Simultaneous detection and serotyping of dengue infection using single tube multiplex CDC Dengue Real-Time RT-PCR from India

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Abstract Four antigenically different dengue virus serotypes (DENV-1, DENV-2, DENV-3 and DENV-4) are known to cause infections in humans. Some of these are known to cause more severe disease than the others. Chances for developing Dengue hemorrhagic fever-dengue shock syndrome (DHF-DSS) increases significantly with history of previous infection with one of the four serotypes. Therefore, early diagnosis, serotyping and providing early warning of dengue fever epidemics to concerned authorities becomes very important for better patient outcome and to curb the rapid spread in the community. During the 2014 outbreak, a total of 100 samples from suspected cases of dengue were collected. NS1 antigen based rapid test was used for serological diagnosis. Dengue complex one step reverse transcription-polymerase chain reaction was performed to look for presence of viral RNA. Single tube multiplex RT-PCR was also performed to look for infecting serotype. CDC Dengue Multiplex Real Time PCR assay was performed for rapid diagnosis and simultaneous serotyping of the dengue virus. Out of the 100 samples screened, 69 were found to be positive by NS1Ag Rapid test. 34 samples were found positive by dengue consensus

RT-PCR assay. 22 samples were found to be positive by single tube Dengue multiplex RT-PCR assay. Serotype DEN-2 was present in maximum numbers followed by DEN-3. 44 samples were found positive by DENV CDC Multiplex Real time PCR assay. DEN-2 was found in maximum numbers followed by DEN-1. Dengue remains to be an important health problem in India and across the globe. Few serotypes of dengue are more dangerous than the others. Rapid diagnosis and serotyping remains the key for better patient management and prevention of disease spreading in the community. Highly sensitive, specific and rapid CDC real time RT-PCR assay was found to be most promising tool among all available molecular diagnostic methods. This will serve a rapid and reliable simultaneous dengue virus detection as well serotyping assay in near future for rapid identification of dengue suspected sample screening.

Keywords Dengue · Virus · Infection · Antigen

Introduction

Among the many diseases caused by arboviruses, dengue fever has emerged as the most dangerous public health problem for mankind. Four antigenically different dengue virus serotypes (DENV-1, DENV-2, DENV-3 and DENV-4) are known to cause infections in humans. Classical Dengue fever and the Dengue hemorrhagic fever-dengue shock syndrome (DHF-DSS), which is the severe form of the disease, both have caused very high morbidity and mortality among the humans. Out of the four dengue serotypes, some of them are known to cause more severe disease than the others. Chances for developing DHF-DSS is very high when there is previous infection with one of

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the four serotypes and second time infection with a heterotypic serotype [8, 11].

It has managed to cause panic in the affected regions almost every season because of the factors like ability of the virus to cause acute rise in cases, non availability of specific therapy or vaccine and ability to cause severe disease on slight delay in diagnosis and management [2]. Therefore, early diagnosis, serotyping of the virus and providing early warning of dengue fever epidemics to concerned authorities becomes very important to curb the rapid spread in the community.

As per CDC (centre for disease control & prevention), 40% of the world's population, which is around 2.5 billion people, today live in high risk zones in this world, having the risk of dengue transmission. Dengue is endemic in at least 100 countries in Asia, the Pacific, the Americas, Africa, and the Caribbean. As per World Health Organization (WHO) estimates, 50–100 million dengue infections occur every year which includes 500,000 cases of DHF and 22,000 deaths, mostly among children.

India witnessed a huge number of dengue cases in the year 2014. Nearly 40,571 cases were diagnosed to have dengue infection and 137 cases have been reported to have died of this infection. There is a possibility of underreporting and the actual number could be very high. Except for three states (Meghalaya, Manipur, and Nagaland), all the remaining states and union territories of India reported cases of dengue. But, deaths because of dengue, was reported by only 18 of the 35 states/union territories. State of Maharashtra alone, were reported 8573 cases and 54 deaths. This is the highest number of cases and deaths reported by a single state in India in 2014. This is the first time in last 5 years since 2010, when Maharashtra is at the top of the list among the Indian states in having the highest number of dengue cases. This is the second consecutive year when Maharashtra is having the highest number of deaths caused by dengue.

The most challenging aspect of dengue infection is the rapid and accurate diagnosis. Isolation of dengue virus is available only in very few centers. Dengue infection may be detected by performing number of serological and molecular assays. Serological techniques, although very rapid and do not require trained personnel, do not provide the serotype information which is very essential in case of secondary dengue infection. Molecular methods like RT-PCR does require trained manpower, relatively costly instruments and reagents, but provides with rapid and important information like presence of viral RNA and type of serotype [9, 10, 12]. In this study, we have used both the serological and molecular methods for the rapid and accurate diagnosis of dengue infection.

