only one serotypes DENV 2 were detected in these samples by PCR. DENV-2 was found to be most common circulating serotype. Samples having only antibodies and no antigen on rapid card test were also positive for virus by PCR.

Conclusion: Prevalence of dengue infections is increasing. Moreover, it is important toscreen for dengue virus in those samples also which do not show NS1Ag on rapid tests andhave either one or both the antibodies. Real-time PCR is found to be more sensitive in detecting infection than Enzyme linked immunoassay.

Keywords:ELISA/rt-PCR, enzyme linked immunoassay,real time polymerase chain reaction

Introduction

Dengue virus infections, transmitted by mosquito bites, mainly Aedes aegypti, are very common and are reported from the majority of countries worldwide. Dengue virus infectionscan result in minor febrile illness in the majority of cases. However, it can also cause severe disease in the form of Dengue Hemorrhagic fever/Dengue shock syndrome (DHF/DSS).

There are Four known dengue virus serotypes, DENV-1, DENV-2, DENV-3, and DENV-4. All of these four serotypes are known to cause human infections. It is observed that one of four serotypes predominantly causes infections in a particular region as compared to the other serotypes. Secondary infections are known to be more severe in nature with more chances of development of DHF/DSS [1].There have been reports suggesting concurrent infection of dengue with more than one serotype [2, 3, 4, 5].However, its association with disease severity is not well understood. The prevalenceof dengue coinfections in India is not known. It is observed that, in hyper endemic regions, chances ofinfection with more than one serotype are much higher when multiple dengue serotypes cocirculate in a population. These cases of infection with multiple serotypes also pose a risk ofthe emergence of recombinant virus strains with distinct properties.

Diagnosis of dengue virus infection can be achieved by various modalities, including virus culture, antigen (NS1Ag), as well as antibody (IgM and IgG) detection with ELISA, immuno chromatographic assays, and molecular tests like RT-PCR, Real-Time PCR, and LAMP [7, 8].However, serological techniques like IgM ELISA do not detect the serotype of thevirus. The “gold standard” for serotyping of the dengue virus is viral culture followed by indirect immuno fluorescence. Serotyping can also be performed by plaque reduction neutralizationtechnique (PRNT) but is very difficult to perform and costly. The molecular methods based on PCR technique not only offer a suitable alternative to conventional viral isolation techniques but can also provide the rapid and reliable detection of serotype of the virus [9, 10].

Objectives: The aim of this study was to find out the predominant dengue virus serotype circulating in Udaipur during the study period and also to look for any infections. The present study was carried out utilizing two different Enzyme linked Immunoassay and molecular methods(Real-Time PCR) on serologically positive dengue cases.

Study Design: Cross-sectional analytic study.

Study Setting: Department of Microbiology at the Pacific Institute of Medical Science (PIMS) in Udaipur, Rajasthan.

Study duration: One year which includes time required for data collection, analysis and reportwriting. October 2021 to October 2022.

Study population: Participants in this study were required to have clinical symptoms that were compatible with dengue, and blood samples were collected from those participants.

Inclusion Criteria: The clinical foundation for diagnosing the patients as having dengue fever was based on standard criteria such as presentation of feverish illness of 2-7 days duration, with symptoms such as headache, myalgia, arthralgia, rash, hemorrhagic signs, and leucopenia.

Exclusion Criteria: Patients with febrile illness but diagnosed other than dengue

Material and methods

Samples

This retrospective observational study was carried out at a tertiary care center in Pecific Institute Medical Science, Udaipur Rajasthan. Institutional Ethics Committee clearance was taken for theuse of anonymized stored samples.All serum samples that were found to be positive for dengue antigen or antibody at the microbiology laboratory during routine clinical testingwere included in this study.

