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Use of NS 3 consensus primers for the polymerase chain reaction amplification and sequencing of dengue viruses and other flaviviruses

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Summary. Consensus primers for the polymerase chain reaction were designed based on conserved motifs within the serine protease and RNA helicase domains encoded by the NS 3 genes of dengue and other flaviviruses. Target fragments of 470 bp were amplified on cDNA templates synthesized from RNAs of dengue types 1, 2, 3, and 4, Japanese encephalitis, Kunjin, and yellow fever viruses using random or specific downstream primers. PCR of oligo(dT)-primed cDNAs from Japanese encephalitis and Kunjin viral RNAs did not yield target bands. As few as 10^3 copies of dengue viral RNA could be detected. Direct DNA sequencing of PCR products of reference strains of dengue 2 (NGC), Kunjin (MRM 61C) and yellow fever (17 D) viruses demonstrated complete concurrence with published data. However, 2 nucleotide differences were observed between our data for dengue 3 H 87 strain and the published sequence, resulting in a single amino acid disparity. Differences at 21, 16, and 11 nucleotide positions were noted between dengue 1 Hawaii and S 275/90; dengue 4 H 241 and 814669; Japanese encephalitis Nakayama and JaOArS 982 viral strains, culminating in only 4, 1 and 1 amino acid residue differences, respectively. These amino acid disparities occurred outside putative active sites of the enzymatic domains, emphasizing the important role of the NS 3 protein in flaviviral replication. This RNA-PCR consensus primer strategy coupled with DNA sequencing represents a valuable tool for the molecular diagnosis and epidemiology of dengue and other flaviviral infections.

Introduction

Outbreaks of dengue virus infection and the associated syndromes of dengue fever, dengue haemorrhagic fever and the potentially fatal dengue shock syndrome constitute major public health problems in tropical and sub-tropical

regions all over the world, including the Asia-Pacific where the *Aedes* mosquito vector is prevalent. In Singapore alone, a record number of 2179 confirmed cases of dengue were reported in 1991.

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 kb encoding the capsid, membrane and envelope proteins, as well as non-structural proteins including three major proteins known as NS 1, NS 3, and NS 5 whose functions are not yet fully elucidated. The NS 3 gene is one of the most conserved genes among the four dengue virus serotypes, and indeed even among other members of the flavivirus family [5, 34]. A putative serine protease domain spans the N-terminal region of the NS 3 protein [2, 6]. 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 [5, 18]. These two domains are involved in processing of the viral polyprotein which is a critical event in viral expression and replication. Furthermore, monoclonal antibodies produced against NS 3 proteins have been shown to confer partial protection in laboratory mice against lethal challenge with dengue viruses [42].

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 [21] and the polymerase chain reaction (PCR) [16]. Since rapid diagnosis of dengue virus infection is critical in patient management and disease surveillance, we adopted the technique of cDNA amplification by PCR for the sensitive identification of dengue viruses. We report here the use of a single pair of NS 3-specific consensus primers for PCR which can detect all four dengue virus types, as well as other related flaviviruses. In addition, sequence data of the PCR products from the various viral templates are compared and analyzed.

Materials and methods

Virus strains

Reference strains of dengue type 1 (Hawaii), type 2 (New Guinea C), type 3 (H 87), and type 4 (H 241), yellow fever (17 D vaccine), Japanese encephalitis (Nakayama), and Kunjin (MRM 61C) viruses tested in this study were propagated in C 6/36 *Aedes albopictus* cells [25]. Dengue virus serotype was confirmed by immunofluorescent staining of viral antigen by type-specific monoclonal antibodies.

Isolation of viral and cytoplasmic RNA

Dengue virions were purified by precipitation in 7% polyethylene glycol (PEG) and 2.3% NaCl, and centrifugation through a 30% sucrose cushion. Dengue viral RNAs were isolated by phenol/chloroform extraction and ethanol precipitation [39].