Materials and methods

Acute phase clinical samples

A panel of 100 dengue suspected acute phase clinical samples were collected during the 2014 outbreak from a tertiary health care centre in Pune. All the samples were kept in proper freezing conditions and investigated for presence of DENV at Division of Virology, Defense Research & Development Establishment, Gwalior. All samples used in this study were collected with informed consent of patients following ethical guidelines.

Dengue virus isolates

Along with the positive controls in CDC real time PCR kit, Indian dengue serotype 1–4 isolates [DENV-1 (GenBankAcc No. JQ917404); DENV-2 (GenBankAcc No. DQ448231); DENV-3 (GenBankAcc No. FJ644564); DENV-4 (GenBankAcc No. HM 237348)], isolated in different outbreaks from India, were also included as positive controls in this study [3, 5, 15]. In addition, closely related alphavirus viz., Ross River virus and flavivirus viz., Japanese Encephalitis virus (JaOArS982), West Nile virus (Eg101) and synthetic gene constructs of alpha viruses including O'NyongNyong virus (Gulu strain), Semliki Forest virus (L10 strain) and Sindbis virus (J02363.1) were also included in the present study for the cross-reactivity.

Serological diagnosis

S/D ICT

The SD Dengue NS1 ICT kit (Standard Diagnostics, Korea) was used for serological diagnosis of dengue samples. Test protocol was followed as per manufactures instruction. Briefly, 100 µL of serum sample was applied to the sample well “S” of the NS1 Ag strips of the device followed by addition of four drops of assay diluents. Results were interpreted after 10 min.

Molecular diagnosis

Extraction of viral RNA

Viral RNA was extracted from the infected serum samples using the QIAamp viral RNA mini kit (Qiagen, Germany), according to the manufacturer's protocol. Briefly 560 µL of buffer AVL was added to 140 µL of infected material (serum) and vortexed for 15 s and the mixture was incubated at room temperature (15–25 °C) for 10 min. After

washing twice, finally RNA was eluted in 70 μL of elution buffer in collection tube and stored at -80°C until use.

Dengue complex one step reverse transcription-polymerase chain reaction (RT-PCR)

The confirmation of dengue specific RNA was carried out by a one step RT-PCR protocol using Sigma's Enhanced avian HS RT-PCR kit (Sigma, USA) following the manufacturer's instruction. The PCR amplification was carried out in a final volume of 25 μL using the viral RNA as template. Briefly PCR mix contain 2.5 μL 10X PCR buffer, 1.5 μL MgCl_2 (3 mM), 0.5 μL dNTP (200 mM each), 0.5 μL Reverse Transcriptase (0.4 units/ μL), 0.5 μL RNase inhibitor (0.4 units/ μL), 0.5 μL TaqDNA polymerase (0.05 units/ μL), 0.5 μL of respective forward (5'-TCAATATGCTGAAACGCGAGAAACCG-3') and reverse primers (5'-TTGCACCAACAGTCAATGTCTTCAGGTTC-3') targeted towards C-prM gene, 5 μL extracted viral RNA and 13 μL of molecular biology grade water. The PCR amplification was carried out in a final volume of 25 μL in a thermal cycler (Bio-Rad, USA). The thermal profile comprised of reverse transcription at 48°C for 45 min, initial denaturation at 95°C for 2 min followed by 35 cycles at 95°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min and final extension at 72°C for 10 min.

Agarose gel electrophoresis

The size and quantity of the PCR amplicon was confirmed by 1% agarose gel electrophoresis. Briefly, agarose gel was prepared with ethidium bromide (final concentration 0.5 $\mu\text{g}/\text{mL}$). 8 μL of the PCR amplicon was mixed with 2 μL of 6X Gel loading dye and loaded along with a known molecular weight marker (1 Kb DNA ladder, Fermentas) in an adjacent well. The electrophoresis was carried out at 80 V for 90 min. After the electrophoresis, the amplicons were visualized in a Gel Documentation system (Bio-Rad, USA) and were photographed.

Single tube multiplex RT-PCR

The dengue multiplex RT-PCR assay was performed. Briefly, the RT-PCR reaction was set up in a 25 μL reaction volume using master mix of Sigma's Enhanced Avian HS RT-PCR kit (Sigma, USA), extracted viral RNA as a template with dengue virus group-specific consensus forward primer (D1), and four serotype specific reverse primers as published in previous studies targeted towards C-prM gene [14].

