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Research Article

PCR Detection of Dengue Virus Serotypes in Samples from Punjab Using RT-PCR by Using Specific Primers

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ABSTRACT

Background: Dengue virus is a positive-stranded encapsulated RNA virus. The size of genomic RNA is approximately 11 Kb in length and it consists of three structural genes which encode the nucleocapsid or core protein (C), a membrane associated protein (M), an envelope protein (E), and seven nonstructural (NS) genes. In Pakistan species or strains of dengue viruses infecting people is not known. Currently available techniques for detection of dengue virus in Pakistan were ELISA, dot blot immunoassay, complement fixation test.
Objectives: The objective of the study was to collect dengue samples from different regions, extract RNA and optimize RT-PCR by using specific primers i.e DEN-I, DEN- II, DEN-III, DEN-IV cloning.
Study Design: Case series
Sampling technique:non probability / convenient sampling
Sample size: 15 blood samples from MyohospitalLahore and Allied hospital Faisalabad.
Data collection and analysis: Reverse transcriptase PCR is rapid and accurate method for diagnosis of dengue virus. Using Reverse transcriptase PCR one is able to detect and differentiate among the four DENV serotypes. Dengue viral RNA can be detected in early phase of infection using reverse transcriptase PCR. Multiplex PCR was used instead of using a single primer set, it will amplify any of the strains in the sample. Restriction digestion by*EcoR1* and *Pst1* and desired clone was obtained.
Results:
Conclusion:

Keywords: Dengue virus, PCR essay, Serotypes.

INTRODUCTION

Dengue is severe viral infection. The word “dengue” is orginate from the Swahili phrase Ka-dingapepo. Dengue fever was known as “water poison” related with flying insects in ancient book in 992. Initially the occurrence of dengue in most regions of world since 1780s. First reported case in 1789 outbreak in Philadelphia “break bone fever” was discovered [1].Occurrence of dengue virus developed significantly in recent years. The disease dengue prevalence of dengue increase day by day around the world and it is predicted that dengue virus causes 50 to 100 million cases of severe febrile disease every year [2].

Dengue virus is most common in tropical regions of the world [3]. Positive-stranded encapsulated RNA virus of dengue genome. Length of dengue genome is 11 kb. Dengue virus consist of three structural genes first gene code for core protein (C), second gene code for membrane associated protein (M), third protein code for an envelope

protein (E), on the otherhand dengue virus has also seven nonstructural (NS) genes [4].

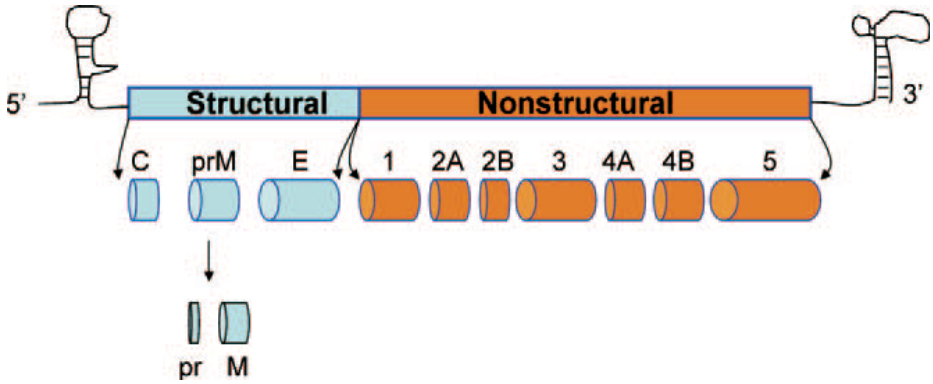

Figure 1.1 Dengue viral genome.

Dengue protein comprised of three thousand amino acids process posttranslational through specific proteases enzyme. RNA genome has single open reading frame which translate into polyprotein. Due to signal and stop-transfer sequences, translocation of protein is directly around the membrane. Aminoacid chain is then cut by two specific enzymes i.e proteinases and glycosyltransferases to give way to proteins [5].

