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Diagnosis and serotyping of dengue using cost-effective high-resolution melting curve based real time PCR

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Abstract---Dengue virus (DENV) is classified into four serotypes: DENV-1, DENV-2, DENV-3, and DENV-4 which are genetically-related but antigenically distinct types. Although primary infection with a serotype of DENV gives lifelong immunity to that serotype, the secondary infection with other serotype leads to the development of dengue hemorrhagic fever and dengue shock syndrome. Serological tests that can detect NS1 antigen on the day one of illness is nonspecific and less sensitive to diagnose dengue in the first week of illness. IgM and IgG antibody detection may not be positive until the later part of first week. Hence it is necessary to develop a nucleic acid-based test kit for detection of DENV in the early phase of the disease such that the complications can be prevented. Simultaneous detection of serotype of DENV helps in understanding the epidemiology of the disease. In the present study, a single primer pair was designed to 3' untranslated region of DENV genome. After amplification, the melting temperature (T_m) for DENV-1, DENV-2, DENV-3 and DENV-4 was found to be 83.96 ± 0.58 °C, 81.99 ± 0.26 °C, 84.54 ± 0.15 °C, and 83.28 ± 0.12 °C, respectively. Results obtained by indigenously developed RT-PCR kit with high resolution melting (HRM) curve analysis were 100% in concordance with commercial serotyping kit.

The percentage of DENV-1, DENV-2, DENV-3 and DENV-4 serotype detected in clinical samples analyzed was 14.98%, 35.31%, 36.38% and 27.82%, respectively. In conclusion, the RT-PCR-HRM analysis using the single primer pair developed was not only able to detect DENV infection in clinical samples but also was able to serotype the DENV with high efficiency. The kit developed is cheaper, sensitive, easy to perform and less time consuming.

Keywords---diagnosis, serotyping dengue, cost-effective high-resolution, real time PCR.

Introduction

The dengue virus (DENV) is a flavivirus that is transmitted mostly by female *Aedes aegypti* mosquito and, to a lesser extent by *Aedes albopictus*. In the last 50 years, there is a surge in number of Dengue cases due to increase in global population, international travel, ineffective mosquito control and inadequate vaccination strategies. After biting the infected human, the extrinsic incubation period of DENV inside mosquito is 8-12 days, later the vector can transmit the virus for the rest of its life. It is estimated that about 390 million people could get DENV infections per year (95% credible interval 284–528 million), of which 96 million (67–136 million) might manifest clinically (with any severity of disease) [1](Bhatt *et al.*, 2013). Although, the risk of infection exists in about 129 countries [2](Brady *et al.* 2012), 70% of the actual burden is in Asia [1](Bhatt *et al.*, 2013). The number of dengue cases reported to WHO increased over 8 fold in the last two decades, from 0.5 million cases in 2000, to over 2.4 million in 2010, and 5.2 million in 2019 (fact sheet from www.who.in). Dengue is classified as dengue with or without warning signs and severe dengue. Severe dengue affects mainly infants, young children and adults. The severe dengue fever can be life-threatening due to plasma leaking, fluid accumulation, ascites, severe bleeding, low platelets and organ impairment [3]. To date, there is no specific treatment for dengue. Hence it is necessary and important to diagnose dengue infection at early stage to prevent it from progressing to severe dengue. In addition, with adequate mosquito control measures, early diagnosis can prevent the spread of DENV via mosquito bites from infected patients during the viremic phase.

