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Diagnosis of Dengue Viral Infection in Haryana by Reverse Transcriptase PCR

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Abstract

Background: Dengue is a common mosquito-borne viral disease in India with both endemic and outbreak forms. It causes hospitalization of about five hundred thousand people with a higher mortality rate. Thus, early and correct diagnosis is very crucial for treatment of Dengue fever. **Methods:** A total of 25 blood samples were obtained from patients within the age range of 5 years to 64 years, who were having symptoms of Dengue illness. The samples were obtained within the 7 days of onset of fever. All the blood samples that were subjected to NS1 ELISA for Dengue diagnosis afterwards they analysed with our RT-PCR test and both the methods were compared. The targeted product size was ~240 bp. **Results:** Among the 25 Dengue virus suspected samples tested, 22 were found positive with RT-PCR assay and 20 with NS1 ELISA (two were equivocal cases). The size of the PCR product obtained after amplification with specific primers was ~240 bp. The amplified product was also verified by restriction digestion with *KpnI* enzyme and the bands obtained were of sizes ~183bp and ~57bp. Sequencing of the 240 bp PCR fragment was also performed to confirm the result. **Conclusions:** RT-PCR based assay was found to be more specific and sensitive as compared to the ELISA assay.

Keywords
Dengue fever, NS1, RT-PCR, Early detection.

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INTRODUCTION

Dengue virus is a member of enveloped, single stranded RNA viruses of the flaviviridae family [1]. The flaviviridae family also includes the West Nile virus, Yellow fever virus, Japanese encephalitis virus, Hepatitis C virus and tick-borne encephalitis virus. Dengue is transmitted by mosquitoes of the genus *Aedes*, mainly of the species *A. aegypti* [2].

The cycle of transmission begins once an adult female *A. aegypti* bites an infected human, acquiring the virus along with the blood meal. When an infected mosquito bites a healthy human, it retransmits the virus to complete the transmission

cycle [3]. Dengue virus is found with prevalence in tropical and subtropical areas throughout the world. About 40 percent or 2.5 billion of the world's population reside in the urban and suburban areas that are at the risk of dengue infection [4].

The DENV genome is about 10.7 kb of positive-sense RNA that codes the three structural proteins (capsid protein C, membrane protein M, envelope protein E) and seven non-structural proteins (NS_1, NS_{2a}, NS_{2b}, NS_3, NS_{4a}, NS_{4b}, NS_5). It also includes short non-coding regions on both the 5' and 3' ends. NS_1 is a conserved protein in the genome of flaviviruses. All non-structural proteins are responsible for regulation and replication of virus [5].

The World Health Organization (WHO) estimates that about one hundred million DENV infections occur each year, causing hospitalization of five hundred thousand people and a death rate of 2.5 percent. Till now, there were only four serotypes of Dengue virus and an additional fifth serotype was isolated in October 2013 [6,7]. Nowadays, Dengue is causing serious illness with their different serotypes. India's population is twice that of Southeast Asia, the region that currently reports the most dengue-related deaths [8]. Infection with one-serotype of Dengue virus results in lifelong immunity to it but there is no cross-protection against the others [9]. There is no specific treatment available for Dengue. Thus, early diagnosis has a major role in the treatment and controls the complications of Dengue fever.

METHODS

Clinical samples

Blood samples were obtained from State Bacteriology Lab, Karnal, Haryana with written permission of the state bacteriologist. All blood samples were obtained with the consent of the patients and following the ethical issues guidelines. A total of 25 blood samples were obtained from patients within the age range of 5 years to 64 years who were having symptoms of Dengue illness. The majority of the patient's age was 23 years to 44 years with male. The samples were obtained within the 7 days of onset of fever (acute phase). Blood samples were previously assayed for DENV by NS1 ELISA at State Health Lab, Karnal, Haryana.

