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Optimization of real time PCR for checking the activity of siRNA in Dengue Serotypes

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Abstract

Dengue, the most prevalent infectious disease among the arbovirus community is emerging as a first order health complication, especially, in the tropics and in subtropical regions. It is untreatable, since, there is no advanced diagnostic method with which tendency of the infection can be traced with great precision in its acute phase. In our previous studies we showed that, siRNA targeting the 5NTR among all the 4 serotypes of dengue successfully inhibited the replication of respective serotypes to the greatest extent as confirmed by the real-time RT-PCR. In the present study, we monitored the relative expression of dengue genomic RNA in treated (rAdsh-5b) and non treated cells and control (rAdsh-N) to reveal the efficiency of the real-time RT-PCR in detecting the viral load in the presence of antiviral agent (rAdsh-5b) to discriminate the disease severity in the presence and absence of antiviral therapeutics. The cDNA for all the samples were prepared and performed real-time RT-PCR with the aid of SYBR green 1 and the post PCR amplicons were analyzed with melting curve analysis to check the specificity and efficiency of the process. The results showed that, the relevant viral RNA expression was found to be less in rAdsh-5b treated sample than the non treated sample as marked by the Ct-values and the melting curve peaks. This indicates that real-time RT-PCR can serve as a gold standard for diagnosing the dengue infection in the acute phase and in distinguishing the relative viral content in the sample treated with antiviral agent.

Key Words: Dengue, siRNA, real-time RT-PCR, rAdsh-5b, rAdsh-N, Ct-values, cDNA.

Introduction:

Dengue, being a fatal infectious disease is spreading around the globe very rapidly due to the uncontrolled migration from the dengue endemic places and lack of mosquito controlling methods. The dengue virus incidences are being increased tremendously in the recent years, expanding severity with the wide array of vector(s). The contagious viral activity is attributed to the fact that, the clinical presentation of the infections is asymptomatic and sometimes undifferentiated. Hence, clinical manifestation is required to halt the progression of the disease. The dengue viral infection can be diagnosed either by direct method or by indirect method which include genome detection, antigen detection, virus isolation and serological methods (aided in IgM and IgG detection) respectively (1-6). Since the virus lasts in the infected tissue for 0-14 days bearing undifferentiated symptoms, the reliability of each method lies mainly on the type of the specimens being used and the stage of the infection.

In the recent years, the field of molecular diagnosis based on the reverse transcription PCR has become the standard tool due to its

sensitivity, reliability and specificity in the detection of acute phase infections of life threatening diseases, which in turn replaced the conventional methods. Since the emergence of RT-PCR, many research laboratories have reported various protocols for the detection of dengue virus in wide range of samples (7-9). In fact, two step nested RT-PCR method discribed by Lanciotti *et al.* and Laue *et al.* (10, 11) and single step multiplex RT-PCR by Henchal et al. (8) were the reference protocols. The conventional PCR studies described so far confirmed the persistence of virus in the disease state by detecting the viral load at a single point of time. Since viral load is a direct marker of the disease severity there is an urge in monitoring disease progress in the real-time by detecting the viral persistence from the acute phase. However, it has been postulated that monitoring the viral replication over time could be an efficient marker of a progressive viral disease rather than determining the absolute viral load at a single point of time (12, 13). This statement limited the conventional PCR methods which are solely depends on the end point analysis. With the development of fluorescent chemistries such as SYBR green, Taqman probes, linear and hairpin oligoprobes and self-fluorescing amplicons (12, 13) in the recent days, many investigators have developed the real time RT-PCR methods for the rapid diagnosis of dengue in clinical samples by reducing the time and cost in real time compare to the previously described RT-PCR and other conventional methods (14-17). There are many real-time RT-PCR methods utilizing different combinations of primers, probes and fluorescent chemistries in detecting, serotyping, and quantification of dengue virus in various clinical samples have been described (18-22). However, SYBR green and Taqman probes were most widely used detection dyes among fluorescent chemistries. The real time RT-PCR protocols reported so far in the present study are based on the consensus sequences of the dengue genome such as NS5b coding region, Capsid region, 3^1 Non-coding and 5^1Non-coding sequences with the ease of either SYBR green1 or Taqman probes as a marker dye (23, 24). In the recent decades, the real time PCR methods expanded their level of detection not only by addressing different clinical specimens, but also by targeting different regions of the dengue viral genome.