There are several serological tests (IgM-based test, IgG-based test, IgM/IgG ratio test, hemagglutination inhibition test, plaque reduction neutralization test) used for diagnosis of DENV infection. However, there are notable downsides of serological tests such as low- sensitivity, time-consuming, labor-intensive, and low-specificity (most of the serological tests are cross-reactive to antibodies of other viruses which belong to Flaviviridae family) [4](Kabir *et al.*, 2021). Assays based on the detection of nonstructural protein 1 (NS1) are also non-specific for DENV infection (can cross react with other Flaviviral infections), but the reported sensitivity of these assays range from 24–93%, which makes it less reliable [5,6] (Tricou *et al.*, 2010; Guzman *et al.*, 2010). Also, the sensitivity of NS1 detection assays during secondary infection decreases to 20% in comparison to primary infection [5–8] (Tricou *et al.*, 2010; Guzman *et al.*, 2010; Chaterji, *et al.*, 2011; Waggoner *et al.*, 2013a). On the other hand, nucleic acid tests (NATs) which

quantifies the DNA/RNA are sensitive and specific with added advantage of detecting the DENV at the early stage of infection. The polymerase chain reaction (PCR) -based assay is considered gold standard for detection of DENV. The viral RNA is transcribed into cDNA, followed by amplification and detection using fluorescence probes. Currently available PCR kits are expensive and hence not affordable by all in India. Though the test can be performed with the available RT-PCR equipment used for diagnosis of Covid-19, they are cost prohibitive.

DENV is classified into four serotypes: DENV-1, DENV-2, DENV-3, and DENV-4 which are genetically-related but antigenically distinct types. The nucleotide sequences of the 4 serotypes of DENV have 63-68% homology between each other. Further, the nucleotide sequences of different virus strains of the same serotype have greater than 95% homology between each other (Weaver and Vasilakis, 2009; Chen and Vasilakis, 2011). A primary infection with one serotype of DENV is found to confer lifelong immunity to the same serotype. However, secondary infection with heterologous DENV is found to augment the infection due to the phenomenon called antibody-dependent enhancement [6,11] (Guzman and Vazquez, 2010; Kulkarni, 2020). The dominant serotype during every large DENV outbreaks swaps. In India, concurrent infection with different DENV serotypes was reported during 2006 dengue outbreak [12] (Bharaj *et al.*, 2008). Hence, precise clinical diagnosis of DENV serotype is required for health care personnel to plan case management and initiating preventive measures to avoid impending epidemic. Currently available PCR tests for serotyping are not affordable due to high cost. Keeping these views, in present study, real time PCR (RT-PCR) based high melting curve (HRM) analysis is developed to distinguish four different serotypes of DENV in a closed tube reaction. Since the method developed targets single genetic region, it would be superior in terms of cost, ease of diagnostic method and turnaround time for identification of different serotypes of DENV. HRM distinguishes DNA sequence variants based on melting temperature (T_m) of PCR product. Alternative diagnostic methods that require, post-PCR processing, enzyme restriction, electrophoresis and TaqMan-probe are time-consuming, laborious or costly. Also, the specificity of HRM based detection is comparable to DNA sequencing [13] (Li *et al.*, 2011), hence the method developed in the present study using RT-PCR-HRM analysis to detect various serotypes of DENV would be superior.

Materials and Methods

Materials

The commercial RT-PCR kit for detection of DENV serotypes was purchased from Helix molecular diagnostics, Germany (Fluoro AMP Dengue serotyping RT PCR KIT). RT enzyme (Invitrogen), QIAamp viral RNA mini kit (For RNA extraction) and Type-it HRM PCR Kit containing the necessary ingredients like dNTPs, Taq polymerase, Eva green and molecular grade water required for the in-house PCR were purchased from Qiagen, Hilden, Germany.

Clinical samples

The serum samples were collected from 151 patients who presented with clinical

features suggestive of Dengue infection (2019) from the JSS Medical College Hospital, Mysore, India. The study was duly approved (Ref No. JSSMC/IEC/1912/04 NCT/2018-19) by the Institutional ethical committee, JSS Medical College, Mysore, India. Before collecting the blood samples, informed consent was obtained from the participants. Among the 151 samples, 107 samples that showed positive results using commercial RT-PCR kit were taken for diagnosis and serotyping using in-house kit designed.