DENV NS1 ELISA PROCESSING

Dengue early ELISA kit (CTK Biotech, USA) was used to determine the presence of DENV NS1 protein in the blood samples. Positive and negative controls were added to the wells according to the manufacturer's instructions which comprised of two negative and two positive controls. Finally, ELISA sample plates were read at 450 nm wavelength with a reference filter of 650 nm. Each sample O.D. value (absorbance) was divided by the cut-off value to obtain index values. Index values < 0.9 were ruled out as negative, between 0.9 and 1.1 as equivocal, and above 1.1 as positive for DENV NS1 detection.

Design of DENV serotype-specific Primers

Oligonucleotide forward and reverse NS1 gene specific primer sequences were fetched using BLASTn (*https://blast.ncbi.nlm.nih.gov/Blast.cgi*) and the melting temperature of the primers were set. The primers were designed in such a way that there was no mispriming with the human DNA. The sequences of forward and reverse primers are given below in the table1: -

Table 1: - Primers used for NS1 gene amplification

Primer name	Nucleotides	Tm(°C)	Length (bp)
DENV Forward	CATGATCATTCCAAAGAGTCTAGCTCCTCC	60	30
DENV Reverse	GGAGGAAGTGTGCACGAGCGGCA	60	23

RNA Extraction

Total RNA was extracted from 0.5ml of blood samples by Trizol reagent method (Ambion, USA). Blood samples were transferred to a 2 ml of eppendorf tube (Tarsons) and RBCs were lysed using lysis buffer composed of 8.29 gm NH_4Cl, 1 gm $KHCO_3$ and 400 µL of 0.5M Na_2.EDTA (ph-8.0) in 1L of water. The cells were treated with lysis buffer until a white pellet was obtained to which 500 µL of Trizol was added. RNA was extracted, pelleted and dissolved in nuclease free water (10 µL/pellet). The quantity of RNA samples was determined using a Nano Drop 1000 spectrophotometer (Thermo Scientific, USA) while the quality was checked by agarose gel electrophoresis. The quantities of RNAs obtained from the samples were within the range of 250-600 ng/µL).

cDNA Synthesis

First strand complimentary DNA was synthesized by using the ***Revert Aid*** First Strand cDNA synthesis kit (Thermo Scientific, USA) using random hexamer primers according to the manufacturer's instructions. 500 ng RNA was taken for each cDNA synthesis.

RT-PCR Amplification

The PCR reaction using DENV specific primers was prepared using Taq Polymerase and Buffer (GeneI) as 2.5 µL 10X Reaction Buffer, 0.5 µL dNTP Mix (2.5mM), DENV FWD & DENV RV primers (10pmol) each, 0.25 µL Taq Polymerase (5U/µL) and 0.5 µL cDNA which was subsequently made to a final volume of 25 µL/ reaction by adding water.

The parameters for PCR cycling included an initial denaturation at 95˚C for 1 min, 35 cycles of denaturation (95˚C) for 10s, annealing (60˚C) for 10s and extension (72˚C) for 10s along with a final extension at 72˚C for 2 min. The PCR products were electrophoresed on 3% agarose gel (Sigma, Aldrich) in prepared in 1X TAE buffer, followed by staining with Ethidium Bromide (EtBr) and then subsequent visualization using the Gel Doc system.

Cloning

The PCR products were purified using PCR clean up kit (MN Nagel) and finally eluted in 12µL nuclease free water. The 60 ng purified of PCR products were cloned using ***InsTAclone*** PCR cloning kit (Thermo Scientific, USA) according to the manufacturer's instructions. The cloned products were then transformed into XL1-Blue competent *E.Coli* cells. The recombinant clones were subsequently screened through PCR.