Though, Lanciotti *et al.* (10) developed nested RT-PCR in which the primers randomly target various regions of the dengue genome to detect and differentiate the dengue serotypes in the clinical samples (21), the experiments concluded with the false negative results due to the cross contamination and multiple steps involved. To avoid these false results, recent methods have been designed in selecting the primers from gene bank targeting only the conserved regions of the genome and profound use of these modified primers against the mutating viral strains (25). In the present study, we selected the consensus primers primer D1 and Primer D2 common to conserved regions of all serotypes of the dengue genome and The severity of virus infection in the presence and absence of the antiviral molecule rAdsh5b which is designed to target the 5^1UTR conserved sequences of the dengue is monitored based on the SYBR Green one real time RT-PCR and specificity of the method is confirmed using melting curve analysis. In an ultimate sense, we aimed at differentiating viremic spike in the infected cell culture and the suppressed state of the same viral entity in response to the antiviral molecule rAdSh5b.

Materials and Methods

***Viruses, Plasmid and Cell lines selection*:** The four Dengue virus serotypes are DENV-1, DENV-2, DENV-3 and DENV-4 (U88535, Nauru Island, AF038403-New Guinea C, M93130-H87, M14931-Dominica) and monkey kidney Vero cell line from ATCC, Virginia, USA for virus infection and rAdsh5b plasmid targeting conserved sequences in the 5^1-UTR region of the above mentioned virus strain's genome (26).

Primer selection based on the 5^1-UTR sequence alignment : The 5^1-UTR of the above DENV strains were aligned using ClustalW

sequence alignment software and the conserved set of nucleotides among all DENV were selected. The consensus primers D1 and D2 were selected based on the criteria that, the primers should have maximum homology with the above selected sequences and must avoid pairing with non-similar sequences to reduce the false results as described in the earlier studies (22).

***Virus infection and RNA extraction*:** The Vero cells were seeded in 12-well plate 48hr before the infection with a seed density of $0.1x10^6$/ml. When the cells attained desirable confluency, spent media was removed and 250 µl of rAdsh-N contained in 2% ΔFCS+1X DMEM was added from B1 to B4 columns, rAdsh5b from C1 to C4 columns and returned to incubator. After 2hrs of incubation, 750µl of 5 ΔFCS+1X DMEM was added to make the final volume to1000µl and placed in the incubator to finish day-1. After 24hrs of the rAdsh infection, DENV infections were setup from column A to C as follows. The medium was removed from each well and 250 µl of virus diluent DENV-1 (A1 to C1), DENV-2 (A2 to C2), DENV-3 (A3 to C3) and DENV-4 (A4 to C4) was added and the plates were returned to the incubator. After 2hs, 750 µl of 5% ΔFCS+1X DMEM was added to make the final volume to 1000 µl and replaced in the incubator to complete day-2. After every 24hrs, designated as day 3, day 4, day 5, day 6, day 7, day 8, day 9 100 µl of cell culture supernatant were collected and stored at -80^{0}C until use. The RNA from each day sample is harvested using Trizol reagent (Invitrogen). In brief, cells were washed with 1ml of 1XPBS and lysed the cells by adding trizol reagent and mixed vigorously by adding chloroform for 15 seconds and then centrifuged. RNA from the aqueous phase was precipitated by adding isopropanol and centrifuged at high speed. The RNA pellet was dissolved in RNase free water and incubated for 10 minutes at 55^{0}C and finally quantified by using Nano drop.