Principles of the procedure

The CDC DENV-1-4 Real-Time PCR Assay is usedon an ABI 7500 Fast Dx Real-Time PCR Instrument. The CDC DENV-1-4 Real-Time PCR Assay includes a set of oligonucleotide primers and dual-labeled hydrolysisprobes for *in vitro* qualitative detection of DENV serotypes 1, 2, 3 or 4 from serum or plasma collected from human patients with signs and symptoms consistent with dengue (mild or severe). The targeted regions of viral RNA are transcribed into complementary (cDNA) and amplified by the polymerase chain reaction (PCR). The fluorescently labeled probes anneal to amplified DNA fragments and the fluorescent signal intensity is monitored by the ABI 7500 Fast Dx instrument during each PCR cycle. Amplification of target is recorded as increase of fluorescence over time in comparison to background signal.

A positive control virus mix is also included, which consists of heat-inactivated DENV-1 Haw, DENV-2 NGC, DENV-3 H87, and DENV-4 H241. A Human Specimen Control (HSC) is a noninfectious cultured human cell material that provides a positive signal in the assay and demonstrates successful recovery of RNA as well as the integrity of the RNA extraction reagent. The human RNase P RNA (RP) is present in cultured cell material and in most clinical samples and detectable by RT-PCR using the primers and probes provided. The CDC DENV-1-4 Real-Time RT-PCR Assay can be run in singleplex (each DENV serotype detected in a separate reaction) or in multiplex (the four DENV serotypes are run in the same reaction). These two formats provide equal sensitivity.

Summary of Dengue Testing RTPCR Process

Resuspend Primers and Probes, aliquot and store -20˚C

↓

Extract Samples RNA and HSC RNA

↓

Prepare Master Mix (20 µL)

↓

Prepare RT-PCR Plate (5 µL RNA)

↓

Run CDC RT-PCR Assay on ABI 7500 Fast

Analyze Data

Report Results

Procedure

Nucleic acid extraction QIAamp Viral RNA mini kit (Qiagen, Germany) was used accordingto the manufacturer's protocol for the Viral RNA extraction from the serum samples. The RNA was eluted in 60 ml of elution buffer. This RNA was subsequently stored at -80˚C till further use.

Conventional multiplex RT-PCR

The samples were subjected to two-step conventional multiplex RT-PCR. In the first step, the viral RNA was converted to cDNA, and subsequently, the multiplex cDNA PCR was carriedOut. High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, California, USA) was used as per the manufacturer's instructions to synthesize cDNA from the RNA extracted from the samples. The reaction was run with a final volume of 20 ml containing 10 ml of the RT master mixand 10µl of RNA.The reaction was carried out with cycling conditions of 25˚C for 10 min, 37˚C for 120 min, 85 _C for 5 min and finally stored at 4˚C for a short time and -80˚C for longer storage.

Table1: DETECTION KIT (PRIMER AND PROBE SETS)

(Store at 2-8 ºC in PCR Reagent Preparation Area) Label	Part #	Description	Quantity/ Tube	Reactions/ Tube
D1-F	SO3504	DENV-1 Forward Primer	5 nmol	200
D1-R	SO3505	DENV-1 Reverse Primer	5 nmol	200
D2-F	SO3507	DENV-2 Forward Primer	5 nmol	200
D2-R	SO3508	DENV-2 Reverse Primer	5 nmol	200
D3-F	SO3510	DENV-3 Forward Primer	5 nmol	200
D3-R	SO3511	DENV-3 Reverse Primer	5 nmol	200
D4-F	SO3513	DENV-4 Forward Primer	5 nmol	200
D4 R	SO3514	DENV-4 Reverse Primer	5 nmol	200
RP-F	SO2669	RNase P Forward Primer	5 nmol	200
RP-R	SO2670	RNase P Reverse Primer	5 nmol	200
D1-Probe	SO3506	DENV-1 Probe	1 nmol	200
D2-Probe	SO3509	DENV-2 Probe	1 nmol	200
D3-Probe	SO3512	DENV-3 Probe	1 nmol	200
D4-Probe	SO3515	DENV-4 Probe	1 nmol	200
RP-Probe	SO3516	RNase P Probe	1 nmol	200