Cytoplasmic RNAs were isolated from virus-infected and non-infected cells by a rapid method described by Gough [19]. Briefly, cells were harvested, pelleted and resuspended in 10 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 1.5 mM $MgCl_2$ and 0.65% Nonidet P-40. After centrifugation, the cytoplasmic lysate was transferred to an equal volume of 7 M urea, 1% SDS, 0.35 M NaCl, 10 mM EDTA, 10 mM Tris-HCl (pH 7.5). The mixture was extracted with phenol/chloroform and precipitated with ethanol.

The RNAs were quantitated with a UV spectrophotometer at 260 nm, where an optical density of 1 corresponds to approximately 40 μg/ml.

Amplification primers

Two 17-base oligonucleotide consensus amplimers, DV 1 (upstream) and DV 3 (downstream) as shown in Table 1, were designed based on highly conserved motifs within the NS 3 gene from the published nucleotide sequences of dengue virus types 2 [13, 20, 26], 3 [36], and 4 [31]. DV 1 was designed on the basis of the conserved Gly-Thr-Ser-Gly-Ser-

Table 1. Nucleotide and deduced amino acid sequences of consensus PCR primers: homology with published dengue and other flaviviral strains

Primer/ strain	Nucleotide sequence (and position)	Amino acid sequence (and location)	Reference
DV1 (+) primer 5′GGRACKTCAGGWTCTCC3′		GXSGX	
D1S275	(4899) **C**A**T******** (4915)	(1607) ***** (1611)	[18]
D2NGC	(4918) *****C********G** (4934)	(1608) ***** (1612)	[27]
D2JAM	(4918) ********************* (4934)	(1608) ***** (1612)	[14]
D2PR159	(4918) ********************* (4934)	(1608) ***** (1612)	[21]
D2PDK	(4918) ********************* (4934)	(1608) ***** (1612)	[3]
D3H87	(4910) ********************* (4926)	(1606) ***** (1610)	[37]
D4814669	(4917) ********************* (4933)	(1606) ***** (1610)	[32]
JES982	(5004) *****A**C**C**A** (5020)	(1637) ***** (1641)	[42]
KNMRM61C	(4987) *****A*****C**G** (5003)	(1638) ***** (1642)	[12]
YF17D	(4976) **C*************** (4992)	(1620) ***** (1624)	[38]
MVE	(5003) **************C** (5019)	(1636) ***** (1640)	[13]
TBEWEST	(4985) *****A*****CAGC** (5001)	(1625) ***** (1629)	[33]
WNV	(4951) **************C** (4967)	(1634) ***** (1638)	[4]
HCV1	(3487) **CT*C**G**GGG*** (3503)	(1163) ***** (1167)	[9]
HCVBK	(3819) **CT****G***GG*** (3835)	(1163) ***** (1167)	[43]
DV3 (−) primer 5′AARTGIGCYTCRTCCAT3′			
DV3 (+) seq. 5′ATGGAYGARGCICAYTT3′		MDEAH	
D1S275	(5352) ********************* (5368)	(1758) ***** (1762)	[18]
D2NGC	(5368) ********************* (5384)	(1758) ***** (1762)	[27]
D2JAM	(5368) ********************* (5384)	(1758) ***** (1762)	[14]
D2PR159	(5368) ********************* (5384)	(1758) ***** (1762)	[21]
D2PDK	(5368) ********************* (5384)	(1758) ***** (1762)	[3]
D3H87	(5363) ********************* (5379)	(1757) ***** (1761)	[37]
D4814669	(5367) ********************* (5383)	(1756) ***** (1760)	[32]
JES982	(5458) ********************* (5474)	(1788) ***** (1792)	[42]
KNMRM61C	(5440) ********************* (5456)	(1789) ***** (1793)	[12]
YF17D	(5432) ********************* (5448)	(1772) ***** (1776)	[38]
MVE	(5456) ********************* (5472)	(1787) ***** (1791)	[30]
TBEWEST	(5444) ****************G (5460)	(1778) ***** (1782)	[33]
WNV	(5404) ********************* (5420)	(1785) ***** (1789)	[4]
HCV1	(3943) TGT******TG*****C (3959)	(1315) C**C* (1319)	[9]
HCVBK	(4275) TGT******TG*****C (4291)	(1315) C**C* (1319)	[43]