Sanger Sequencing

Sanger's di-deoxy sequencing was performed and run on Genome Lab™ GeXP Genetic Analysis System (Beckman Coulter). The DTCS quick start cycle sequencing kit (Beckman Coulter) was used for preparing sequencing master mixture according to manufacturer's instructions. A 100 fmol DNA was taken as template. 5 pmol of either M13 forward or M13 reverse universal primers used for a single sequencing reaction. Finally, 8 µL of quick start master mix was added to make final reaction volume of 20µL. 30 cycles of denaturation (96°c for 20 sec.), annealing (50°c for 20 sec.), and extension (60°c for 4 min.) were performed at Veriti™ 96-Well Thermal Cycler (Thermo Fisher Scientific). The cycling sequencing product was purified with "ethanol precipitation" method and vacuum dried. The dried products were reconstituted with 40 µL SLS solution (supplied with the kit), loaded into 96 well sequencing plate and layered with mineral oil. The sequencing plate was run into Genome Lab™ GeXP Genetic Analysis System (Beckman Coulter) along with buffer plate. The sequencing data was analyzed after the run was completed.

RESULTS

ELISA Assay

Among the 25 Dengue virus suspected samples tested, 20 (80.0%) were found positive with CTK-Biotech NS1 ELISA kit. There were 2 (8%) equivocal cases whose results were within the range of 0.9 to 1.1 according to the reference value. Negative samples with NS1 ELISA had values ranging from 0.034 to 0.046 and the positive samples 3.56 to 10.8. The exact observed values of 2 equivocal cases were 0.91 and 1.0 as shown in the wells 4A & 4B of the Fig 1.

Absorbance NS1CTK Plate:01 Date:18-11-20 Time:18:34 (450nm)

	01	02	03	04	05	06	07	08	09	10	11	12
A	0.000	0.045	0.000	0.275	0.000	0.000	0.000	0.000	0.004	0.000	0.000	0.000
B	0.000	0.036	0.000	0.301	0.001	0.000	0.000	0.000	0.000	0.000	0.000	0.000
C	0.000	1.146	0.000	0.046	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
D	0.000	1.223	0.000	0.046	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
E	0.000	0.034	0.000	1.338	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
F	0.000	1.070	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
G	0.000	1.191	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
H	0.000	1.255	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Fig 1. Optical density values of suspected cases for Dengue NS1 carried out through ELISA using 450nm wavelength. In lane 2A & 2B were the negative controls, 2C & 2B were the positive controls; 2E, 4C & 4D were negative for Dengue; 2F, 2G, 2H & 4E were positive for Dengue while 4A & 4B were the equivocal for Dengue which ranges 0.9 to 1.0.

RT-PCR Assay

RNA yield from blood WBCs was found better than serum such that only WBCs from blood of patients were used to extract RNA. In the process of RT-PCR test, the size of the PCR product obtained after amplification with DENV specific primers was ~240 bp. The PCR product obtained was further confirmed by restriction digestion with ***KpnI*** (10U/µL) enzyme and then fully confirmed by sequencing. Sequencing results were then subjected to NCBI BLASTn to validate the data. It was found to be DENV sequence with 100% identity. After restriction digestion, the bands obtained were of sizes ~183bp and ~57bp. Out

of the 25 samples tested, 22 (88.0%) were found positive with this assay.

All controls were uniformly negative with our RT-PCR assay. There was no false positive or false negative test noted during this study with our RT-PCR assay. The gel electrophoresis of the PCR products and the restriction digestion products are shown in the Fig 2,3,4,5.

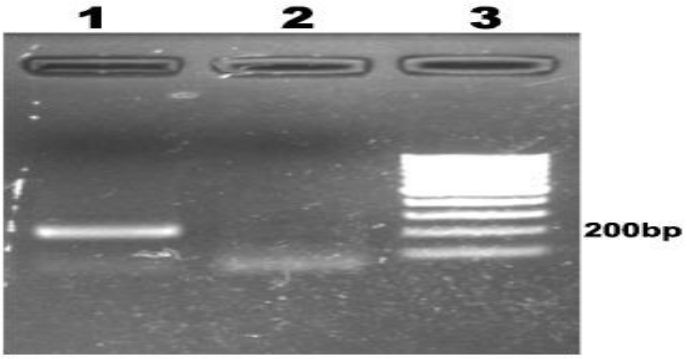


Fig2 Gel electrophoresis of PCR products using DENV-3 primers. Sample in Lane 1 shows positive for Dengue virus corresponding to size ~240bp while Lane 2 was negative for the virus. 100bp ladder was loaded in lane 3.