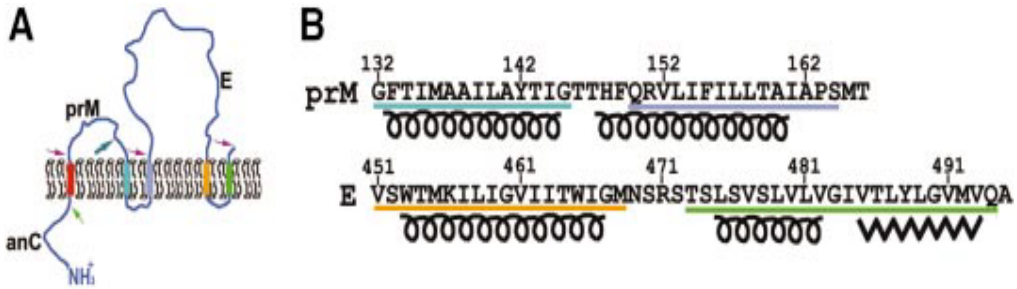

Figure 1.2A and B Structure of transmembrane protein

The serotypes of dengue virus are main factor of multiple infection as a result of enhancement of antibody dependence. Mechanism of dengue hemmorhagic fever / dengue shock syndrome factors are: viral virulence, genetic background of host, activation of T-cell, viral load, and autoantibodies. Replication cycle of dengue virus includes following steps i.e after translation genomic RNA is converted into proteins, positive and negative stranded RNAs is synthesize, assembly and mature virions is release [6].For the detection of virus, the techniques for isolation of virus through cell culture, but on the otherhand it is not reliable, because this technique takes many days for diagnosing the dengue virus. Recently two techniques have been discovered for diagnosing of dengue virus i.e RT-PCR and ELISA [7]. RT-PCR is rapid and accurate method for diagnosing the dengue virus as compared to other techniques. Dengue virus infections initially start from mild fever and flu and then leads to severe form resulting in fatality [8].

The study focuses on optimization of reverse transcriptase PCR for dengue virus and rapid extract the dengue virus through reverse transcriptase PCR and establish the protocol for dengue virus detection.

OBJECTIVES:

The objectives of the study were to

- Collection of dengue samples from two different regions of Punjab, Faisalabad and Lahore and Optimize dengue samples by RT-PCR by using specific primers i.e DENV-I, DENV-II, DENV-III, DENV-IV for all strains and GAPDH primer was used as internal control primer.

MATERIAL AND METHODS:

15 blood samples of Dengue infected patients from different regions of PUNJAB (Allied Hospital of Faisalabad and Mayo Hospital of

Lahore) were collected. Fresh blood was collected in EDTA coated cortex blood collection tubes. 3ml blood was taken in each EDTA coated cortex blood collection tubes. Before doing RNA isolation it was necessary to wear gloves and sterilize the benchtop with Lysol or any disinfectant.

All media and solutions were prepared in distilled water and autoclaved at 121oC at 15lb/inch2 for 15 min. Glasswares were washed properly and oven dried before use. All the chemicals were of analytical grade. Vortex, EDTA coated cortex blood collection tubes, micropipettes, microtips, Dengue primers (DENV-I, DENV-II, DENV-III, DENV-IV), refrigerated centrifuge, incubator, eppendorfs, beaker, fridge (-40oC, -4oC), PCR tubes, PCR machine. Prepared 0.5% of bromophenol blue in ultrapure water. 10ml total. 1ml of dye and 9ml formamide was taken. Mix and store it at -20oC. RNA extraction using leukolockTM total RNA isolation, whole blood using tempusTM blood RNA tube and RNA isolation from whole blood by trizol reagent was done. RNA quantified on a spectrophotometer and the concentration of RNA is calculated by using formula equation.

1OD at 260nm= 40µg/ml

Absorbance at 260nm (A260) and absorbance at 280nm (A280) were also known. The OD of RNA 1.8 to 2.0. Negative control was important in reverse transcriptase polymerase chain reaction to access for genomic DNA contamination of the RNA sample. The control reverse transcriptase reaction contains every reagent for the reverse transcription reaction except for the reverse transcriptase enzyme. In negative control equal amount of water was added as in other eppendorfs in place of water RNA was added. PCR product was purified through phenol chloroform extraction method. Transformation and mini preparation of plasmid DNA was done and finally confirmation of positive construct was done by restriction with EcoR1 and Pst1 which gave a fragment of 221bp.

RESULTS:

Table 1 Concentration and purity of total RNA isolated from different blood samples of dengue infected patients.