Master Mix Preparation/Plate Setup: Prepare Master Mix according to the following:

Table2: MULTIPLEX DENV-1,2,3,4 REACTION

Reagent	Volume	Volume/rx	Total Number Reactions (N)	Total Volume
Nuclease-free Water	2.2 µL	N x 2.2 µL	N = 10 + 1 = 11	24.2 µL
2X premix	12.5 µL	N x 12.5 µL	N = 10 + 1 = 11	137.50 µL
Primer D1-F	0.5 µL	N x 0.5 µL	N = 10 + 1 = 11	5.5 µL
Primer D1-R	0.5 µL	N x 0.5 µL	N = 10 + 1 = 11	5.5 µL
Primer D2-F	0.25 µL	N x 0.25 µL	N = 10 + 1 = 11	2.75 µL
Primer D2-R	0.25 µL	N x 0.25 µL	N = 10 + 1 = 11	2.75 µL
Primer D3-F	0.5 µL	N x 0.5 µL	N = 10 + 1 = 11	5.5 µL
Primer D3-R	0.5 µL	N x 0.5 µL	N = 10 + 1 = 11	5.5 µL
Primer D4-F	0.25 µL	N x 0.25 µL	N = 10 + 1 = 11	2.75 µL
Primer D4-R	0.25 µL	N x 0.25 µL	N = 10 + 1 = 11	2.75 µL
Probes (DENV-1-4)	0.45 µL	N x 0.45 µL	N = 10 + 1 = 11	4.95 µL
SuperScriptTM III RT/Platinum®Taq Mix	0.5 µL	N x 0.5 µL	N = 10 + 1 = 11	5.5 µL
Total Volume	20 µL	N x 20 µL	220 µL	

Prepare Mix on ice and add template.

a) Place PCR plate on ice.

b) Add 20 µL of Master Mix to each well.

c) Add 5 µL of extracted sample, including HSC samples.

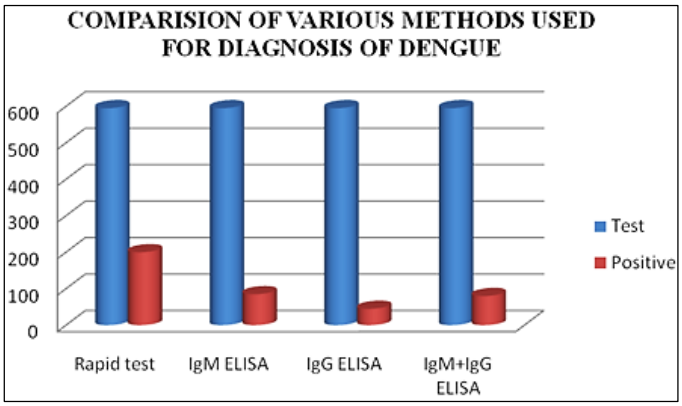
d) Seal with optical 8-cap strip and place plate in **Applied Biosystems 7500 Fast DX** Real-time PCR System.

REASULT

- In the present study Rapid dengue duo cassette method, IgM ELISA, and IgG ELISA were done for 595 samples (Table-3,4). Rapid test was positive total 200 samples (33.61%), IgM ELISA in 85 samples(14.28%) and IgG ELISA in 45 (7.56%) samples and both IgM+IgG ELISA in 80(13.44%). The detection of dengue cases was more specificity and sensitivity by RT-PCR method when compared to the other two methods in this study.

