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Insilico primer designing of structural region of Dengue virus for molecular diagnostic

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Abstract

Dengue has reemerged significantly from the past two decades for the public health problems. Clinicians require a diagnostic test with high sensitivity, which is applicable during early symptomatic phase. PCR is a specific and sensitive molecular technique to detect the gene for monitoring possible Dengue outbreaks and circulating viral serotypes. Bioinformatics tools were used to design and verify the novel Dengue subtype specific primers. In this investigation, 24 primers targeting the structural region of four variants of dengue virus were designed through in silico. There is no cross reactivity found with the primers designed with any other virus. All these sets of primers of dengue were highly specific and may perhaps be helpful for the molecular diagnostic of Dengue viruses.

INTRODUCTION

Dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) are a major public health problem globally found mostly in tropical and subtropical countries (Rigau-Pérez et al 1994; Gubler, 1998). These infections are of considerable public health importance because of the high morbidity and mortality associated with their worldwide distribution. The known geographic range of dengue includes the Americas, Africa, Asia and the South Pacific determined by the distribution of its mosquito vectors. Movement of dengue in about 2/5 of the world population among different geographic area is an important element in the epidemiology of the disease (WHO, 2002). During a Dengue virus epidemic, infection rates among those susceptible to the virus are generally 40-50% but could potentially reaches as high as 80-90%.It is estimated that 5 lakh cases of DHF require hospitalization and a minimum of 2.5% of them resulted in death. The fatality rate of DHF, if untreated, is around 20% but proper treatment could reduce them to about 1%.At present it is estimated that each continent has 5% fatality rate for those infected with Den V, with children and young adults being the groups which are most susceptible to fatalities from infection.(WHO, 2002).

The dengue virus is a single-stranded positive-strand RNA virus. The four serotypes of dengue virus exists viz DEV I-IV. The genome size of all four dengue virus was approximately 11kb in length (Halstead et al 1977). Genome is encoded within a single polyprotein. It consists of capsid (C), premembrane (prM), envelope protein (E), and seven nonstructural (NS) protein genes (Deubel et al 1988). However, both 5` and 3` ends of the open reading frame are flanked by an untranslated region (UTR). The genome organization is 5'UTR-C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3'UTR.

The current strategy was prepared to identify and characterize the dengue virus in the laboratory through the cell culture technique. However, this requires cell culture facilities which are difficult to maintain in laboratories (Harris et al 1998). A viral growth usually takes long time and always in the risk of contamination. Two different methods have been used in dengue diagnostics, immunological ELISA and PCR. Polymerase chain reaction is a sensitive, specific and rapid molecular assay to identify and characterize the virus. The dengue suspected person blood sample was extracted and isolation of the RNA was done and it was converted into the cDNA. The cDNA will be used as a template for specific molecular diagnostic of dengue viruses. The detection of RNA virus with molecular technique viz reverse transcriptase polymerase chain reaction (RT-PCR) (Alcon et al 2002).

Bioinformatics provide an innovative strategy to design and validate the primers. Currently, several insilico tools are available freely for designing and validating the primers and probes. In this study, we have selected tools for designing

the primers against the structural region of four variants of dengue virus. This structural region helps in contributing the vital role in the pathogenesis. These virulence factors contribute to its pathogenicity and provide ability to attach in the host cells for the development of disease. These factors can be used for detection and characterization of the virus. PCR is used to amplify a precise fragment of DNA from complex mixture of starting material usually template genomic DNA or cDNA. The aim of this study was to design and verify the primers of structural region of dengue virus (DENV I-IV) by insilico methods. This analysis possibly will use for the molecular identification and characterization for early diagnosis and thus, prevention of the severe infection of dengue in humans.

MATERIAL AND METHODS

COLLECTION OF SEQUENCES

The complete nucleotide sequences of structural region of envelope, capsid and premembrane of Dengue virus I, II, III, IV were extracted from biological database, viz. National Centre for Biotechnology Information (NCBI) cited at http://www.ncbi.nlm.nih.gov

STRATEGIES FOR DESIGNING OF PCR PRIMERS

The open reading frame of structural gene was prepared from the whole genome of dengue virus. These nucleotide sequences were aligned by ClustalX 1.83. Conserved region present in these nucleotide were selected manually. Primers were designed with Oligo 4.0, Gene runner and DNASTAR (lasergene). The main characteristic features of these primers comprise high GC content; length of nucleotides, loop delta G value and melting temperature were recorded. The designed primers were then simultaneously compared to the other sequences in the Genbank database to verify their identity and similarity with other organisms.

BASIC LOCAL ALIGNMENT SEARCH TOOLS (BLAST)

The relatedness of sequences deposited in databases was evaluated by BLAST (Basic Local Alignment Search Tool), (Altschul et al., 1990) implemented via the NCBI website [www.ncbi.nlm.nih.gov/blast/] against the complete training dataset which is extracted from Genbank database. The Blastn (nucleotide query – nucleotide database comparison) in which conditional composition score adjustment having no filters of BLOSUM 62 matrix with threshold expect value 10 were used. Specificity and cross reactivity was check by PSI-BLAST.

RESULTS AND DISCUSSION

Total four serotype of known dengue virus used in the study was given (Table 1).