CDC dengue multiplex real time assay

For the detection and serotype identification of the dengue virus, Taqman based multiplex real time RT-PCR assay was performed in ABI 7500 Fast Dx Real time PCR instrument using the dengue specific primer and probes provided by CDC, USA (Catalog No KK0128, Package insert available at <https://www.cdc.gov/dengue>) [13]. The assay includes a set of oligonucleotide primers and dual labeled 5' fluorescent (Taqman) probe for detection of DENV 1–4. Targeted gene for DEN-1 was NS5 gene; for DEN-2, it was E gene; for DEN-3 and DEN-4, it was prM gene. Appropriate positive and negative controls were taken in each reaction during sample investigation. These positive and negative controls were provided along with CDC kit and reaction was performed according to the manufacturer's instruction. 5 μL of extracted RNA samples, including HSC were added in wells. Plate was sealed with optical tap film and placed in Applied Biosystems 7500 Fast DX Real-time PCR system where fluorescent signal intensity was monitored during each PCR cycle. Assay was run in multiplex format (the four dengue serotypes are in same reaction) with each serotype specific probe labeled with different probes: DENV 1-FAM; DENV 2-HEX; DENV 3-TEXAS RED; DENV 4-CY5; RNase P-FAM. Thermal cycling conditions were RT 50°C for 30 min, RT inactivation at 95°C for 2 min, fluorescence detection at 95°C 15 s, 60°C 1 min for 45 cycles. Target amplification is recorded as increase accumulation of fluorescence over time in contrast to background signal.

Purification of PCR products

Representative PCR products from Dengue complex one step reverse transcription-polymerase chain reaction (RT-PCR) were purified for preparing template for sequencing reaction using QIAquick gel extraction kit (Qiagen, Germany) following manufacturer's protocol.

Nucleotide sequencing

The sequencing of the gel purified PCR products were performed using ABI PRISM Big Dye terminator v3.1 cycle sequencing ready reaction kit (Applied Biosystems, USA) and the same set of primers as used in RT-PCR following manufacturer's protocol.

Briefly, sequencing reaction was carried out in a final volume of 10 μL by mixing the Big Dye terminator mix containing the thermo stable AmpliTaq DNA polymerase, dNTPs and four dye-labelled dideoxy nucleotide terminators (ddNTPs) and 25 ng of purified PCR product, and either upstream or downstream primer in a PCR tube. The thermal profile comprises denaturation 96°C for 1 min, 25

cycles of denaturation primer annealing and chain termination at 96 °C for 10 s, 55 °C for 5 s, 60 °C for 4 min. These products were purified using Centri-sep columns (Applied Bio systems, USA) as per manufacturer instructions.

Analysis of sequences

The nucleotide sequence of positive sample was deciphered. The nucleotide sequences were subjected to BLAST to find closest possible match from available sequences in GenBank at <http://www.ncbi.nlm.nih.gov/BLAST>.

Results and discussion

Clinical features of dengue outbreaks

Dengue fever is an arboviral disease of paramount importance. India is a Dengue endemic country reporting many thousand cases every year [3–5, 15]. Many hundred people lost their lives because of this dreaded public health problem in a country where there is abundance of the disease transmitting mosquitoes and high density of people living close to each other. So far, there is no specific antiviral drug available for this infection. Non availability of effective vaccine makes this even more dangerous and leave the population susceptible to this preventable and potentially fatal disease. Dengue can be caused by any of the four closely related dengue 1–4 serotypes. Importantly, Infection with one dengue serotype does not protect against infection by other serotype. In fact, sequential infections by different dengue serotypes put people at greater risk for severe forms of the disease; dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). All these factors make the correct identification of virus and the infecting serotype, of paramount importance, for better patient outcomes and for effective control of dengue outbreaks.

Large number studies which came out with the finding that infection with certain serotypes is more dangerous, have more chances of development of DHF/DSS as compared to other serotypes. Severe clinical manifestations, abdominal pain and hepatomegaly are comparatively higher in DEN-2 patients as per majority of the published reports. Dengue hemorrhagic fever (DHF) is also most commonly caused by Den-2 (35%) serotype followed by DEN-3 (31%), whereas DEN-1 is associated with less severe disease [7]. Infection by DENV-2 and DENV-4 more commonly occurs as secondary infection whereas infection by DENV-1 and DENV-3 is more common as primary infections [8, 17]. Infection with DENV-3 is

associated with more number of febrile days than infection with DENV-2 [17]. All these factors make early and correct diagnosis of infecting dengue serotype very important and relevant for better patient management and outbreak management.

Diagnosis of dengue virus is based on viral culture, serology and viral RNA detection by molecular methods. Classical method like virus culture is time consuming, complex, less economical and requires expertise. It is available only with very few laboratories. In addition, these techniques are successful only during the viraemia phase of infection, which is transient (less than a week) in nature. Serological methods (NS1 antigen based), although very rapid, cost effective, requires least expertise to perform and interpretation, do not provide serotype information. Off late, a large number of diagnostic kits based on IgM capture ELISA, indirect ELISA, dipstick, ELISA and immunochromatography principles have been developed and validation of these kits revealed variable results owing to the format and the nature of antigen incorporated in the kit [6, 16]. In addition these kits are highly expensive and have failed to meet the demand at the time of epidemic [1]. Therefore, we need methods which are highly sensitive and specific, rapid, easily available methods to timely manage patients and curb the spread of this potentially explosive problem.

