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Research Article

An Evaluation of Usage of Specific Primers and Detection of Dengue Virus

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ABSTRACT

Purpose: the purpose of the study was to evaluate the the usage of specific primers and detection of dengue virus.

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.

Sampling technique: non probability / convenient sampling

Sample size: 15 blood samples from Myohospital Lahore 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.

Keywords: usage, specific primers, detection, dengue virus, PCR essay, Serotypes.

INTRODUCTION

Dengue virus is cause for a large number of illnesses, ranges from imperceptible illness, unpredictable fever and mild flu which tends to severity resulting in depression and increase in death rates. Dengue fever symptoms are abrupt fall in platelets count, bleeding and abnormal changes in liver functioning. If these symptoms not controlled as per normal ranges, then it leads to critical syndrome, like blood clotting inside the blood vessels of the human body [1]. At present there is no vaccination of dengue fever except treatment through different therapies. 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. 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. Dengue virus is most common in tropical regions of the world [2].

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 [3]. 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. Severity of dengue disease increase day by day. There are chances of getting reinfection due to dengue hemorrhagic fever /dengue shock syndrome. In India 1870s cases of dengue were reported [4].

MATERIAL AND METHOD

Prior to the year 2011, the world health organization described that classification of dengue virus on the basis of various symptoms and their severity caused by dengue viruses on human beings. However, presently the classification of dengue virus is based on clinical administration, but still it may not identify the mechanism which cause disease. The production, growth and propagation of dengue virus is generally dependent on conditions like moisture, temperature and humidity [5]. The most important factors, determining the spatial spread and persistence are temperature, rainfall and humidity. Beside other climatic factors seasonal variation is important factor for incubation time of dengue and the transmission capacity to spread easily. For the transmission of dengue it is not necessary to provide only favourable climatic condition for spreading the dengue virus. In the transmission cycle of dengue, dengue itself is considered the third component of transmission but on the other hand the availability of human host based on population density and immunity level [6].

Transmission of dengue virus depends upon the self resistance and immune system of human body. It is directly proportional to increase or decrease of dengue population. Significant factor for determination of intensity of dengue virus are inherited occurring of a cell, changes within species of viruses and order of infection. Factors also effect transmission mechanism of dengue virus and enhance the level of disease resulting in increase the concentration of virus [7]. This disease transferred from different region of world i.e Vietnam, Thailand and Cambodia, showing the change in viral life cycle. These factors related to occurrence of severe dengue. For the identification of dengue virus infection specific different assay are implied which are associated with the health of patient. During the onset of symptoms it is necessary for the quantification of RNA on the other hand the time period for the implication of immunoglobulin M between 4–6 days. For clinical diagnostic different kit methods are available as well as different techniques are also available for isolation of RNA by virus culture, second technique for isolation of RNA through nucleic acid and third technique by detection of NS1, or IgM. Still there is no approval of test for diagnosis of dengue fever. With the collaboration of two organization Centers for Disease Control and World Health Organization, panels decided to justify and establish guidelines as diagnostic point of view [8].

Movement of human from urban to rural areas can create rapidly spreading of dengue virus in Southern Vietnam and different other urban areas of Ho Chi Minh City. In the extrinsic mechanism of incubation period increase in temperature is directly proportional to growth of larvae, larvae converted into adult stage, increase in biting while on the other hand increase in temperature is inversely proportional to virus replication within the vector. If intensity of temperature has positive impact on abundance of vector. Many phenomenas directly relate the temperature with growth of *Aedes aegypti* and rate of incidence. Many statistical approaches concentrate on the parameter of temperature and divide the temperature ranges (i.e mean, maximum and

minimum). Due to not constant rainfall when outdoors or indoor containers which are filled with rain water it provide favourable environment for growth of vector and increasing the population but on the other hand it may have negative effect on breeding time period of dengue vector. The habitat of Aedes vectors is that it breeds on clean or still water, dwellings, in spoil tyres, poor sanitation, outdoor and indoor containers of water. Occurrence of dengue virus globally due to most important climatic factor i.e annual vapor pressure this phenomena is approved by many scientist [9].

RESULTS

Blood samples of dengue infected patients were collected from Myo hospital Lahore and Allied hospital Faisalabad. Sterilized syringes, ice box, cotton, spirit and EDTA coated vials were used during sample collection. The blood samples were stored in ice box. Vials containing the blood samples were inverted 2 to 3 times for homogenization and isolation of total RNA was done. The blood sample of dengue patients was sticky and smelly and disinfectant was used for washing. Care was taken in handling of the sample, because of the chance of getting the infection. RNA isolation was done in safety cabinet 3. After the isolation of total RNA from blood samples by three different methods i.e. Leuko lock™ total RNA method, Tempus™ blood RNA tube method, Trizol method. The samples run on 0.8 % agarose gel for confirmation of RNA. From three methods of total RNA isolation maxiumum concentration of RNA by Leuko lock™total RNA method was 3 µg/ml, and from Tempus™ blood RNA tube method was 9 µg/ml and from Trizol method was 90 µg/ml as shown in table 4.1. From the agarose gel the upper band was of 28S rRNA and second band was 18S rRNA and third band was 5S rRNA. The agarose gel containing RNA was run for 1 hr on 100V.