RT-PCR-HRM analysis of DENV serotyping

RNA from 107 DENV positive Serum samples was extracted using a QIAamp viral RNAmini kit as per Manufacturer's instructions. They were then subjected to RT-PCR by the in-house kit using Rotor-Gene Q 5plex RT-PCR cycler (Qiagen, Hilden, Germany). The reaction mixture consisted of reverse transcriptase enzyme, HotStarTaq Plus DNA Polymerase, dNTP mix, EVA green dye, buffer and in-house synthesized primers for DENV. The primer sequence used for DENV is - forward primer: 5'GACTAGHGGTTAGWGGAGAC- 3' and reverse primer: 5'-AGACAGCAGGATCTCTGGTC-3'. The temperature profile for PCR reaction is 50 °C for 15 min (reverse transcription step), 95 °C for 10 min (enzyme activation), followed by 40 cycles of 95 °C for 10 sec, 55 °C for 30 sec. The fluorescence emitted was captured at the end of the extension step of each cycle. Finally, a HRM ramping from 65 °C to 95 °C was performed. Fluorescence data were acquired at 0.1 °C increments every 2 sec to generate specific melting curves. Data analysis was performed using Rotor-Gene Q Software 2.3.5

Results

The amplification curve of DENV serotypes using commercial kit is shown in figure 1. The DENV serotype, DENV 1 (Figure 1A), DENV 2 (Figure 1B), DENV 3 (Figure 1C) and DENV 4 (Figure 1D) was detected using four different probes labeled with fluorescence dye namely, FAM, VIC, ROX and Cy5, respectively.

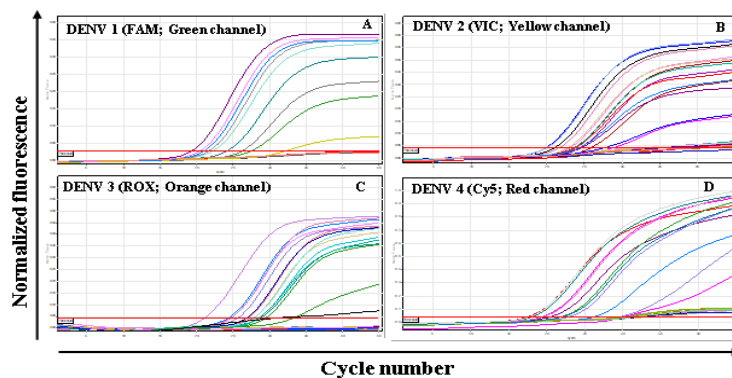


Figure 1. Amplification curve of DENV serotyping of clinical samples using commercial kit (FluoroAMP dengue serotyping kit, Helix Molecular Diagnostics, Germany).

The in-house HRM assay clearly differentiated the serotypes of DENV in the 107 DENV positive clinical serum samples tested. Figure 2, 3, 4 and 5 shows the

HRM graphs including melting curve, normalized melting curve and difference plots corresponding to the RT-PCR-HRM of the DENV 1, DENV 2, DENV 3 and DENV 4 serotypes from clinical samples, respectively. In the difference plot of DENV 1 (Figure 2C), there is no difference in the clinical samples with respect to known DENV 1 positive sample. However, in figure 3C, 4C and 5C the difference in fluorescence is visualized in comparison to DENV 1 positive sample for the serotypes DENV 2, DENV 3 and DENV 4, respectively.