S/D ICT

A total of 100 Clinical samples suspected of dengue infection were collected during an outbreak in Pune, Maharashtra in 2014. All these samples were screened for antigen (NS1) detection using Standard Diagnostics, Korea ICT Kit, at a tertiary health care centre in Pune. 69 samples were found to be positive for NS1 antigen test.

Dengue complex one step reverse transcription-polymerase chain reaction (RT-PCR)

Viral RNA was extracted from all 100 acute phase samples and was used to perform dengue complex RT-PCR assay using dengue complex specific consensus primers revealing characteristic 511 bp amplicon (Fig. 1a). A total of 34 samples were found positive by dengue consensus RT-PCR assay.

Single tube multiplex RT-PCR

All 100 samples were also subjected to Dengue multiplex RT-PCR assay for dengue serotype identification (Fig. 1b). A total of 22 samples were found to be positive for one of the four dengue serotypes by this assay. DENV Multiplex RT-PCR assay revealed DENV-1 (1), DENV-2 (16),

Fig. 1 a Agarose gel electrophoretic analysis of DEN samples by RT-PCR: Lane 1—O' gene ruler™ Express 1 Kb DNA ladder (Fermantas Cat # SM 1163), Lane 2–16: amplified products of clinical samples, positive samples showing 511 bp. **b** Agarose gel electrophoretic analysis of DEN samples by conventional Multiplex RT-

PCR: Lane 1—O' gene ruler™ Express 100 bp DNA ladder (Fermantas Cat # SM 1143), Lane 2–9: positive samples showing different serotype of DENV-D1—483 bp, D2—119 bp, D3—288 bp, D4—392 bp

DENV-3 (3), DENV-4 (2) serotype positivity among 100 samples. Results obtained by serological and different molecular diagnostic assay are given as supplementary data.

CDC dengue multiplex real time assay

All 100 samples were subjected to CDC Dengue multiplex real time qRT-PCR assay for dengue serotype identification. The assay was performed in multiplex format as each probe was labeled with different fluorophore-color, helping in simultaneous detection of dengue serotype in each of the positive samples. The positive controls provided in kit, contains DENV1 -Haw, DENV 2-NGC, DENV3- H87, DENV4 -H241 standard strain, showed positive amplification for all the four serotype. Here also an internal control reaction was run for each of the sample as mentioned in the CDC kit instructions. 44 samples were found positive for dengue virus infection. Three samples had co-infection by two serotypes. DENV CDC Multiplex Real time PCR assay revealed DENV-1 (16), DENV-2 (20), DENV-3 (5), DENV-4 (6) serotype positivity among 100 samples. Similarly, cross reactivity was also checked for closely related *Flaviviruses* and *Alphaviruses*, where no amplification was observed.

Nucleotide sequencing

Representative positive samples, amplified by dengue consensus primers having size of 511 bp, were gel purified and cycle sequencing was performed. NCBI- BLAST analysis resulted in 100% concordance for dengue virus.

The superiority of high NS1 positivity during sample investigation is due to the early collection of all the samples right after the onset of fever and not delayed by more than 03 days, which is a limitation in case of molecular diagnostics available now a days. But still during investigation a late infection cases which is only possible through these molecular based methods crucial for the DHF/DSS is not possible through NS1 screening. Another advantage of rapid detection and simultaneous serotyping is that the method is 100% confirmatory and serves as gold standard for rapid diagnosis. Diagnosis of dengue viraemia, during first 5 days of illness, definitely needs a molecular method. CDC DENV 1–4 real time RT-PCR assay not only facilitates early dengue diagnosis, but also provides the serotype conformation. It is a simple and quick assay [13]. This is a first study of rapid detection and serotyping of dengue samples from India using CDC Real time RT-PCR assay. Among the three molecular methods used over here, highest number of cases was picked up by dengue CDC real time RT-PCR. Other two methods, single tube multiplex RT-PCR assay and Dengue complex specific RT-PCR, were seen to miss few cases as compared to the CDC real time PCR method. There was 100% correlation for serotyping between the dengue conventional single tube multiplex RT-PCR and CDC real time PCR assay.

In conclusion, Dengue remains to be an important health problem affecting geographies across the globe. Few serotypes are more dangerous than the others. Rapid diagnosis and serotyping remains the key for better patient management and prevention of disease spreading in the community. Highly sensitive, specific and rapid CDC real time

RT-PCR assay is promising tool among all available molecular diagnostic methods.

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