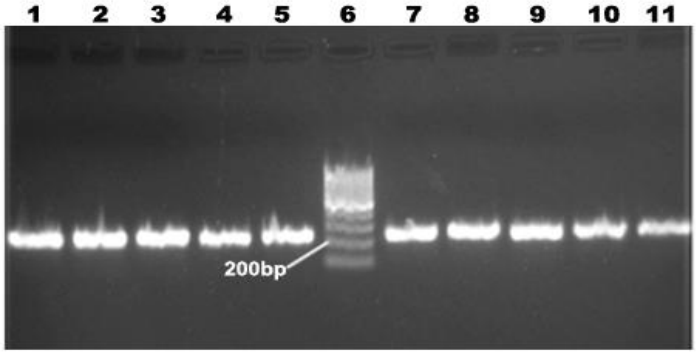


Fig3 Gel electrophoresis of PCR products using DENV-3 primers. Lane 6 contained 100bp ladder. Lanes 1-5 and 7-11 showed PCR products at ~240bp indicating that all the samples tested were positive for Dengue virus.

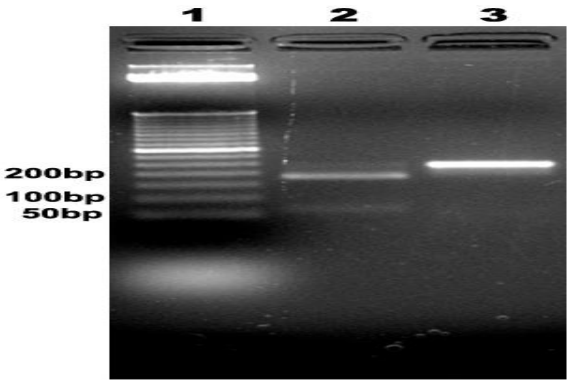


Fig4 Gel electrophoresis of products formed after the restriction digestion of the PCR products using DENV-3 primers. Lane 4 contained 50bp ladder. Lanes 1-3 and 5-7 showed restriction digestion products with one band near 200bp size while the other band near 50bp size, further confirming that the samples tested were positive for Dengue virus.

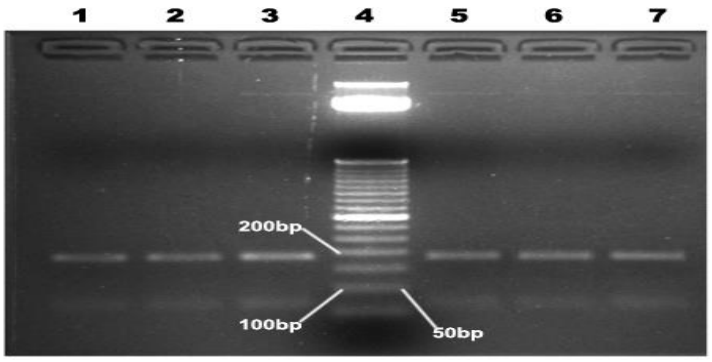


Fig5 Gel electrophoresis of products formed after the restriction digestion of the PCR products using DENV-3 primers. Lane 1 contained 50bp ladder. Lane 2 contained digested product while. Lane 3 contained undigested PCR product. This confirms the band near the 50bp size is digested product and not the primer dimers that often arise during PCR process.