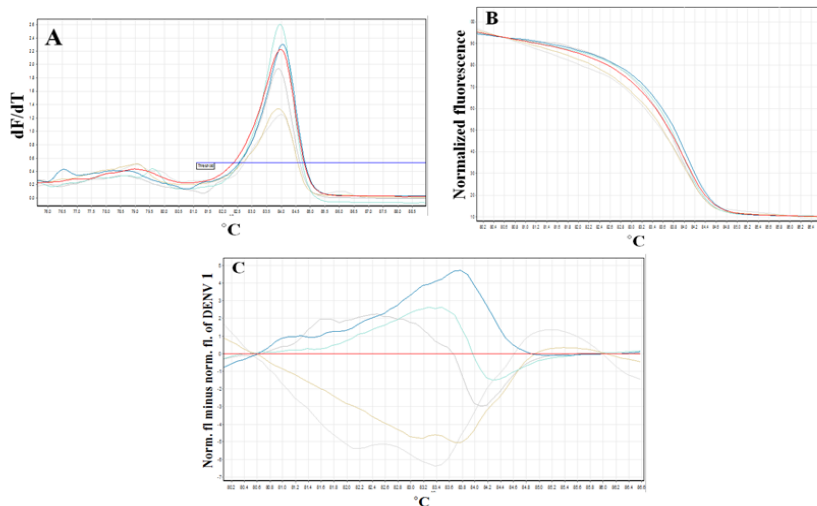


Figure 2. Graphs of RT-PCR-HRM analysis of DENV 1 serotyping of clinical samples. A. Melting curves of the HRM step; B. Normalized plot; C. Difference plot in relation to DENV 1 serotype.

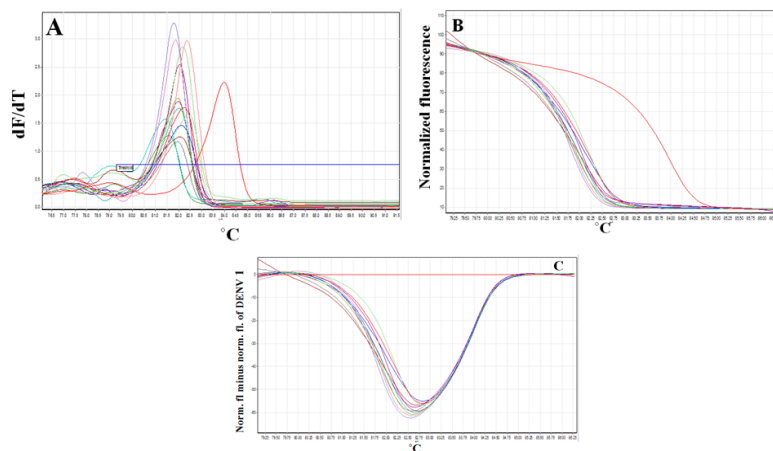


Figure 3. Graphs of RT-PCR-HRM analysis of DENV 2 serotyping of clinical samples. A. Melting curves of the HRM step; B. Normalized plot; C. Difference plot in relation to DENV 1 serotype

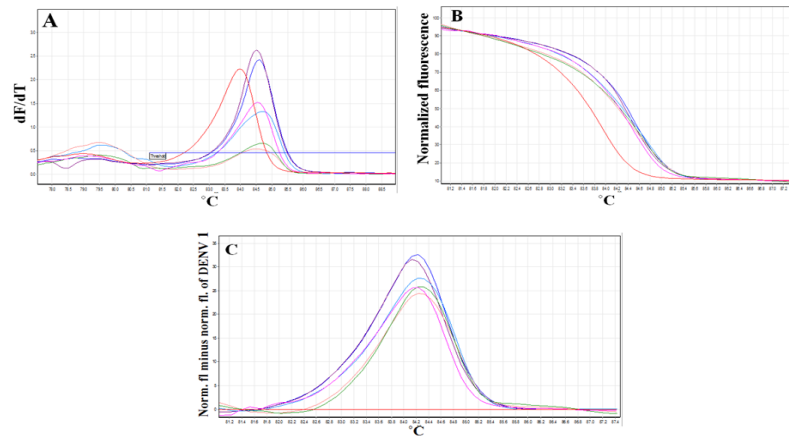


Figure 4. Graphs of RT-PCR-HRM analysis of DENV 3 serotyping of clinical samples. A. Melting curves of the HRM step; B. Normalized plot; C. Difference plot in relation to DENV 1 serotype