(+) Sense orientation; (−) anti-sense orientation
I Inosine; *K* G/T; *R* A/G; *W* A/T; *Y* C/T
X Any amino acid
Asterisk indicates match

Pro sequence (amino acids 1608–1613 of dengue virus type 2) which lies in the serine protease domain [2, 5, 6]. DV 3 was synthesized based on the Met-Asp-Glu-Ala-His-Phe motif (amino acids 1758–1763) corresponding to conserved segment II of the nucleotide triphosphatase/RNA helicase domain [5, 18]. The DV 1/DV 3 primer pair flanks a 470 bp target region.

cDNA synthesis

Synthesis of viral cDNA templates was performed using a GeneAmp RNA PCR kit (Perkin-Elmer Cetus) in a 10 µl reverse transcription reaction mixture consisting of 1 ng dengue viral RNA or 1 µg cytoplasmic RNA from virus-infected cells; 1 × buffer containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$; 0.5 or 1 mM each of the four deoxyribonucleoside triphosphates; 1 U/µl RNase inhibitor; 0.5 or 0.75 µM DV 3 downstream primer or 2.5 µM random hexamers or 2.5 µM oligo(dT) primers and 2.5 U/µl Moloney murine leukaemia virus reverse transcriptase; incubated at room temperature for 10 min [random hexamers or oligo(dT) primers only], 42 °C for 15 min, 99 °C for 5 min, and 4 °C for 5 min.

Polymerase chain reaction

To the 10 µl synthesized cDNA volume was then added 40 µl of a PCR mix giving final concentrations of 1 × PCR buffer containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$; 0.1 or 0.15 µM DV 1 upstream primer [or both DV 1 and DV 2 primers for cDNAs primed with random hexamers or oligo(dT) primers]; and 2.5 U/100 µl *Taq* polymerase. This mixture was subjected to an initial 95 °C for 1 min, followed by 30 cycles of 95 °C denaturation for 0.5 min, 50 °C annealing for 1 min, 72 °C extension for 1 min, with a 1 min ramp between each step of every cycle, and a final extension of 72 °C for 10 min. 20% of each PCR product was resolved by electrophoresis on an ethidium bromide-stained 2% agarose gel. Serial ten-fold dilutions of purified dengue viral RNA ranging from 1 ng to 0.01 fg in 10 µg/ml of carrier yeast tRNA were subjected to cDNA synthesis and PCR as described to ascertain detection sensitivity. 1 pg of purified viral RNA was estimated to be approximately 10^5 copies, derived from the calculation of 1 mole (or 11 000 × 330 g) of dengue viral RNA being equivalent to 6×10^{23} molecules. Stringent precautions were taken to avoid cross contamination of specimens from "carryover" DNA [27].

Isolation and amplification of PCR products from agarose gels

To obtain specific templates for direct sequencing, PCR products of size 470 bp were excised from agarose gels, DNA eluted and reamplified. Briefly, the cut bands were left in Ultrafree-MC tubes with 0.45 µm filters (Millipore) at − 80 °C for 10 min, thawed and centrifuged at 14 000 rpm for 5 min. 100 µl of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) were added to the centrifugate, then incubated at 37 °C for 15 min and centrifuged at 14 000 rpm for 5 min. The DNA in this mixture was extracted thrice with butanol and twice with ether, ethanol precipitated, washed and resuspended in TE. Appropriate dilutions of the eluted DNA were reamplified by PCR (without ramping) and electrophoresed as described above.