No. of samples	Leukolock^{TM}total RNA method		Tempus^{TM} blood RNA tube method		Trizol method	
	Concentration of RNA (µg/ml)	A_{260}/ A_{280}	Concentration of RNA (µg/ml)	A_{260}/ A_{280}	Concentration of RNA(µg/ml)	A_{260}/ A_{280}
1	3	0.7	6	0.01	40	1.5
2	2	0.6	5	0.02	60	1.6
3	1	0.5	4	0.03	70	1.7
4	2	0.4	8	0.04	80	1.8
5	1	0.3	7	0.05	90	1.9
6	2	0.2	9	0.06	50	2.0

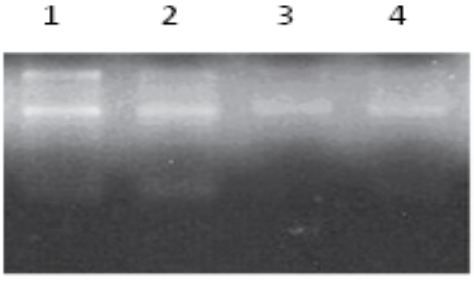

Figure 1: Total RNA extracted from the blood of dengue infected patient by Leukolock^{TM} total RNA method. Lane 1,2,3,4: total RNA from sample 1, 2, 3 and 4 respectively.

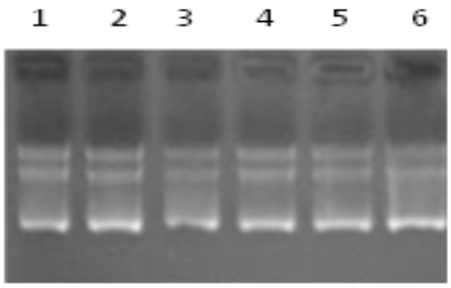

Figure 2: Total RNA extracted from the blood of dengue infected patient by Tempus^{TM} blood RNA tube method. Lane 1,2,3,4,5,6: total RNA from sample 1, 2,3,4,5 and 6 respectively.

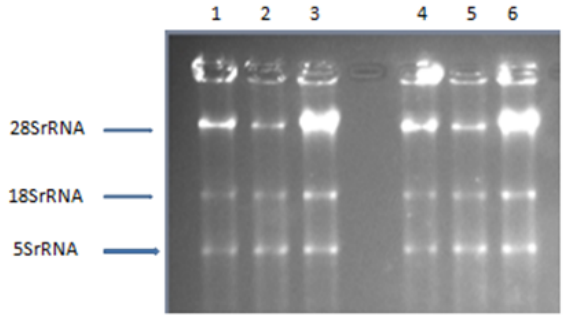

Figure 4.3: Total RNA extracted from the blood of dengue infected patient by Trizol method. Lane 1,2,3,4,5,6: total RNA from sample 1, 2,3,4,5 and 6 respectively.

4.3Reverse transcription polymerase chain reaction (RT-PCR)

In RT-PCR, RNA was converted to cDNA. There were two steps of RT-PCR i.e first step was cDNA synthesis, second step was PCR amplification. In RT PCR the first sample was of negative control, the other four samples were of human GAPDH gene (reverse) primers and the last one was ladder. GAPDH primers were used in RT-PCR as an internal control primer. The gene of GAPDH is a house keeping gene, that is expressed constitutively and stably in cells and tissues, GAPDH is used as control for RT-PCR. 0.5 µg of RNA isolated 4 different samples of dengue patients were subjected for RT-PCR. GAPDH primers were used to check the amplification quality of total RNA. Lane 2 shows positive amplification from the control GAPDH RNA (Fermentas). For RT-PCR reaction RT-PCR Fermentas kit was used. 0.5 ug of DNA Ladder was loaded on the gel gel as shown in fig 4.4.