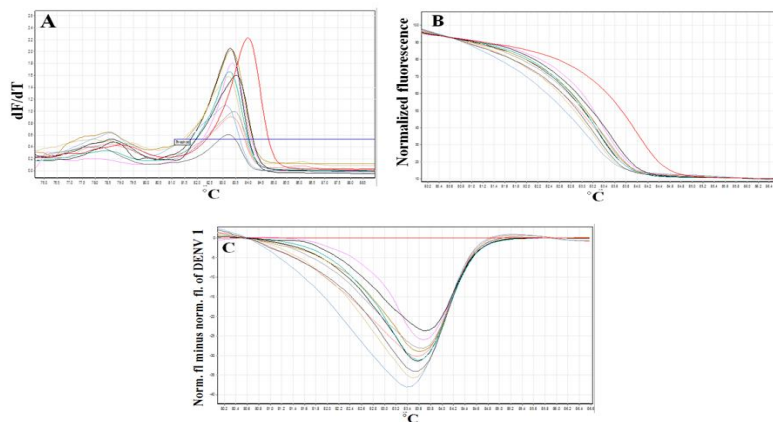


Figure 5. Graphs of RT-PCR-HRM analysis of DENV 4 serotyping of clinical samples. A. Melting curves of the HRM step; B. Normalized plot; C. Difference plot in relation to DENV 1 serotype

The representative HRM graphs including amplification curve, melting curve, normalized melting curve and difference plots corresponding to the RT-PCR-HRM of all DENV serotypes from clinical samples are demonstrated in Figure 6. The RT-PCR-HRM analysis using in-house designed primers were able to significantly categorize ($P < 0.01$) the amplicon into the four distinct groups and identified the DENV serotypes with confidence interval of $> 95\%$. The DENV 1, DENV 2, DENV 3 and DENV 4 yielded average T_m of 83.96 ± 0.58 °C, 81.99 ± 0.26 °C, 84.54 ± 0.15 °C, and 83.28 ± 0.12 °C, respectively. The T_m is adjusted according to the T_m of control. The serotyping of DENV using in-house designed primers in RT-PCR-HRM analysis was 100% in concordance with the commercial kit. The percentages of DENV 1, DENV 2, DENV 3 and DENV 4 serotype detected in clinical samples analyzed were 14.98%, 35.31%, 36.38% and 27.82%, respectively.

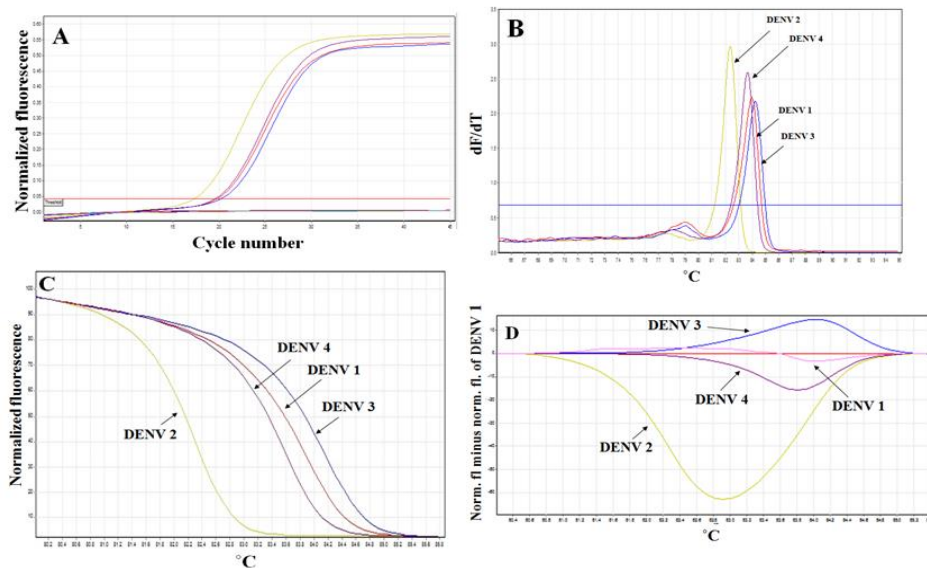


Figure 6. Representative graphs of RT-PCR-HRM analysis of DENV serotyping. A. Amplification curve; B. Melting curves of the HRM step; B. Normalized plot; C. Difference plot in relation to DENV 1 serotype