Tabel3: COMPARISION OF VARIOUS METHODS USED FOR DIAGNOSIS OF DENGUE

Methods	Tested	Positive	Percentage(%)
Rapid test	595	200	33.61
IgM ELISA	595	85	14.28
IgG ELISA	595	45	7.56
IgM+IgG ELISA	595	80	13.44



Tabel4: ANTIBODY RESULTS IN EARLY & LATEFEBRILE PERIOD (n=210)

Duration	IgM ELISA	Percentage%	IgG ELISA	Percentage%	Both Positive	%
1-5 days (n=100`)	70	70	10	10	20	20
6-10 days (n=70)	5	7.14	15	21.4	50	71.4
>10 days (n=30)	10	25	20	50	10	25

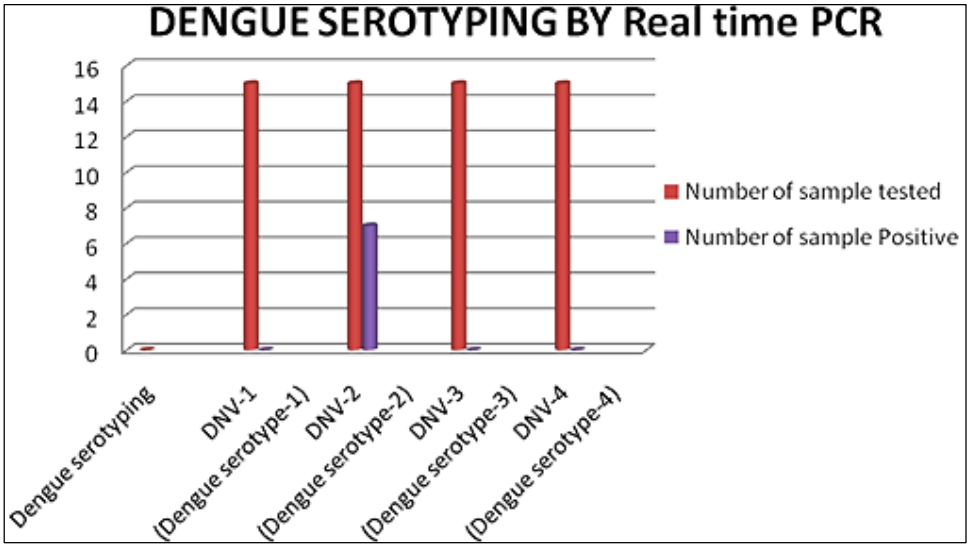
Total % of Antibody detection	IgM-40.47%	IgG-21.4%	Both-38.09%

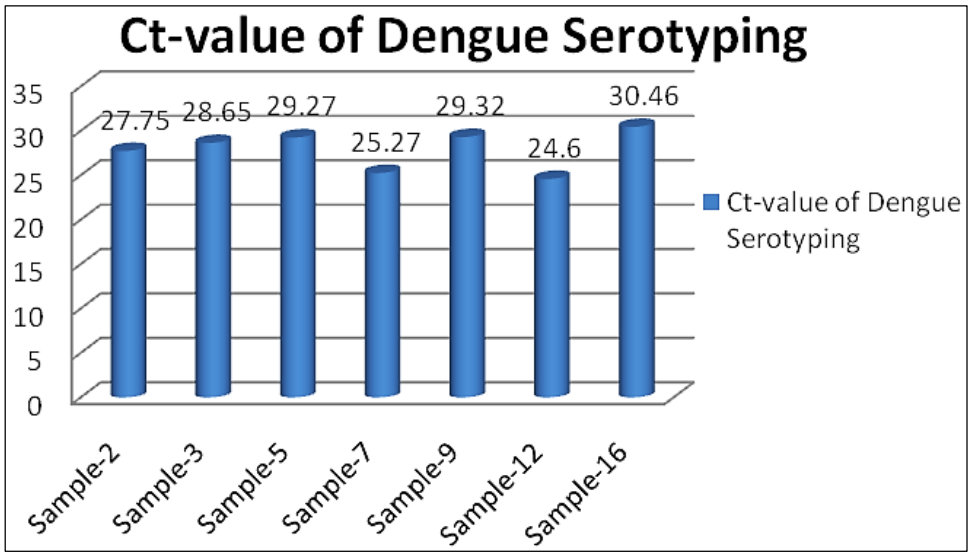
Real-time multiplex RT-PCR assay-

- Out of the total of 210 dengue antibodypositive samples, serotyping was conducted on 15 infection dengue sample,amongtotal 7serum samples were detected positive for DENV 2 Serotype, remaining samples were undetermined.(Table-5)All samples were positive for RNase-P internal control.