Table 4.1 Concentration and purity of total RNA isolated from different blood samples of dengue infected patients.

No. of samples	Leuko lock™total RNA method		Tempus™ 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

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) °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

DISCUSSION

Dengue fever is divided into two groups i.e dengue fever and dengue hemorrhagic fever. Especially in children dengue dengue hemorrhagic fever is common [10]. 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.

Discovery of reverse transcriptase PCR leads to revolution as compared to other technique [11]. Detection of PCR base on following steps: expression of gene define therotically and practically, 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 [12].

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 fermentas kit.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 1 mM, 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 ^{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. Maximum ampilification was with 55 ^{o}C. 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. 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.

CONCLUSION

Dengue virus is the most common arbovirus in subtropical and tropical regions of the world. 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. The focus of study was to collect dengue samples from different regions, extract RNA, optimize RT-PCR by using specific primers i.e DENV-I, DENV- II, DENV-III, DENV-IV and cloning. Currently available techniques for detection of dengue virus in Pakistan were ELISA, dot blot immunoassay, complement fixation test. 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. Restriction digestion by *EcoR1* and *Pst1* and desired clone of 250bp fragment was obtained.

REFERENCES

1. Reyes-Ruiz, J. M., Osuna-Ramos, J. F., Cervantes-Salazar, M., Guillen, A. E. L., Chávez-Munguía, B., Salas-Benito, J. S., & Del Ángel, R. M. (2018). Strand-like structures and the nonstructural proteins 5, 3 and 1 are present in the nucleus of mosquito cells infected with dengue virus. *Virology*, *515*, 74-80.
2. York, A. (2018). Viral infection: A fungus boosts dengue virus in the mosquito gut. *Nature Reviews Microbiology*.
3. Lindsey, N. P., Staples, J. E., Powell, K., Rabe, I. B., Fischer, M., Powers, A. M., ... & Hancock, W. T. (2018). Ability To Serologically Confirm Recent Zika Virus Infection in Areas with Varying Past Incidence of Dengue Virus Infection in the United States and US Territories in 2016. *Journal of clinical microbiology*, *56*(1), e01115-17.
4. Carrington, L. B., Tran, B. C. N., Le, N. T. H., Luong, T. T. H., Nguyen, T. T., Nguyen, P. T., ... & Le, D. T. (2018). Field-and clinically derived estimates of Wolbachia-mediated blocking of dengue virus transmission potential in Aedes aegypti mosquitoes. *Proceedings of the National Academy of Sciences*, *115*(2), 361-366.
5. Flechas, M. C., Ocazionez, R. E., & Stashenko, E. E. (2018). Evaluation of in vitro Antiviral Activity of Essential Oil Compounds Against Dengue Virus.
6. Moi, M. L., Takasaki, T., & Kurane, I. (2018). Detection of Virus-Antibody Immune Complexes in Secondary Dengue Virus Infection. In *Hemorrhagic Fever Viruses* (pp. 331-337). Humana Press, New York, NY.
7. Reid, D. W., Campos, R. K., Child, J. R., Zheng, T., Chan, K. W. K., Bradrick, S. S., ... & Nicchitta, C. V. (2018). Dengue virus selectively annexes endoplasmic reticulum-associated translation machinery as a strategy for co-opting host cell protein synthesis. *Journal of Virology*, JVI-01766.
8. Gurugama, P., Jayarajah, U., Wanigasuriya, K., Wijewickrema, A., Perera, J., & Seneviratne, S. L. (2018). Renal manifestations of dengue virus infections. *Journal of Clinical Virology*.
9. Koo, C., Xu, H., Tien, W. P., Lai, Y. L., Ng, L. C., & Hapuarachchi, H. C. (2018). Clade Replacement Associated with Epidemic Transmission of Dengue Virus Type 2. *World Academy of Science, Engineering and Technology, International Journal of Medical and Health Sciences*, *5*(12).
10. Hashem, A. M., Sohrab, S. S., El-Kafrawy, S. A., El-Ela, S. A., Abd-Alla, A. M. M., Farraj, S. A., ... & Madani, T. A. (2018). First complete genome sequence of circulating dengue virus serotype 3 in Jeddah, Saudi Arabia. *New Microbes and New Infections*, *21*, 9-11.
11. Gao, M., Daniel, D., Zou, H. Y., Jiang, S., Lin, S., Huang, C. Z., ... & Chen, S. (2018). Rapid detection of a Dengue virus RNA sequence with single molecule sensitivity using tandem toehold-mediated displacement reactions. *Chemical Communications*.
12. Li, Y., Lee, M. Y., Loh, Y. R., & Kang, C. (2018). Secondary structure and membrane topology of dengue virus NS4A protein in micelles. *Biochimica et Biophysica Acta (BBA)-Biomembranes*, *1860*(2), 442-450.