Figure 1

Table 1: Dengue virus serotype used in this study

Nucleotides	Description /Accession No.
Dengue virus 1	Dengue virus Type 1 NC_001477
Dengue virus 2	Dengue virus type 2 NC_001474
Dengue virus 3	Dengue virus type 3 NC_001475
Dengue virus 4	Dengue virus type 4 NC_002640

The total genome size of dengue studied was about 10 kb and encodes the structural and non structural region. Structural region of genome are helpful in the coating of capsid, envelope and membrane glycoprotein. These regions are essential for the virus replication and helpful in the infection in the hosts. The structural regions are very important for the molecular based diagnostic of dengue virus. In this investigation, all the three important structural region of dengue virus have been used to design the dengue specific primers. The primers were also verified for their cross reactivity with other organisms, resultant there was no cross reactivity observed within the dengue virus or other virus. The GC contents, melting temperature, expected size of PCR product and position of primers pair in the genome of dengue virus was given (Table 2).

Figure 2

Table: 2 Primer designing for specific amplification of Dengue virus.

Gene designation	Primers Designation	Primer sequences	Position within the gene	Product size (bp)	GC content	Melting temperature (Tm)
Dengue 1 Capsid ancher	DNV1F	5'ACAACCAACGGAAAAAGACGG'3	1- 341	341 bp	47%	62°
	DNV1R	5' GGGCTGTGGGCATCATCA '3			58 %	61°
Membrane glycoprotein	DNV1F1	5'TTCCATCTGACCACCCGAGGG '3	1-497	497 bp	61.9%	68°
	DNV1R1	5'ATGGCATGTGCTAGAAAAAGGGCT '3			45.8%	70°
Envelope protein	DNV1F2	5'CATGCGGTGCGTGGGAATAGG 3'	1-1484	1484 bp	61.9%	68°
	DNV1R2	5'ATCTCATTAAGTCTAGCCCTGTT 3'			37.5	66°
Dengue 2 Capsid ancher	DNV2F	5'AACCAACGGAAAAAGGCGAAA 3'	1-299	299 bp	42%	60°
	DNV2R	5'CCTTCCAATCTCTTTCCTGA 3'			45%	58°
Membrane glycoprotein	DNV2F1	5'GAAGGGGCCTGGAAACATG 3'	1-224	224 bp	57.9%	60°
	DNV2R1	5'CACCCTTACCCTGACCTCTG 3'			64%	60°
Envelope protein	DNV2F2	5'AACAGAAGCCAAACAGCCT 3'	1-1484	1484 bp	45%	58°
	DNV2R2	5'TATTTTTGTCTTCGGTTTGTCG 3'			36.4%	60°
Dengue 3 Capsid ancher	DNV3F	5' ACAACCAACGGAAGAAGCG 3'	1-299	299 bp	52.6%	58°
	DNV3R	5'ACTTGTTGGTTGCCTTCTTCT 3'			42.9%	60°
Membrane glycoprotein	DNV3F1	5'TCAGTGGCGTTAGCTCC 3'	1-224	224 bp	58.8%	54°
	DNV3R1	5'AATTACGACCAGTGGGG 3'			52.9%	52°
Envelope protein	DNV3F2	5'AATGAGATGTGTGGGAGTAGGAAA 3'	1-1478	1478 bp	41.7%	68°
	DNV3R2	5'CCAACTGCACCACGAGCTCG 3'			65.0%	66°
Dengue 4 Capsid ancher	DNV4F	5'AGAACCACCTTTCAATATGCT3'	1-338	338 bp	40.0%	56°
	DNV4R	5'GAATGCCTACCACGATCCG3'			55.6%	56°
Membrane glycoprotein	DNV4F1	5'GTAGCTTTAACACCACATTCAGG 3'	1-224	224 bp	43.5%	66°
	DNV4R1	5'CCTAAGGGCGAGAACCGTCC3'			65.0%	66°
Envelope protein	DNV4F2	5'AGACACCCATGCAGTAGGAAAT3'	1-1478	1478 bp	47.8%	68°
	DNV4R2	5'AGTTAACTGTTTCTCTACCGTCTTT3'			36.0%	68°

The primers might be suitable for the mutagenesis of dengue virus. These primers may be helpful for PCR amplification complete structural gene and also for the expression of all three regions. The expressed protein might help in the immunodiagnostic of all four dengue virus. The non structural region of DNA is helpful in the stability of the genome and virus. These non structural regions may be used in the taxonomy and classification of dengue virus. In our previous study, the conserved domains present in the genome of Flavivirus were used for the taxonomy and reclassification (Data not shown). The phylogenetic analysis of all four dengue virus have been reported previously (Somvanshi and Seth 2008). The proteomes analysis of the structural region of all four dengue virus have been reported (Somvanshi and Seth 2008).

There was no cross reactivity observed in the primers within the dengue virus and other organisms. As the NCBI BLASTN is an online program use for the homology search. In this study, all these primers were confirmed with this programme. There was no primers homology match with the other organisms. Dengue I primers were found matching with the dengue I sequences database. And other dengue II, III and IV were harmonized with the II, III and IV respectively. Therefore, we can state primers are specific and does not cross react with others organisms. In this study, different set of PCR primers have been used to identify the dengue. The molecular techniques are able to directly assess the presence of viral RNA in plasma/serum samples from subjects with suspected disease. Actually, reverse transcriptase PCR was found extremely practical in the recent outbreak of severe acute respiratory syndrome, when medical staff could quickly avoid the adoption of quarantine measures for dengue RNA-positive patients (Barkham et al 2006). Several RT-PCR methods for dengue RNA detection includes both conventional and real-time PCR have been described (Norman et al 2001).

In conclusion, we report the development of specific primers for molecular level diagnostic of structural region of dengue viruses. The primers may be a support for the diagnostic of dengue and dengue hemorrhagic infection in humans. PCR based techniques will be useful for early molecular detection and control of Dengue viruses and in this way it provide the support for human health management.

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