Purification and direct sequencing of PCR products

The reamplified products were extracted with chloroform and ether, precipitated with PEG, NaCl and washed with 80% ethanol. About 0.5 to 1 pmol of each PCR product template was sequenced at least twice and in both directions with ^{32}P 5′ end-labelled DV 1 and DV 3 primers using a dsDNA cycle dideoxy sequencing system (Bethesda Research Laboratories) according to the manufacturer's instructions with modifications. *Taq* polymerase was employed to catalyse 20 linear amplification cycles each at 95 °C for 0.5 min, 50 °C for 1 min

and 72 °C for 1 min without ramping. The sequencing reactions were electrophoresed in 8% polyacrylamide gels which were dried and autoradiographed.

Computer analysis

Nucleotide and amino acid sequences were aligned and compared using the DNASIS (eighth version) and PROSIS (fourth version) software programmes (Hitachi).

Results

PCR amplification of NS 3 fragments of dengue and other flaviviruses

Using primers DV 1 and DV 3, target fragments of expected size 470 bp could be successfully amplified on cDNA templates synthesized from purified RNAs of dengue virus types 1, 2, 3, and 4 as well as from cytoplasmic RNAs of cells infected with each of the four dengue, Japanese encephalitis, Kunjin and yellow fever viruses using specific downstream primers (Fig. 1 A) or random primers

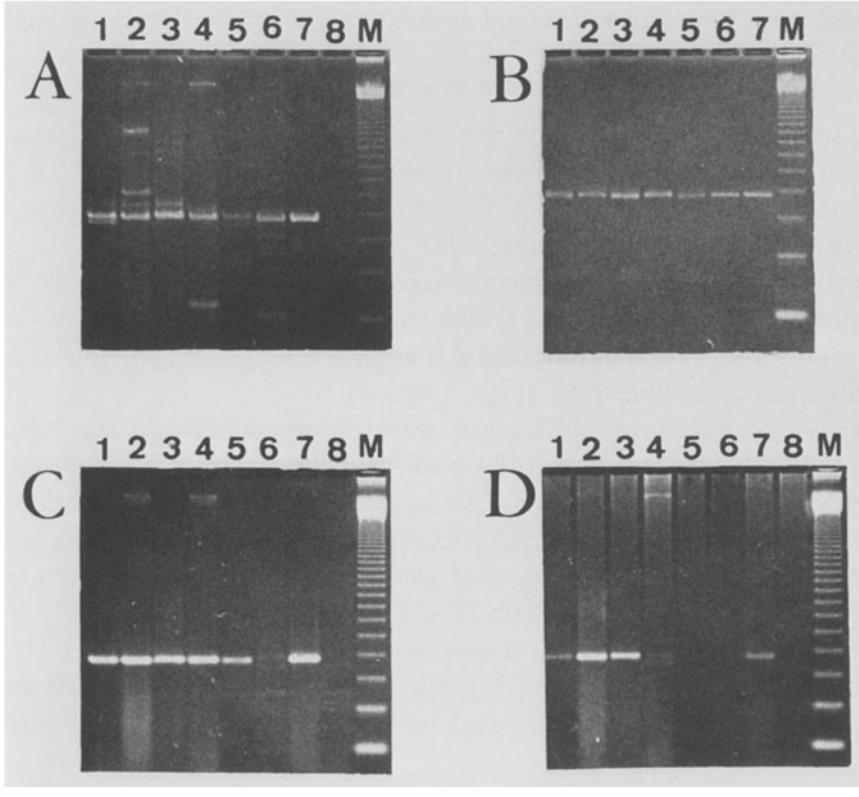

Fig. 1. Gel electrophoresis of PCR products of 470 bp amplified from cDNAs synthesized using **A** specific downstream, **C** random, or **D** oligo(dT) primers from cytoplasmic RNAs of C 6/36 cells infected with dengue types 1 (*1*), 2 (*2*), 3 (*3*), 4 (*4*), Kunjin (*5*), Japanese encephalitis (*6*), yellow fever (*7*) viruses; and of non-infected cells (*8*). *M* 123 bp marker. **B** Reamplification of excised 470 bp bands from **A** yields sharp target bands suitable for direct sequencing

(Fig. 1 C). In contrast, using cDNA templates derived from oligo(dT) primers, PCR yielded clearly discernible bands for all the viruses tested except Japanese encephalitis and Kunjin viruses (Fig. 1 D).