Table 5: DENGUE SEROTYPING (N-15)

ID	**Sample ID**	**Ct-value of Dengue Serotyping**	**Serotype**
1.	Sample-1	--	Undetermined
2.	Sample-2	27.75	DENV 2
3.	Sample-3	28.65	DENV 2
4.	Sample-4	--	Undetermined
5.	Sample-5	29.27	DENV 2
6.	Sample-7	25.27	DENV 2
7.	Sample-8	--	Undetermined
8.	Sample-9	29.32	DENV 2
9.	Sample-10	--	Undetermined
10.	Sample-12	24.6	DENV 2
11.	Sample-13	--	Undetermined
12.	Sample-15	--	Undetermined
13.	Sample-16	30.46	DENV 2
14.	Sample-17	--	Undetermined
15.	Sample-18	--	Undetermined
16.	PC	9.65 7.56 12.75 8.65	DENV1($2*10^8$copies) DENV2($2*10^8$copies) DENV3($2*10^8$copies) DENV4($2*10^8$copies)





Discussion

Enzyme-linked immunosorbent assaytest for IgM or IgG antibodies against dengue virus is commonly used for diagnosing dengue virus infection. However, antibody formation up to detectable levels may take some time after the onset of the illness. Thereby antibody-based tests do not provide immediate information for the management of the patients. As dengue patients often present within three days after onset of symptoms, false-negative results with antibody-basedassays remain a concern [11].ELISAs take a relatively long time for the result. Hence, rapidtests are being used as they provide the results within a short time. Simple, rapid tests that can give the results within 15 to 30 min and can be used as point-of-care tests are the need of the hour, especially in resource-limited settings [11].

In this study, only one serotypes DENV 2 were found in this study from samples dengue positive.Reddy *et al*. have also reported circulation of all the four dengue virus serotypes from Kerala, a southern Indian state, in the year 2013. However, in the years 2014 and 2015, they found only three serotypes (except DENV-4) to be circulating [12].Similarly, Shrivastava *et al*. from Pune have reported circulation of all four dengue virus serotypes in the year 2016. Arshi *et al*. have reported circulation of all the serotypes in Delhi in 2015 [13].Other authors have reported circulation of only DENV-1 and DENV-2 in Delhi in the year 2012 with a maximum number of cases caused by DENV-1. Another study from Delhi, which included samples between 2011 and 2014, demonstrated the circulation of three serotypes, including DENV-1, DENV-2, and DENV-3 with DENV-2 being the most common serotype [14].Other large cities like Pune in Maharashtra have also reported DENV-2 as the most common serotype in 2014 [14].

Conclusion

Dengue virus infections are increasing every year in almost all the states of the country. All four dengue virus serotypes are now found circulating in the same season. Multiple DENV causing infection are now reported more frequently than ever and need to be considered with more clinical data and outcomes. Multiplex Real-time RT-PCR is found to be more sensitive as compared to other dengue testin detection and serotyping of dengue virus infections. As thereis no vaccine available in India against dengue presently, while there is an abundance of the vector responsible for the transmission of the disease, effective surveillance becomes very important for the appropriate and timely management of cases and the necessary public health response.

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Conflict of interest: None.

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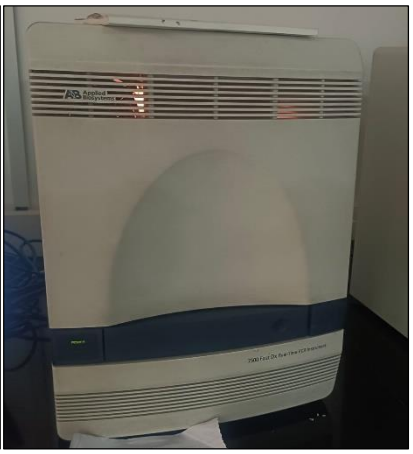

TimePCR Machine

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