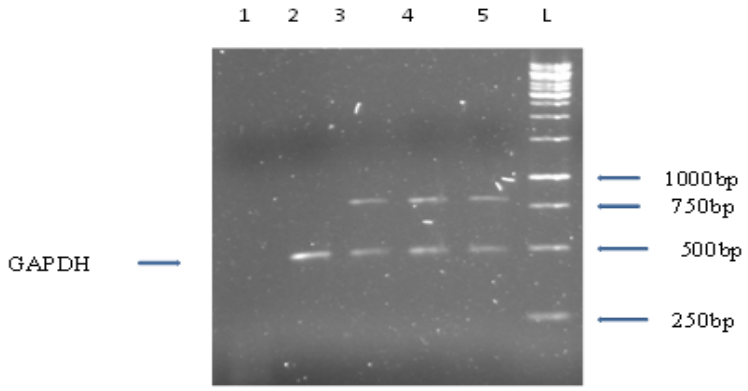

Figure 4: RT-PCR of total RNA isolated from dengue infected patients. Lane1: negative control; Lane 2: positive control GAPDH RNA (Fermentas). Lane 2,3,4,5: positive amplification from the control GAPDH RNA (Fermentas). Lane L: DNA ladder of 1Kb.

4.4 PCR (Polymerase Chain Reaction)

After RT-PCR, PCR amplification was done. According to the dengue serotype, 4 sets of primers were designed and ampilification was done with 4 sets of primers in uniplex PCR. PCR was positive for DENV2 serotype as shown in fig. 4.5. The PCR ampilified fragment of DENV2 serotype is of 221bp.

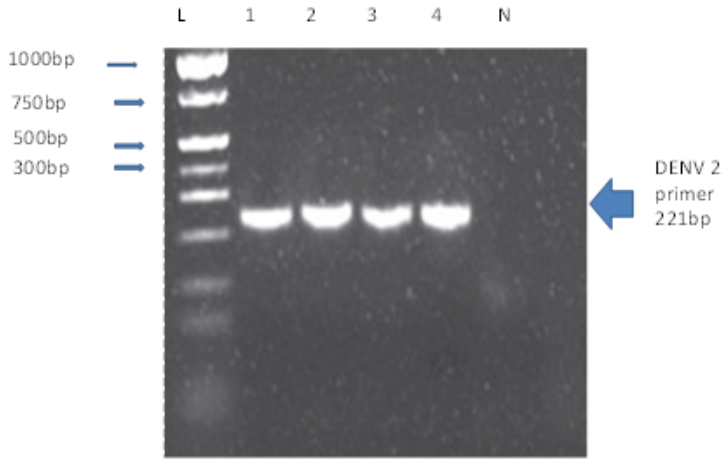

Figure 4.5: Multiplex-PCR ampilification by using dengue serotype specific primers DENV1, DENV2, DENV3 and DENV4. Lane 1,2,3,4: the ampilification with only DENV2 primer respectively; Lane L: DNA ladder; Lane N: Negative control.

4.5 Optimization of conditions for multiplex PCR

4.5.1 Optimization of annealing temperature

Conditions were optimized for multiplex PCR by changing the annealing temperature of the PCR reaction. The four sets of primers has melting temperature in the range of 48 ^{o}C -55 ^{o}C. Multiplex PCR was optimized with all the four sets of primer pair by using annealing temperature of 43 ^{o}C, 45 ^{o}C, 47 ^{o}C, 49 ^{o}C, 50 ^{o}C, 52 ^{o}C and 55 ^{o}C. PCR was positive with DENV2 serotype by using annealing temperature of 50 ^{o}C, 52 ^{o}C and 55 ^{o}C but the maximum PCR ampilification was at 55 ^{o}C as shown in fig 4.6.

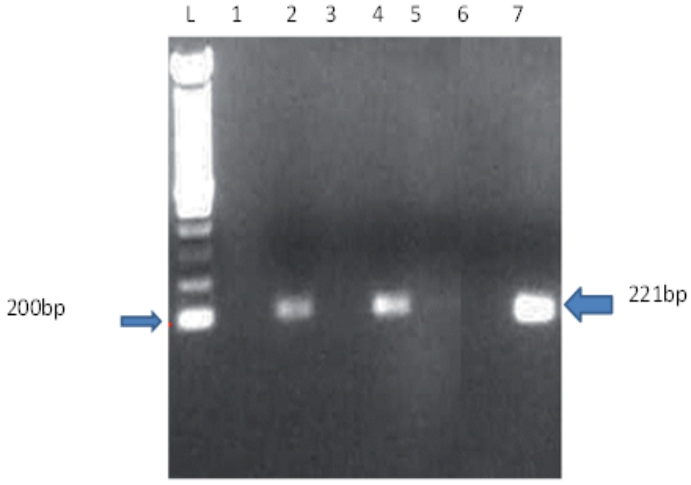

Figure 4.6 Optimization of annealing temperature on 0.8% agarose gel. L: DNA ladder. Lane 1: annealing temperature 43^{0}C, Lane 2: annealing temperature 50^{o}C, Lane 3 : annealing temperature 45^{o}C, Lane 4: annealing temperature of 52^{o}C, Lane 5 : annealing temperature 47^{o}C, Lane 6: annealing temperature 49^{0}C, Lane 7:annealing temperature 55^{o}C.

