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TITLE

Detection and Identification of Dengue and Other Flaviviruses Using NS3 Primers

This invention concerns the detection and identification of dengue viruses and other related flaviviruses by the polymerase chain reaction (PCR) using a single pair of consensus NS3 primers and four type-specific NS3 primers.

Dengue viruses comprise four serologic types (types 1, 2, 3, and 4) and belong to the family Flaviviridae whose other related members include Japanese encephalitis, Kunjin, Murray Valley encephalitis, St. Louis encephalitis, tick-borne encephalitis, West Nile, yellow fever, and hepatitis C viruses. The dengue virus consists of a single positive-stranded RNA genome of approximately 11 kilobases encoding the capsid, membrane and envelope proteins, as well as non-structural proteins including three major proteins known as NS1, NS3 and NS5 whose functions are not yet fully elucidated. The NS3 gene is one of the most conserved genes among the four dengue virus serotypes, and indeed even among other members of the flavivirus family. A putative serine protease domain spans the N-terminal region of the NS3 protein. Lying C-terminal to the protease is a nucleotide triphosphatase/RNA helicase domain consisting of 7 conserved segments numbered I, Ia, II-VI thought to be the nucleotide triphosphate binding sites. These two domains are involved in processing of the viral polyprotein which is a critical event in viral expression and replication.

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Furthermore, monoclonal antibodies produced against NS3 proteins have been shown to confer partial protection in laboratory mice against lethal challenge with dengue viruses.

Cell culture and immunological assays such as neutralization and haemagglutination inhibition tests are established and widely employed for the diagnosis and typing of dengue viruses. However, these tests are relatively time-consuming and considerably less sensitive than newer molecular biologic methods such as nucleic acid hybridization and PCR.

Synthetic Oligonucleotide Primers

This invention envisages a process for detecting and identifying all four dengue virus types, as well as other related flaviviruses. Two 17-base oligonucleotide consensus primers, DVCU (upstream) and DVCD (downstream) as shown in the table below, were designed based on highly conserved motifs within the NS3 gene of dengue virus types. DVCU was designed on the basis of the conserved Gly-Thr-Ser-Gly-Ser-Pro sequence which lies in the serine protease domain. DVCD was synthesized based on the Met-Asp-Glu-Ala-His-Phe motif corresponding to conserved segment II of the nucleotide triphosphatase/RNA helicase domain. The DVCU/DVCD primer pair flanks a 470 base pair target region.

In addition, four unique type-specific primers for the 4 dengue virus types were designed as defined in the table below, in order to type and sequence dengue viruses. When used in

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conjunction with DVCU primer in PCR, these type-specific primers designated DSP1, DSP2, DSP3 and DSP4 (specific for dengue types 1, 2, 3 and 4 respectively) yield PCR products of diagnostic sizes of 169, 362, 265 and 426 base pairs, respectively. These PCR products can be readily distinguished by agarose gel electrophoresis.

Being chemically-synthesized oligonucleotides with most of them containing degenerate bases or the unnatural inosine base, these new primers (DVCU, DVCD, DSP1, DSP2, DSP3, DSP4) are thus not naturally-occurring entities.

Other workers have reported PCR of dengue and other flaviviruses using type-specific primers [references 1, 2, 4, 6] and consensus primers [references 3, 5] for regions outside of NS3, often accompanied by sequence-specific probe hybridization. References 1 to 6 are hereinafter defined.

Table 1 Abbreviations

A = adenine; C = cytosine; G = guanine; T = thymine; I = inosine.

Degenerate bases: K = G/T; R = A/G; W = A/T; Y = C/T.

Table

DVCU consensus upstream primer: 5' GGRACKTCAGGWTCTCC 3'

DVCD consensus downstream primer: 5' AARTGIGCYTCRTCCAT 3'

DSP1 dengue 1 type-specific primer: 5' AGTTTCTTTTCCTAAACACCTCG 3'

DSP2 dengue 2 type-specific primer: 5' CCGGTGTGCTCRGCYCTGAT 3'

DSP3 dengue 3 type-specific primer: 5' TTAGAGTYCTTAAGCGTCTCT 3'

DSP4 dengue 4 type-specific primer: 5' CCTGGTTGATGACAAAAGTCTTG 3'

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Note

Reference strains of dengue type 1 (Hawaii), type 2 (New Guinea C), type 3 (H87) and type 4 (H241), yellow fever (17D vaccine), Japanese encephalitis (Nakayama), and Kunjin (MRM61C) viruses were tested. All these viruses are flaviviruses. Viral cDNA synthesis and amplification are performed by reverse transcription followed by PCR using the primers. To obtain specific templates for direct sequencing, PCR products are excised from agarose gels, DNA eluted and reamplified. Each purified PCR product template is directly sequenced in both directions using the primers for DNA sequencing.

According to the present invention there is provided a process for identifying viral infection comprising the use of DVCU and DVCD consensus primers as defined herein by the table to detect the presence of dengue or other flaviviruses by amplifying by a polymerase chain reaction target fragments from biological or clinical samples containing cDNA templates synthesised from RNAs of any of the four dengue viruses or other related flaviviruses.

According to another aspect of the invention there is provided pairs of oligonucleotide primers comprising DSP1, DSP2, DSP3 and DSP4 primers in conjunction with DVCU and DVCD primers, the primers in conjunction with DVCU and DVCD primers, the primers being as defined herein by the table. According to a further aspect of

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the invention there is provided the use of the said pairs of primers for specifically determining the dengue virus type (that is, type 1, 2, 3 or 4) in biological or clinical samples.