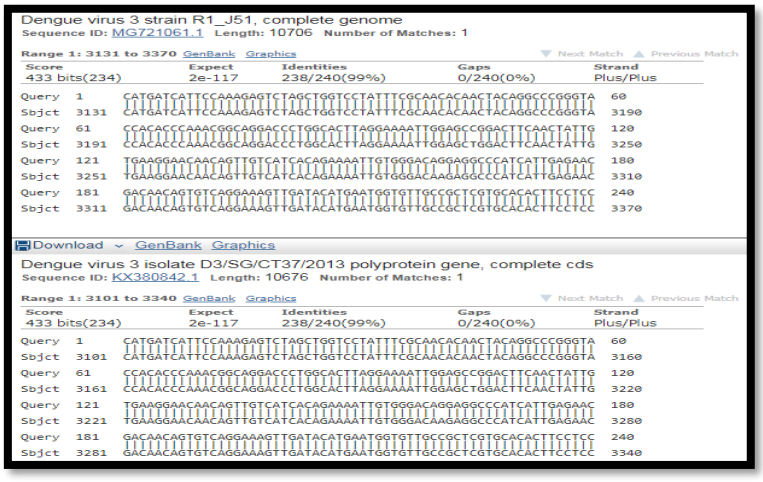
DISCUSSION

Dengue fever is a worldwide tropical disease that causes significant number of deaths each year. A proper method for diagnosis is still great challenge in developing countries like India due to lack of resources, infrastructure and skilled manpower. A recent study published shows that every year the prevalence of dengue fever is increasing [10]. This disease is a major health problem in India and needs to be diagnosed and treated in early phase of the disease to avoid any associated morbidity and mortality [11,12].

We targeted NS1gene because the titter level of NS1and its duration is always found greater at the time of primary infection than secondary infection [13]. So, it is becoming a most targeted tool in the early diagnosis. In our study, NS1 ELISA and RT-PCR based assay for diagnosis of Dengue virus were compared with each other in terms of robustness and sensitivity. It was found that the diagnostic performance of RT-PCR was better than the Dengue ELISA kit based test because 2 of the equivocal cases found in ELISA were clearly found to be positive using the RT-PCR method as shown in Table 2 such that the RT-PCR method was more robust as compared to the ELISA assay. The sequencing result for the cloned PCR product showed to have more than 99% identity with Dengue virus-3 serotype as shown in Figure 6.

Table 2: -

Total number of cases	Number of control cases	Number of positive samples by RT-PCR	Number of positive samples by ELISA
25	03	22	20 positive & 2 equivocal



The sequencing result for the cloned PCR product showed to have more than 99% identity with Dengue virus as shown in Figure 6.

Through this work, we provide a scope for more robust and sensitive assay than the ELISA method for diagnosis of Dengue virus. The specificity and sensitivity of this assay makes it more robust compared to the antibody based detection methods which could lead to higher chances of false positives. The antibody based tests can only work if the viral antigen is present in the serum when the sample is being procured which is not with the case of RT-PCR method as the presence of viral RNA is enough for its detection or in other words, the virus can be detected even before it starts its protein synthesis. So, this nucleic acid based method proves to be useful in early diagnosis of Dengue Virus within the day one of the onset of symptoms which could in turn, help in reducing the risk of complications such as DHF or DSS, especially in the endemic areas such as India.

The assay requires at least five hours completing which can be further reduced by introducing certain modifications. It may not require for all Dengue infected cases. However, it is required in some critical, special or equivocal cases to be confirmed by such nucleic acid based diagnosis method.

CONCLUSION

In this report, we describe the development of a highly specific nucleic acid based assay for Dengue virus detection. Two equivocal cases by ELISA were found clearly positive with our RT-PCR based assay which makes it the method of choice over the ELISA. So accuracy, specificity and sensitivity of this assay make it robust compared to antibody based detection methods. This assay requires reasonable amount of time about five hours. The advantage of this assay is that all steps have been performed without use of any detection kit. This method can also be used to detect other fatal viruses like Chikungunya virus, Zika virus, etc.

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Nil.

CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interests regarding the Publication of this article.

ETHICAL APPROVAL

The study was approved by the University Ethics Committee of School of Life and Basic Sciences, Jaipur National University, Jaipur (Monday, Dec.11, 2017). Written informed consent was obtained from each patient.

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