***cDNA synthesis using iScript select cDNA synthesis kit*:** The cDNA were synthesized from the RNA extracted from day 1 to day 7 using iScript select cDNA synthesis kit (Bio-rad). The RNA from the cell culture supernatant sample were mixed with 4 µl of 5X iScript select reaction mix, Gene specific primer (5 picomoles/µl), GSP enhancer solution (1 µl) and Reverse transcriptase enzyme (1 µl) and finally RNase-free water was added to make the final volume to 20 µl. The program consisted of 25^{0}C for 5 minutes, 42^{0}C for 60 minutes, followed by 85^{0}C for 5 minutes. The cDNA product thus obtained was stored at -20^{0}C until further use.

***SYBR Green 1 based real time RT-PCR and Melting curve analysis*:** The real-time RT-PCR (Bio-rad) based on SYBR Green 1 (Bio-rad) was carried out using different dilutions of the cDNA (1:100 and 1:1000) product synthesized from the iScript cDNA synthesis kit. The reaction mixture consisted of 5 µl of cDNA product, 12.5 µl of SYBR Green mix, 0.5 µl of D1 primer, 0.5 µl of D2 primer and 6.5 µl of sterile H_2O to make a final volume of 25 µl. The SYBR Green 1 real time RT-PCR amplification reaction conditions includes 95^0 C for 5 min of pre incubation, 95^{0}C for 30 sec (denaturation), 60^{0}C for 30 sec (annealing) followed by 68^{0}C for 1 min (extension). The reaction continued for 29 cycles with different dilutions of cDNA (1:10 and 1:1000), and the Ct-value for each of the dengue strains were checked using amplification graphs. Since, we incorporated the SYBR Green, a non–specific probe, which binds to the amplified double stranded DNA during real-time RT-PCR, The melting curve analysis was done in the mean time to confirm the amplified product by determining its melting temperature (T_m). The melting curve analysis involves heating the amplified product to 95^{0}C for 1 min and then cooling to 60^{0}C and finally, increasing to 95^{0}C with a transition rate of 0.5^{0}C/30 sec. The samples were run on 1.5% agarose gel to confirm the specific product by size.

***Standardization and normalization of real-time RT-PCR reactions*:** The standardization and normalization of real-time RT-PCR reaction was done in two separate reaction mixture using DENV-4 and GAPDH respectively. The Vero cells

seeded at 0.1x10^6 cells/ml were infected with DENV-4 and the 7th day supernatant collected for RNA extraction and different concentrations (Neat, 1:10, 1:100 and 1:1000) of cDNA (5 µl) was used with D1 and D2 primers and ultimately real-time RT-PCR was performed as above said. In the same way, GAPDH gene was used to synthesize the cDNA and real-time RT-PCR protocol was repeated to normalize the reaction.

Primers used for the Study:

Name of the Primer Primer sequence

D1 5'-TCAATATGCT GAAAC GCGCGAGAAACCG-3'
D2 5'-TTGCACCAACA GTCAATGTCTTCAGGTTC-3'

Results

***Primer selection using 5^1-UTR alignment of four DENV serotypes*:** Although DENV serotypes are antigenically similar, genetically vary among the serotypes and among the strains of the single serotype. Since each serotypes comprises different strains exhibiting distinct genomic variability. To this end, we aligned the 5^1-UTR of the all DENV serotypes and the primer pair common of these conserved sequences was selected. The rAdsh5b construct harboring the shRNA sequences to target the conserved sites in 5^1-NTR was utilized to combat the DENV replication (Table-1).

Specificity of real-time RT-PCR: The specificity of any primer set can be determined by the respective product obtained in an amplification of specified cDNA. It has been shown that, cDNA can be transcribed in the absence of specific primers due to the false priming of RNA during the reverse transcriptase step (27, 28). Hence, investigators have started tagging the primer ends to differentiate the correctly primed once with false primed cDNA using SYBR Green dye (29). In contrast to these, the specificity of our selected primers was verified by the threshold value of the

Table 1. The 5'UTR region of all the four DENV were aligned and set of sequences (shown in red) selected for constructing rAdsh5b were shown and the last underlined sequences(DENV-4) were more similar with the selected construct and hence is more sensitive compared to other prototypes.