Among the 107 clinical samples tested, the dengue severity was classified as per the guidelines issued by the Ministry of Health and Family Welfare, Government of India by the year 2020. In accordance to the guideline, the dengue was considered mild if the patient is presented with symptoms such as fever for 2 to 7 days with any of the symptoms such as nausea, headache, vomiting, rash and myalgia. Moderate dengue was considered if the patients had recurrent vomiting, abdominal pain, rapid pulse, hypotension and rapid fall in platelet count. Further, the case was considered severe if one or more of the symptoms such as severe bleeding; shock or severe/multiple organs was involved. In accordance to the guideline, the number of clinical samples tested in the present study with mild, moderate and severe dengue among different DENV serotype is given in Figure 7. Among the different serotypes, moderate and severe dengue was found among DENV 2 and DENV 3 infected patients.

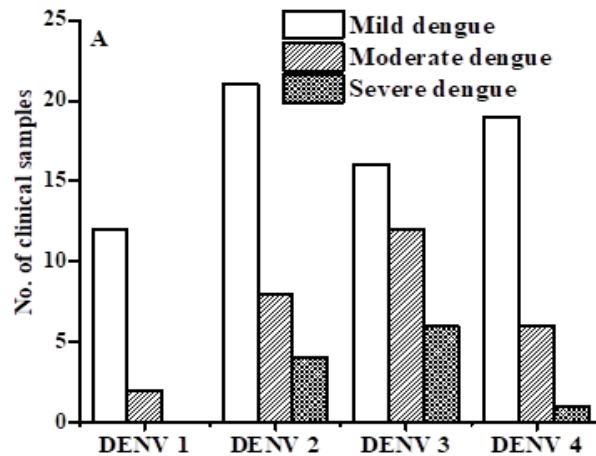


Figure 7. Detail of clinical samples with mild, moderate and severe dengue among different DENV serotypes

Discussion

The in-house primer designed targeted the 3' Untranslated region (3'UTR) region of DENV and was found to have 97.18% sensitivity and 98.75% specificity. The advantage of the primer designed was that the same set of primers was not only able to detect DENV positive in the clinical samples but was also able to distinguish four serotypes of DENV efficiently by HRM analysis as shown in the results of the present study. Sequence variations in double-stranded DNA will generate different melting curve profile and this principle is applied in HRM analysis. The sequence with T_m difference of more than 0.5 °C can be easily distinguished by HRM analysis. Altogether, HRM analysis is simple, inexpensive and rapid method for serotyping [14](Wittwer, 2009).

HRM analysis has been previously reported to differentiate species of bacterial pathogens such as *Brucella*, *Campylobacter*, *Cronobacter*, *Listeria*, *Mycobacteria*, *Pasteurella*, *Staphylococcus*, *Salmonella*, *Shigella*, and *Yersinia* isolated from food and clinical samples [15–25] Also, HRM has been previously used to serotype bacterial pathogens such as *Salmonella* and *Streptococci* and viruses such as astroviruses, influenza A, bronchitis virus and fowl adenovirus (Lin *et al.*, 2008; Steer *et al.*, 2009; Hata *et al.*, 2014; Poonchareon *et al.*, 2019; Ababneh *et al.*, 2020; Scherrer *et al.*, 2020).