The presence of extraneous bands in PCR products using specific downstream primers for viral cDNA synthesis (Fig. 1 A) may be attributed to non-specific priming of the downstream primer during reverse transcription at a lower temperature of 42 °C compared with 50 °C for PCR annealing. However, reamplification of excised 470 bp bands (in Fig. 1 A) produced sharp target fragments (Fig. 1 B) which subsequently yielded authentic viral sequence data.

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 yielded target bands without significant non-specific bands (Fig. 1 C and D). PCR of human genomic DNA or of cDNA from non-infected C 6/36 cells produced no target nor background bands (Fig. 1 A, C, and D, lane 8).

Sensitivity assays indicated that at least 1, 0.1, 0.01, and 1 pg equivalent to 10^5, 10^4, 10^3, and 10^5 copies of dengue types 1, 2, 3, and 4 viral RNAs, respectively, could be detected by gel electrophoresis of PCR products (Fig. 2).

Sequence analysis of NS 3 of dengue and other flaviviruses

Clearly readable stretches of ~ 420 nucleotides (encoding 140 amino acid residues) within the NS 3 target fragment of dengue 2 (New Guinea C), Kunjin (MRM 61C) and yellow fever (17 D vaccine) virus strains were identical with the corresponding published sequences [11, 26, 37].

However, 2 nucleotide differences were noted within this fragment between our sequence for dengue 3 (H 87 strain) and the previously published sequence for the same strain [36], culminating in a single conservative hydrophobic amino acid difference at residue 1709 (Figs. 3 and 4).

Twenty-one nucleotide differences were observed between the NS 3 fragments of the Hawaii strain and the published Singapore S 275/90 strain [17] of dengue 1 virus (Fig. 3). These lead to 4 disparities at amino acid residues 1688, 1690, 1691, and 1729, located between the conserved segments I, I a and II which are nucleotide binding sites within the putative nucleotide triphosphatase and RNA helicase domain (Fig. 4).

Differences at 16 nucleotide positions were seen between the H 241 strain and the published 814669 strain [31] of dengue 4 virus, with only one resultant polar versus hydrophobic amino acid replacement at residue 1644 within the serine protease domain.

The Nakayama strain and the published JaOArS 982 strain [40] of Japanese encephalitis virus differed at 11 nucleotide positions, resulting in only one amino acid difference emerging within the serine protease domain at residue 1662. Comparison of the Nakayama strain with the attenuated vaccine SAA strain [1] revealed an additional disparity at amino acid residue 1739 located between segments I a and II of the RNA helicase domain (Figs. 3 and 4).