	Sh-5b
DENV-1	GTTCTAACAGTTTTTT--ATTAGAGAGCAGATCTCTGATG---
DENV-2	GTTCTAACAGTTTTTT-AATTAGAGAGCAGATCTCTGATG---
DENV -3	GTGCTGACAGTTTTTT--ATTAGAGAGCAGATCTCTGATG---
DENV -4	GTTCTAACAGTTTGTTTGAATAGAGAGCAGATCTCTGGAAAA

Table 2. The specificity of the SYBR green 1 based RT-PCR assay with the selected primers was determined by conducting parallel reactions with and without primers, to check the viral spike in alternative conditions has shown the differential threshold values in both conditions.

Sample name	Den1/ VC	Without primer	Den-2/ VC	without primer	Den-3/ VC	without primer	Den-4/ VC	without primer
Ct value (1:10)	16.37	20.68	15.84	20.20	16.70	20.47	19.47	N/A
Ct value (1:1000)	19.94	18.48	19.11	24.73	19.59	20.40	19.08	22.02

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corresponding real- time RT-PCR reactions involving the panel of DENV amplification with and without the primer. The threshold value was more and was nil in some reaction without the primers D1 and D2 but in the presence of the same primers the representative DENV cDNA were amplified as evidenced by the threshold value compared to the without primer value (Table-2). This strongly implies that the selected primers are specific for the selected serotypes of the DENV.

***Sensitivity of real-time RT-PCR*:** The sensitivity of the SYBR Green 1 based real-time RT-PCR was determined by the Ct-value and the melt curve for each of the DENV serotypes. The threshold value within the same DENV serotype varied widely with different dilutions (1:10 and 1:1000) and the corresponding melting curve is also depicted the decrease in the viral load upon dilution represented by the shortening of the melting peak. The DENV-4 serotype is more sensitive when compared to the other serotypes. This is because the specificity of the consensus primers D1 and D2 towards the 5^1-UTR was more for DENV-4 than other serotypes. Hence, standardization of the assay was done with DENV-4 against the mock infected sample (Fig. 3).

***The DENV viremic spike differentiation in the presence of rAdsh5b using SYBR Geen 1 based real-time RT-PCR*:** The real-time RT-PCR diagnosis of dengue based on 3^1-UTR has already been performed utilizing SYBR Green 1 dye which binds to the amplified DNA product and ultimately detects the specific dengue serotypes using melting curve analysis (21). However, there is a paucity of detection methods which are based on 5^1-UTR of representative dengue prototypes. In the present experiment, despite utilizing the real-time RT-PCR based on 5^1-UTR for detecting the dengue types, it is employed as a tool for differentiating the disease severity while treated with rAdsh5b anti-viral regimen. The complementary DNAs (cDNAs) synthesized utilizing the consensus primers D1 and D2 common to the 5^1-UTR conserved regions were used for the real-time RT-PCR in different dilutions (1:10 and 1:1000) showed characteristic differences in the Ct-value among the DENV serotypes. The threshold value (Ct) differences in the amplification graph represent the viral load in each dilution of the specific DENV serotypes (Fig.1 and 2). The amplification of specific DENV stereotypes was confirmed using melting peaks and subsequently by the gel electrophoresis. The clear band observed

Table 3. In an parallel RT-PCR reaction with DENV1, 2, 3 and 4 alone and along with rAdsh5b regimen at different dilutions (1:10 and 1:1000) have yielded the observable differences in the threshold values. The increase in the threshold value in each dilution indicates the inhibitory effect of respective rAdsh5b against all DENV selected.