According to a still further aspect of the invention there is provided a diagnostic kit for viral infection comprising the use of DVCU, DVCD, DSP1, DSP2, DSP3 and DSP4 primers as defined herein by the table as sequencing primers for the DNA sequencing of flavivirus-derived DNA fragments within the relevant NS3 region of the flavivirus genome.

PCR Amplification of NS3 Fragments of Dengue and Other Flaviviruses

Using DVCU and DVCD primers, target fragments of expected size 470 base pairs can be successfully amplified on cDNA templates synthesized from RNAs of each of the four dengue, Japanese encephalitis, Kunjin and yellow fever viruses using DVCD downstream primers or random primers. In contrast, using cDNA templates derived from oligo(dT) primers, PCR yields clearly discernible bands for all the viruses tested except Japanese encephalitis and Kunjin viruses. PCR results are thus achieveable within hours using reliable cDNA templates.

Reamplification of excised 470 base pair bands produces sharp target fragments which subsequently yield authentic viral sequence data.

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PCR products generated from random-primed and oligo(dT)-primed cDNAs as well as those obtained from PCR of viral cDNAs spiked with human genomic DNA yield target bands without significant non-specific bands. PCR of human genomic DNA or of cDNA from non-infected C6/36 cells produces no target nor background bands.

Assays indicate high sensitivity of at least 1, 0.1 , 0.01 and 1 picogram equivalent to 10^5, 10^4, 10^3 and 10^5 copies of purified dengue types 1, 2, 3 and 4 viral RNAs, respectively, detectable by agarose gel electrophoresis of PCR products alone.

The ability of our consensus primers to amplify this NS3 fragment of dengue and other flaviviruses demonstrates the highly conserved nature of the consensus motifs of the enzymatic domains within NS3. Computer search for consensus primer homology with flaviruses such as Murray Valley encephalitis and West Nile viruses indicates matching of high percentage suggesting potential amplification.

Using DVCU upstream primer with the type-specific DSP1, DSP2, DSP3 and DSP4 downstream primers, PCR products of 169, 362, 265 and 426 base pairs are generated from dengue virus types 1, 2, 3 and 4 respectively, but not from other flaviviral templates.

Sequence Analysis of NS3 of Dengue and Other Flaviviruses

Clearly readable stretches of ~420 nucleotides (encoding

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140 amino acid residues) within the NS3 target fragment of dengue 2 (New Guinea C), Kunjin (MRM61C) and yellow fever (17D vaccine) virus strains are identical with the corresponding published sequences. This complete concurrence confirms the authenticity and reproducibility of the RNA-PCR primer technique coupled with DNA sequencing.

Twenty-one nucleotide differences are observed between the NS3 fragments of the Hawaii strain and the published Singapore S275/90 strain of dengue 1 virus. Differences at 16 nucleotide positions are seen between the H241 strain and the published S14669 strain of dengue 4 virus. The Nakayama strain and the published JaOArS982 strain of Japanese encephalitis virus differ at 11 nucleotide positions.

Stringent analyses by multiple sequencing in both directions of amplified cDNA templates derived from random, specific and oligo(dT) primers are consistently compatible.

Practical Applications

These new primers are feasible for use in the RNA-PCR technique coupled with DNA sequencing for the molecular diagnosis, epidemiology and vector surveillance for infections with dengue and other flaviviral strains. Some insight into the structure-function analysis of NS3 can also be gained from the resulting sequence data.

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CLAIMS

1. Process for identifying viral infection comprising the use of DVCU and DVCD consensus primers as defined herein by the table to detect the presence of dengue or other flaviviruses by amplifying by a polymerase chain reaction target fragments from biological or clinical samples containing cDNA templates synthesised from RNAs of any of the four dengue viruses or other related flaviviruses.

2. Pairs of oligonucleotide primers comprising DSP1, DSP2. DSP3 and DSP4 primers in conjunction with DVCU and DVCD primers, the primers in conjunction with DVCU and DVCD primers, the primers being as defined herein by the table.

3. Use of the pairs of primers in accordance with Claim 2 for specifically determining the dengue virus type (that is, type 1, 2, 3 or 4) in biological or clinical samples.

4. The use of DVCU and DVCD consensus primers as defined by the table to detect the presence of dengue or other flaviviruses by amplifying by PCR, target fragments from biological or clinical samples containing cDNA templates synthesized from RNAs of any of the four dengue viruses or other related flaviviruses.

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5. The use of DSP1, DSP2, DSP3 and DSP4 primers in conjunction with DVCU and DVCD primers as defined herein by the table for specifically determining the dengue virus type (that is, either type 1, 2, 3 or 4) in biological or clinical samples.

6. The use of DVCU, DVCD, DSP1, DSP2, DSP3 and DSP4 primers as defined herein by the table as sequencing primers for the DNA sequencing of flavivirus-derived DNA fragments within the relevant NS3 region of the flavivirus genome.

7. A diagnostic kit for viral infection comprising the use of DVCU, DVCD, DSP1, DSP2, DSP3 and DSP4 primers as defined herein by the table as sequencing primers for the DNA sequencing of flavivirus-derived DNA fragments within the relevant NS3 region of the flavivirus genome.

8. The invention substantially as herein described.