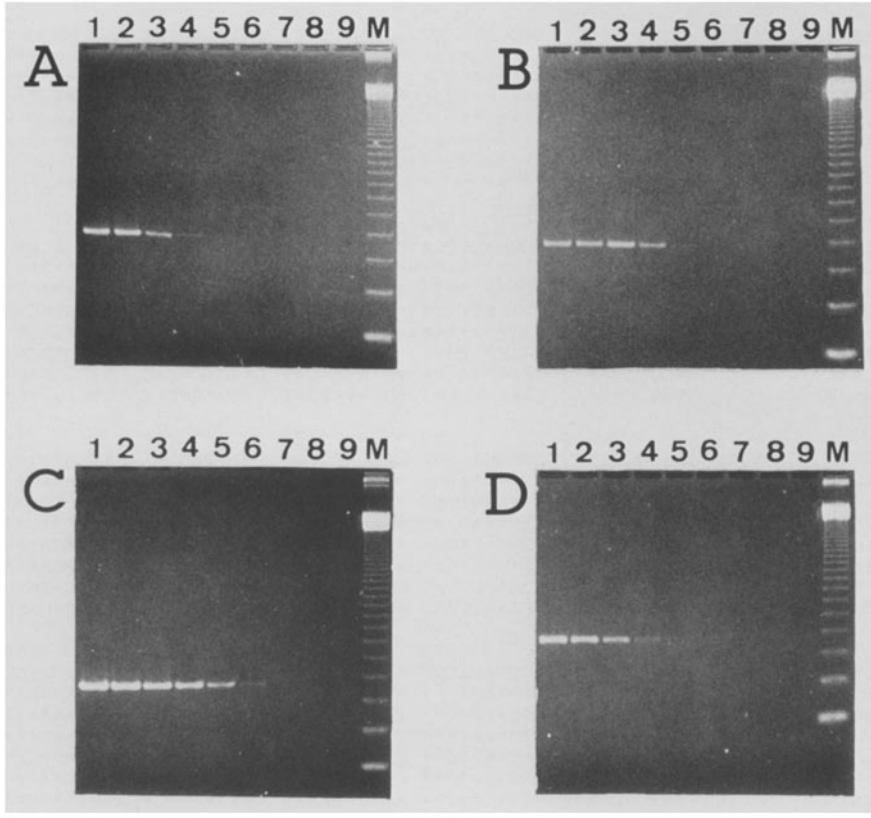

Fig. 2. PCR products amplified from ten-fold serial dilutions of purified viral RNAs of dengue virus **A** type 1, **B** type 2, **C** type 3, **D** type 4. cDNA templates for PCR were synthesized from starting RNAs of 1 ng, 0.1 ng, 0.01 ng, 1 pg, 0.1 pg, 0.01 pg, 1 fg, 0.1 fg, 0.01 fg (*1–9*) using random primers. *M* 123 bp marker

Discussion

Using a single pair of NS 3 consensus amplimers, we are able to detect all four dengue virus types, as well as Japanese encephalitis, Kunjin and yellow fever viruses by reverse transcription of viral RNA and/or cytoplasmic RNA of virus-infected cells, followed by PCR, achievable within hours. cDNAs synthesized from random and specific downstream primers served as reliable templates for PCR. Amplification of oligo(dT)-primed cDNAs yielded target products for all the viruses tested except for Japanese encephalitis and Kunjin viruses. Successful PCR from oligo(dT)-primed cDNAs from viral templates implies the presence of polyadenylated stretches within their genomes. The high detection sensitivity of the PCR protocol using these consensus primers is evident, as little as 0.01 pg or 10^3 copies of viral RNA being detectable on an agarose gel alone.

Even though these NS 3 amplimers were not tested against other related flaviviruses, computer search for primer homology with hepatitis C [9, 41],

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                      10         20         30         40         50
D1HAW                 GTGAACAGAG AGGGAAAAAT AGTAGGTCTT TATGGAAATG GAGTGGTGAC
D1S275      (4920)    ********** *A******** ********** ********** ****A*****
D3H87V                A*A******* *******GG* ***G**A**G *****C**** *******T**
D3H87       (4931)    A*A******* *******GG* ***G**A**G *****C**** *******T**
D4H241                ......**GA *A******G* TA*C**A**C **C******* ****A**C**
D4814669    (4944)    ......**GA *A******G* CA*C**A**C ********** ****A**T**
JENAK                 ...G*TTCTA *T***G*C** CA****C**A **C**C**** ****T*A*CT
JES982      (5028)    ...G*TTCTA *T***G*C** CA****C**A **C**C**** ****T*A*CT