Various other studies have reported the detection of DENV serotypes using RT-PCR based methods [8,26–29]. (Waggoner *et al.*, 2013a; Alm *et al.*, 2015; Chakravarti *et al.*, 2016; Simmons *et al.* 2016; Kim and Hwang, 2020). Multiplex diagnosis of DENV serotypes using TaqMan probe has been reported previously by Callahan *et al.*, [30]2001; Ito *et al.*, 2004; Chien *et al.*, 2006; Lai *et al.*, 2007; Gurukumar *et al.*, 2009; Conceição *et al.*, 2010; Waggoner *et al.*, 2013a; Alm *et al.*, 2015; Kim *et al.*, 2015; Simmons *et al.*, 2016; and Kim and Hwang, 2020.

Nevertheless, one of the previous studies has reported the use of hybridization probe (HybProbe) in the detection of DENV serotypes (Chakravarti *et al.*, 2016). Also, few studies have already reported the HRM analysis for DENV serotyping using primers designed for targeting capsid and premembrane (C- prM) gene sequence (Naze *et al.*, 2009; Chen *et al.*, 2015; Salles *et al.*, 2017). Although there are other studies that have reported methods developed in DENV serotyping, these methods have disadvantages such as the cost of probes (TaqMan and HybProbe) that is high and most of the HRM analysis reported are multiplexed (i.e. these techniques require two or more pair of primers that are distinguished from one another and multiple targets are detected simultaneously in a single reaction well). However, the present study uses single pair of primers to detect the DENV serotypes which would be advantageous in terms of simplicity of the assay and cost effectiveness.

Although there are commercially available kits for the detection of DENV serotypes, the sensitivity of the kit has been reported to be less. The results of in-house multiplex RT-PCR kit by Levi *et al.*, 2007, concurred with the results of Real art-Artus-QuiaGen Germany. But the test was carried out on DENV 3 and occasionally DENV 2 samples only. The centers for disease control DENV-1-4 realtime kit approved by US food and drug administration has been shown to be less sensitive than a laboratory-developed assay, particularly for DENV1 (Santiago *et al.*, 2013; Waggoner *et al.*, 2013b). In another study, four commercial kits such as Simplexa™ dengue RT-PCR assay (Focus Diagnostics, Cypress, CA), RealStar dengue RT-PCR kit 1.0 (Altona Diagnostics, Hamburg, Germany), Dengue virus general type real-time RT-PCR kit Liferiver™ (Shanghai ZJ Bio-Tech Co, China), Geno-Sen's dengue 1-4 real-time RT-PCR kit (Genome Diagnostics Pvt, New Delhi, India) were evaluated and found that only Simplexa™ dengue RT-PCR assay kit was sensitive, however it is based on TaqMan probe which would be a disadvantage in terms of cost which makes it unfeasible for large-scale use (Najioullah *et al.*, 2014). A study on the international external quality control assessment for the molecular diagnosis of dengue infections in which about 37 laboratories (from Europe, Middle East Asia, Asia, the Americas/Caribbean, and Africa) participated has found that only 10.9% data sets met all criteria with optimal performance and 8.7% with acceptable performance whereas 80.4% reported results showed the need for improvement to accomplish appropriate dengue molecular diagnosis. The failures were found to be because of low sensitivity and low specificity (Domingo *et al.*, 2010). In such a scenario, the method developed in the present study would be useful; however, there is a requirement for testing the efficiency of this method in large- number of clinical samples.

Among the 151 positive samples for dengue infection tested, the prevalence of serotype was found to be DENV3 (36.38%) > DENV2 (35.31%) > DENV4 (27.82%) > DENV1 (14.98%). The clinical samples used in the present study are collected during 2019 dengue outbreak in Karnataka, India. The results obtained in the present study was in concordance to the previous report which was carried out using the clinical samples (106 patients) collected during the same period (2019) from Dakshina Kannada district of Karnataka. The study found that about 52.8% of cases were affected with DENV 3 serotype and the least common serotype found was DENV 1 (Rao *et al.*, 2020). The important point to be noted is, all the