Sample Name	Den-1/ VC	Den-1 /rAdsh5 b	Den-2/ VC	Den-2 /rAdsh5 b	Den-3/ VC	Den-3 /rAdsh5 b	Den-4/ VC	Den-4 /rAdsh5 b
Ct value (1:10) dilution	16.37	18.14	15.84	17.46	16.70	18.79	19.47	23.07
Ct value (1:1000) dilution	19.94	20.66	19.11	20.75	19.59	21.75	19.08	22.72

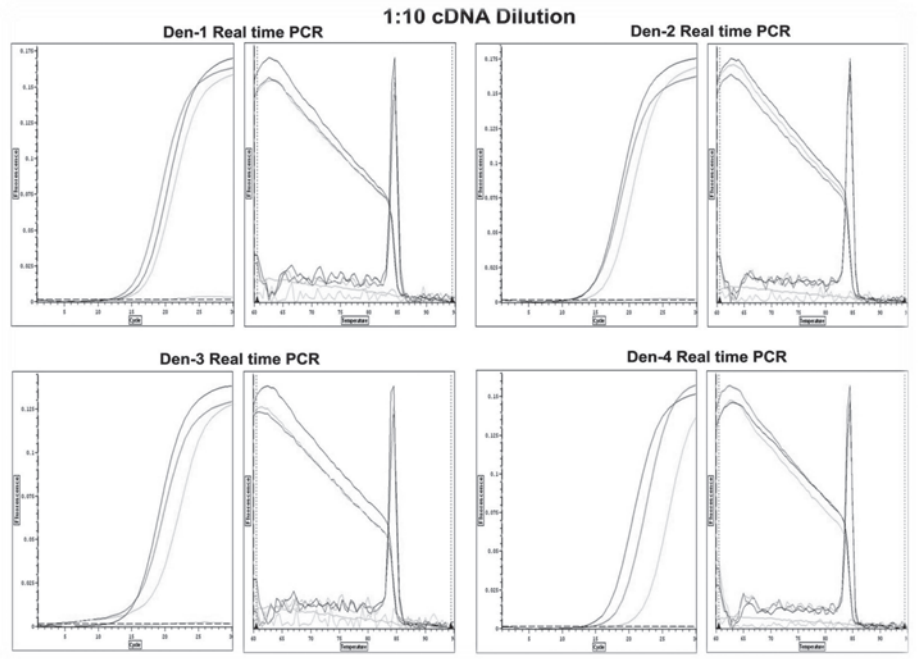

Fig. 1. The amplification graph represents the real time PCR of the 1:10 diluted cDNA of DENV1, 2, 3 and 4 and along with the rAdsh5b, rAdshN, and without primer. The graph indicates the variations in the threshold value of the rAdshN and without primer, and the rAdsh5b against each of the DENV with the fixed dilution of 1:10 of cDNA. The threshold value was more in the rAdsh5b treated sample compared to VC of each of DENV but it was less than the without primer sample implies that, rAdsh5b hindered the replication of DENV ,and hence due to the less DENV specific nucleotides, the Ct value increased significantly compared to VC.

corresponding to 512 mb band indicates amplification was specific for representative serotypes of the DENV (Fig. 4). If the copy number of the target nucleic acid is more in the sample, sooner is the threshold value and subsequent increase in the fluorescence. The threshold value increased in the rAdsh5b treated samples when compared to the each DENV serotypes in both the dilutions implies that, rAdsh5b successfully inhibited the replication of all the four DENV serotypes (Table-3).

Discussion

Since dengue serotypes exhibit variant genetic strains, many real-time RT-PCR methods have been deduced in the recent decades with the concerted effect of advanced detection chemistries and updated molecular information about the emerging dengue viruses. All the assays described in the literature so far have shown that the DENV can be detected and serotyped using real-time RT-PCR employing the primer or probes directed against the conserved regions of the dengue genome such as 3¹-UTR and 5¹-UTR (24). However, there have been efforts to utilize the RT-PCR to quantify the immunodeficiency virus type1 viral load in disease progression and as well in response to the antiviral regimen in the plasma sample (30). In contrast, this is the first report describing the utilization of real-time RT-PCR to detect the dengue serotypes whilst in the disease progression and viral load with response to antiviral regimen. In the present study we showed that SYBR Green 1 based real-time RT-PCR method utilizing the consensus primers common to the 5¹-UTR conserved sequences