                      60         70         80         90         100
D1HAW                 AACAAGTGGA ACCTACGTCA GTGCCATAGC TCAAGCTAAA GCATCACAAG
D1S275                ********** ********** ********** C*****C*** **********
D3H87V                **AG*A**** GG***T**T* ***GA***** G***A*A**T ***GA**C**
D3H87                 **AG*A**** GG***T**T* ***GA***** G***A*A**T ***GA**C**
D4H241                T*A*TCA**T GAT******* ****T***A* G*****CG** AG*A*TGGT*
D4814669              C*A*TCA**T GAT******* ********A* G*****CG** AG*ATTGG**
JENAK                 TGGCG****C T*A******* *C*****C*T G**G*G*G*C CGTCAGG*G*
JES982                TGGCGA***C T*A******* *C*****C*T G**G*G*G*C CGTCAGG*G*

                      110        120        130        140        150
D1HAW                 AAGGGCCTCT ACCAGAGATT GAGGACGAGG TGTTTAGGAA AAGAAACTTA
D1S275                *******C** ********** ********** ********** **********
D3H87V                *T**A**GAC *******T*G **A**A***A ****C*AA** GC****TC**
D3H87                 *T**A**GAC *******T*G **A**A***A ****C*AA** GC****TC**
D4H241                *GCCAGA*TA TGA**T*GA* ******---A *T***C*A** G*A**GA***
D4814669              *GCCAGA*TA TGA**T*GA* ******---A *T***C*A** G*A**GA***
JENAK                 *GCCAGTC*C *GA**CTTAC ACCCCAA*CA ****G**A** G***C*GA*G
JES982                **CCAGTC*C *GA**CTTAC ACCCCAA*CA ****G**A** G***C*GA*G

                      160        170        180        190        200
D1HAW                 ACAATAATGG ACCTACATCC AGGATCGGGG AAAACAAGAA GATATCTTCC
D1S275                ********** ********** ********** ********** **********
D3H87V                **C******* *T**T***** T**G**A**A **G**GC*G* A**********
D3H87                 **C******* *T**T***** T**G**A**A **G**GC*G* A**********
D4H241                **T******* **T****C** C***G*C**A **G****A** **AT***C**
D4814669              **T******* **T****C** C***G*T**A **G****A** **AT******
JENAK                 **TG*GC*A* *TT*G**C** ***T**A*** *****C**G* A*AT***G**
JES982                **TG*GC*A* *TT*G**C** T**C**A*** **G**C**G* A*AT***G**

                      210        220        230        240        250
D1HAW                 AGCCATAGTC CGTGAGGCTA TAAAAAGGAA GCTGCGTACG CTAATCTTGG
D1S275                ********** ********C* ***G****** CG****C**A *****T****
D3H87V                ***T**T**T A*A******A* *C**G**ACG CT*AA*G**T *****T****
D3H87                 ***T**T**T A*A******A* *C**G**ACG CT*AA*G**T *****T****
D4H241                *T*A****** A*A**A**CT *********G ******A**C T*G**T****
D4814669              *T*A******G A*A**A**CT *********G ***A**A**T T*G**T**A*
JENAK                 *CAA***A*T AAG**C**** *TC*GCA*CG C**AA*A**A GCTG*G****
JES982                *CAG***A** AAG**C**C* *CC*GCA*CG C**AA*A**A GCTG*GC***

                      260        270        280        290        300
D1HAW                 CTCCCACAAG AGTTGTCGCT TCTGAAATGG CAGAGGCGCT CAAGGGAATG
D1S275                ********** G********* **C******* ********** **********
D3H87V                *A**A***** G**A******A G****G**** A***A**AT* G**A**GC*C
D3H87                 *A**A***** G**A**T**A G****G**** A***A**AA* G**A**GC*C
D4H241                *******G** ***G**G**G G*C**G**** A******C** ACGT***C**
D4814669              *******G** ***G**G**G G*C**G**** A******C** ACGT***C**
JENAK                 *A**G**GC* G**G**A**A G*A******* ****A**TT* G*GA**GC*C
JES982                *A**G**GC* G**G**A**A G*A******* ****A**TT* G*GA**GC*C