Table 4.2: Four sets of primers used in multiplex PCR along with their sequence and melting temperature.

Primer pair	Primer sequence 5′ Forward 3′ 5′ Reverse 3′	Dengue serotype	Melting temperature (TM) ^{0}C
S1	CAAACCATGGAAGCTGTACG TTCTGTGCCTGGAATGATGCT	DENV1	51.8 52.4
S2	CAAACCATGGAAGCTGTACG TTCTGTGCCTGGAATGATGCT	DENV2	51.8 52.4
S3	GAGTGGAGTGGAAGGAGAAGGG CCTCTTGGTGTTGCTCTTTGC	DENV2	58.6 54.4
S4	CAGACTAGTGGTTAGAGGAGA GGAATGATGCTGTAGAGACA	DENV1	52.4 49.7
S5	ATATGCTGAAACGCGTGAG CATCATGAGACAGAGCGAT	DENV3	48.9 48.9

4.5.2 Optimization of $MgCI_2$ concentration

Multiplex PCR was optimized by using different concentration of $MgCI_2$ such as 1 mM, 2 mM, 2.5 mM, 3mM. PCR was positive with 2.5m M and 3 mM concentration of $MgCI_2$ in fig 4.7.

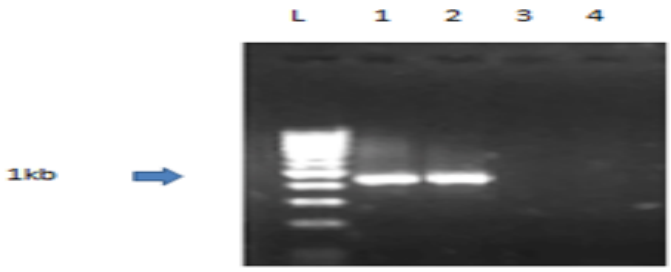

Figure 4.7 Optimization of $MgCI_2$ concentration on 0.8% agarose gel. L: DNA ladder of 1kb. Lane 1: $MgCI_2$ concentration of 2.5mM, Lane 2 : $MgCI_2$ concentration of 3mM, Lane 3 : $MgCI_2$ concentration of 1mM, Lane 4: $MgCI_2$ concentration of 2mM.

4.6 Cloning of PCR

The PCR product was directly ligated to pTZ57R/T vector (Fermentas) after transformation in *E.Coli*. The white colonies were selected for plasmid isolation. The colonies were confirmed by restriction digestion with *EcoR1* and *Pst 1* enzyme. The 250 bp fragment showed the positive clone having insert of 221 bp as shown in fig 4.8.

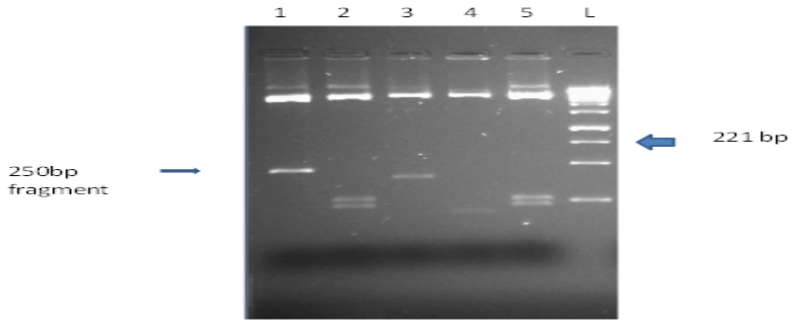

Figure 4.8: 1 % agarose gel showing the restriction of ligated pTZ57R/T vector with *EcoR*1 and *Pst*1. Lane 1: positive clone having 250 bp insert; Lane 2-5: Clone having no 250 bp insert; Lane L: DNA ladder.