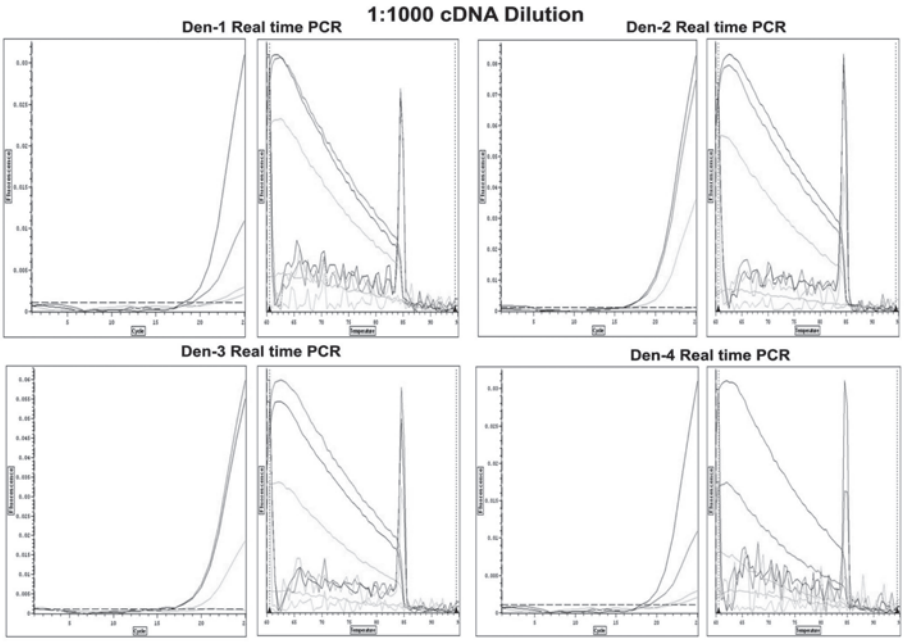

Fig. 2. The amplification graph represents the real time PCR of the 1:1000 diluted cDNA of DENV1, 2, 3 and 4 and along with the rAdsh5b, rAdshN, and without primer. The graph indicates the variations in the threshold value of the rAdshN and without primer, and the rAdsh5b against each of the DENV with the fixed dilution of 1:1000 of cDNA. The threshold value was more in the rAdsh5b treated sample compared to VC of each of DENV but it was less than the without primer sample implies rAdsh5b hindered the replication of DENV ,and hence due to the less DENV specific nucleotides, the Ct value increased significantly compared to VC. With comparison to 1:10 dilution, the Ct value in this dilution have comparably hiked indicating the efficiency detection limit of the real time PCR method with higher dilution sample selected.

can be used to detect all the four dengue serotypes and in fact, confirmed by melting peaks of RNA from the cell culture sample infected with rAdsh5b antiviral regimen. The efficiency of the real-time RT-PCR in discriminating the viremic spike in the presence and absence of antiviral molecule has proved that each DENV serotypes spiked according to their genetic variability as indicated by the threshold values. Since, threshold value depends on the compatibility of the viral RNA sequences with available primer and nucleotides, the specific viremia in the sample can be detected according to its threshold value. The sample infected with DENV 1, 2, 3, and 4 alone at different dilutions showed the lower threshold value compared to the samples infected with rAdsh5b along with all four serotypes and amplification of specific dengue serotypes in both the cases has confirmed using the melting peak shown at 84.5^{0}C which represents the 512 mb in the agarose gel electrophoresis (Fig. 4). The difference in the Ct-value is due to the fact that, rAdsh5b successfully inhibited the replication of all the dengue serotypes by targeting the 5^1-UTR regions which is common to consensus primers D1 and D2. Hence, due to the scarcity of dengue specific viral RNA in the rAdsh5b treated sample the primer spared as unused. However, there is a detectable threshold value in all the samples and in both the dilutions ascribed to the fact that the homology among different dengue genomes may decrease towards the 5^1-UTR region in the same way as reported in the 3^1-NTR (31).