serotypes existed during the study period, and DENV 3 is most prevalent in this geographical region. In Western and Northern India, prevalence of DENV-3 was found to be more common. Previous study from other part of India such as Maharashtra has found predominant circulation of DENV 3 along with DENV 1 during 2017 outbreak (Patil *et al.*, 2018). Also, a study conducted using clinical samples collected during 2003-2005 in Delhi has found the predominance of DENV 3 serotype in the patients (Gupta *et al.*, 2006). In yet another study from Central India (Madhya Pradesh), the samples collected during the year 2016 has showed the prevalence of DENV 3 in 47% of the cases tested (Barde *et al.*, 2019). Other studies have shown that, although DENV 2 is predominant, the prevalence of DENV 3 is high compared to other serotypes in states such as Uttar Pradesh, central Karnataka and Delhi (Kumaria, 2010; Vinodkumar *et al.*, 2013; Mishra *et al.*, 2015). Altogether, DENV 3 serotype has been found to be implicated with major dengue epidemic in India (Dash *et al.*, 2005, 2006; Gupta *et al.*, 2006; Kumaria, 2010; Kukreti *et al.*, 2010; Vinodkumar *et al.*, 2013; Mishra *et al.*, 2015; Patil *et al.*, 2018; Barde *et al.*, 2019). The DENV variants with high replicative rate would spread more rapidly displacing the variant with lower fitness, hence, intra-host/vector diversity of DENV has found to play a role in transmission (Guzman and Harris, 2015; Novelo *et al.*, 2019). Large outbreak of dengue in Cairns, Australia in 2008/2009 was attributed to the very short extrinsic incubation period of the DENV 3 strain in the mosquito (Ritchie *et al.*, 2013). This might be the case even in India which requires further scientific evaluation. There are controversial reports regarding the association of DENV serotypes with the dengue severity. One study has reported the absence of link between dengue severity and serotypes (Rao *et al.*, 2020). Few other studies argue that dengue severity is associated with DENV serotype (Vaughn *et al.*, 2000; Kalayanarooj and Nimmannitya, 2000; Endy *et al.*, 2002; Nisalak *et al.*, 2003; Kumaria, 2010; Vicente *et al.*, 2016; Rajesh *et al.*, 2020; Gupta *et al.*, 2021). In particular DENV-3 serotype has been found to be associated with severe dengue in many studies (Kalayanarooj and Nimmannitya, 2000; Endy *et al.*, 2002; Nisalak *et al.*, 2003; Kumaria, 2010). In other studies DENV-2 was found to be associated with dengue severity (Vicente *et al.*, 2016; Rajesh *et al.*, 2020; Gupta *et al.*, 2021). Since, both these serotypes, DENV 3 and DENV 2 was prevalently detected in the samples collected in the present study from Karnataka, India, the health care personnel's should be prepared for severe illness if any outbreak ensue in the future. All these studies point to the fact that it is important to identify the DENV serotype precociously to understand the epidemiology and severity of DENV infection during an outbreak in future in India. In such case, the in-house RT-PCR-HRM kit developed in the present study would be of greater use.

In the present study, HRM was used successfully to type DENV with the use of single pair of primers. DENV serotyping is important because primary infection by one DENV type does not protect against infection with another serotype. Also, secondary infection with different DENV serotype may lead to the development of complications like dengue hemorrhagic fever and dengue shock syndrome (Soo *et al.*, 2016). The cost-effectiveness and simplicity of the developed assay due to the use of single pair of primers would be advantageous during epidemiological surveillance and screening of blood donors, where large number of samples have to be tested. As the assay takes lesser time for testing, it will be of a great help in

screening during large outbreaks of Dengue like the one that occurred during 2017 and 2019 in Karnataka (Rao *et al.*, 2020; Rai *et al.*, 2021)

Conclusion

In conclusion, RT-PCR-HRM analysis using one primer pair in the present study has been shown to be highly efficient and specific for diagnosis and serotyping DENV in clinical samples. The method developed is a simple, sensitive, specific, rapid and economical in diagnosis and serotyping of DENV that can be useful even during large outbreaks.

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