DISCUSSION:

In recent 12 years the ratio of dengue disease increase. Every year dengue virus cases is about 50-100 million [9].The high ranking technique for the detection and quantification of RNA is RT-PCR due to following conditions: post PCR processing is not required, more than 10^7 fold of RNA level is detected, this technique gives both types of data i.e qualitative and quantitative [10].Dengue fever is divided into two groupsi.e dengue fever and dengue hemorrhagic fever. Especially in children dengue dengue hemorrhagic fever is common. Factors of occurrence and reoccurrence of dengue epidemics are: behavioural change in climate, nature of vector, deficiency of resources. Reverse transcription polymerase chain reaction (RT-PCR) is molecular biology technique used all over the world. First step of RT-PCR is cDNA synthesis. Comparison of RT-PCR and simple PCR, RT PCR provide quantification of RNA while simple PCR only simple amplification.Cloning of gene is by specific enzyme reverse transcriptase. After the cDNA synthesis, PCR step is done. Inspite of northern blot RT-PCR is novel technique for rapid detection and give accurate results [11].

Discovery of reverse transcriptase PCR leads to revolution as compared to other techniques(Schmittgen*et al*., 2000). Detection of PCR base on following steps: expression of gene definetherotically and practically[12], amplification of RNA sample,degradation of RNA in RT-PCR is avoided.RNA purified from the culture media of DEN2 NGC-infected cells was used as a template for the four RT-PCRs. Interestingly, for the reactions to make cDNA fragments A, B, and C, no exogenous primer was added during the RT step. The success of this approach was surprising, but not only did it work, it also resulted in cleaner PCR products than those obtained by using primed RT reaction mixtures. Direct PCR of viral RNA without an dominant RT step yielded no products. Presumably, therefore, either the viral RNA is contaminated with primers or the reverse transcriptase can initiate cDNA synthesis from the 39-terminal hairpin structure [13].

The cDNA products were digested with the appropriate restriction enzymes and cloned first in *E. coli* into the high-copy-number plasmid pGEM11Zf1 and subsequently into the low-copy-number plasmid pCL1921. In theory, these clones could be used to assemble a clone containing full-length cDNA. On the other hand, in practice, efforts to make such full-length clones in *E. coli* failed.In this project RNA was extracted from whole blood by 3 different methods by LeukoLock^{TM}total RNA method, TEMPUS^{TM} blood RNA tube method, Trizol method. In first method the absorbance of RNA was 0.7 from second method absorbance was 0.06 and from third method the absorbance was 1.8-2. From trizol method there was no DNA protein contamination.

The efficienct RNA yield was obtained by trizol method. Sucessfully RNA was isolated from 6 human dengue patients by trizole method then after isolation RT-PCR reaction was done by fermentaskit.Different primers were used DENV-I, DENV- II, DENV- III, DENV- IV amplify all strains. The sequence of primers were taken from Journal. In RT-PCR GAPDH primer was used as internal control primer. One-step reverse transcription (RT)-PCR assay was develope using a universal primer for the rapid detection of the viral RNA of all dengue serotypes. Universal primers of dengue were used. Only DENV2 serotype was positive in all the samples. Multiplex-PCR is optimized with different concentration of $MgCl_2$i.e 1mM, 2 mM, 2.5 mM, 3 mM. PCR was optimized with 2.5m M and 3 mM concentration of $MgCI_2$. Multiplex PCR was optimized with all the four sets of primer pair by using annealing temperature of 43 ^oC, 45 ^oC, 47 ^oC, 49 ^oC, 50 ^oC, 52 ^oC and 55 ^oC. PCR was positive with DENV2 serotype by using annealing temperature of 50 ^oC, 52 ^oC and 55 ^oC. Maximum ampilification was with 55 ^oC. For further confirmation cloning was done. Cells were ligated in pTZ/57RT vector and restricted with *EcoR1* and *Pst1*. The 250 bp fragment showed the positive clone having insert of 221 bp.

CONCLUSION:

In all the collected samples DENV2 serotype is positive in Pakistani dengue patients, which showed the prevalence of serotype DENV2 in Pakistani patients. This research showed that the best method of total RNA extraction is Trizol method. In this study Multiplex-PCR is optimized for rapid identification of different serotypes of Dengue virus in a single reaction. Such optimization conditions will be used in the diagnostic lab for the rapid identification of different serotypes of Dengue virus.

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