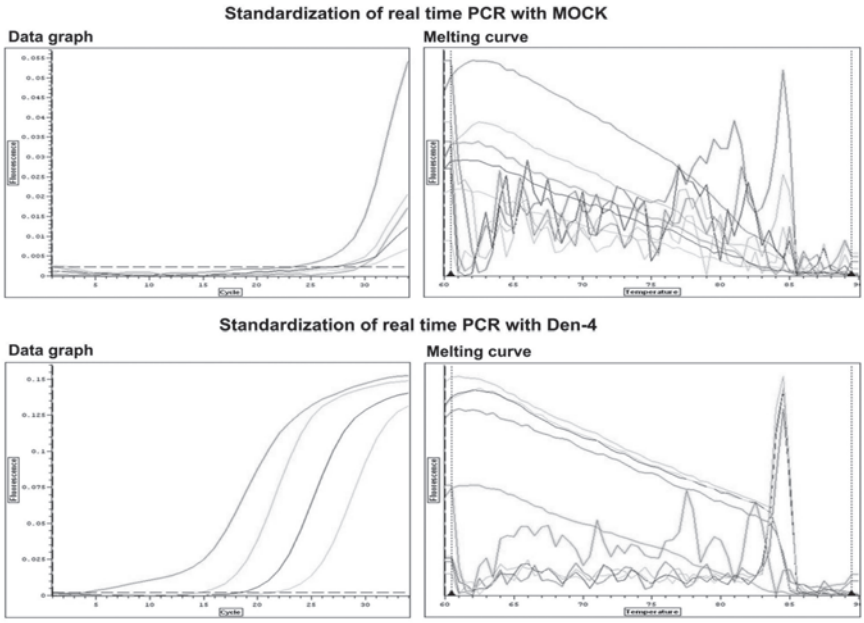

Fig. 3. The standardization of the real time PCR assay done with DENV 4 and with mock infected sample. Since, DENV 4 showed more similarity with the selected construct, the sensitivity criteria are being considered. The amplification graph obtained with the different dilutions neat, 1:10, 1:100, 1:1000 showed marked Ct values compared to the mock infected sample.

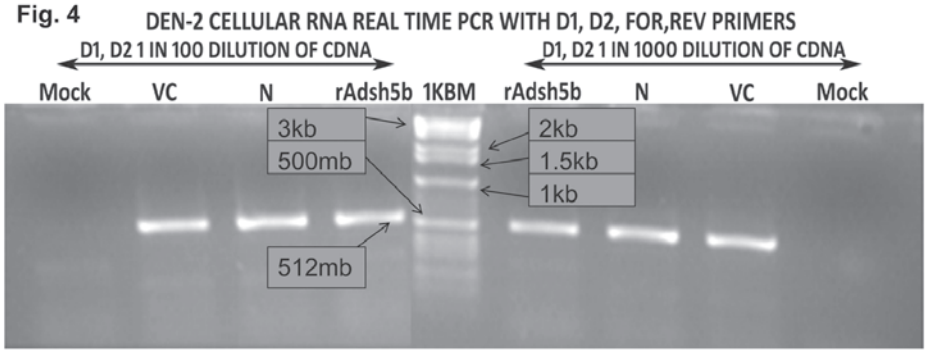

Fig. 4: The agarose gel from the melting curve analysis samples showing the clear 512 kb band indicates that, the real time PCR was specific with the SYBR green 1 dye. The single band in the gel confirms the absence of primer dimers in the reaction.

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Conclusion

The results obtained from our study have explained that SYBR Green 1 based real-time PCR method can be used efficiently to detect and differentiate the viral RNA spike in the presence of antiviral regimen rAdsh5b and hence the assay can be utilized as rapid, sensitive and efficient detection method to check the dengue in the presence of antiviral regimen. Though we utilized the consensus genome coding regions of the dengue for the real-time PCR to identify the different dengue serotypes, the effort was not successful since, the homology among selected dengue serotypes was not completely conserved. This may be because of the variation in the dengue genome either due to the mutation among genotypes, and the strains within a single serotype. Hence, advanced dengue genome scanning methodologies will need to emerge in the near future to identify the most conserved sequences and as well sequences prone to mutation. This effort can be helpful to tag the primer sequences specific for conserved sites and mutation sequences to track the viral RNA degeneracy.

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