                      310        320        330        340        350
D1HAW                 CCAATAAGGT ATCAGACAAC AGCAGTGAAG AGTGAACACA CAGGAAGGGA
D1S275                ********** *C**A***** ********** ********** ******AA**
D3H87V                ********** ****A***** T***ACA**A TC******** *******A**
D3H87                 ********** ****A***** T***ACA**A TC******** *******A**
D4H241                *****CC*T* *******CC* ***T*****A TCA******* *******A**
D4814669              *****CC*T* *******CC* ***T*****A TCA******* *******A**
JENAK                 ***G**C*A* ****A**TT* *******C** **A**G***C A*........
JES982                ***G*GC*A* ****A**TT* *******C** **A**G***C A*........
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                  360        370        380        390        400
D1HAW        GATAGTTGAC CTTATGTGCC ATGCCACTTT CACCATGCGT CTCCTGTCTC
D1S275       ********** **C*****T* *C******** ********** **********
D3H87V       ***T******T **A*****T* *C**A**G** ***A******C T*G*****A*
D3H87        ***T******T **A*****T* *C**A**G** ***A******C T*G*****A*
D4H241       ***C**A*** **C*****T* ****A**C** ***A*CAA*A **TT*A**AT
D4814669     ***T**A*** **C*****T* ****A**C** ***A*CAA*A **TT****AT
JENAK        .......... .......... .......... .......... ..........
JES982       .......... .......... .......... .......... ..........

                  410        420        430
D1HAW        CCGTGAGAGT TCCCAATTAC AACATG....
D1S275       ********** ********** ******....
D3H87V       *A**C**G** ***A**C*** ..........
D3H87        *A**C**G** ***A**C*** ..........
D4H241       *AACC**G** ***A****** ***C*CATA.
D4814669     *AACC**G** ***A****** ***C*TATA.
JENAK        .......... .......... ..........
JES982       .......... .......... ..........
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Fig. 3. Comparison of our nucleotide sequences within the NS 3 genes of dengue 1 Hawaii (*D1HAW*), dengue 3 H87 (*D3H87V*), dengue 4 H241 (*D4H241*) and Japanese encephalitis Nakayama (*JENAK*) strains with the published sequences of correspondingly-related dengue 1 S275/90 (*D1S275*), dengue 3 H87 (*D3H87*), dengue 4 814669 (*D4814669*) and Japanese encephalitis S982 (*JES982*) strains. Numbers within parentheses correspond with published positions. Asterisks denote base matching with dengue 1 Hawaii strain, while dashes are introduced for alignment. (Clearly readable ladders were obtained for only 338 upstream bases of the Nakayama strain)

tick-borne encephalitis [32], Murray Valley encephalitis [12, 29], and West Nile [4] viruses indicated matching of high percentage with the latter two suggesting potential amplification (Table 1).

Other workers have reported PCR amplification of dengue viruses and other flaviviruses using type-specific primers [14, 15, 24, 35] and consensus primers [22, 28] for regions outside of NS 3, often accompanied by sequence-specific probe hybridization. PCR of these RNA viruses using our technique and the assays of other groups is comparable in terms of sensitivity, specificity, and simplicity to PCR assays for DNA viruses such as human papillomaviruses [10, 43].

The complete concurrence of our NS 3 fragment sequence data with the published sequences for the corresponding strains of dengue 2, Kunjin, and yellow fever viruses confirms the authenticity and reproducibility of this RNA-PCR consensus amplimer technique coupled with DNA sequencing.

Stringent analyses by multiple sequencing in both directions of amplified cDNA templates derived from random, specific and oligo(dT) primers were consistently compatible. This thus validates the fidelity of *Taq* polymerase for target fragments of less than 500 bp, its previously-verified low error rate being comparable to enzymes with proof-reading ability such as Vent DNA polymerase [23].

Interestingly, the majority of the base substitutions found between different strains of the same virus were transitions which are known to be the commonest class of point mutations, which occur at relatively high frequency among RNA

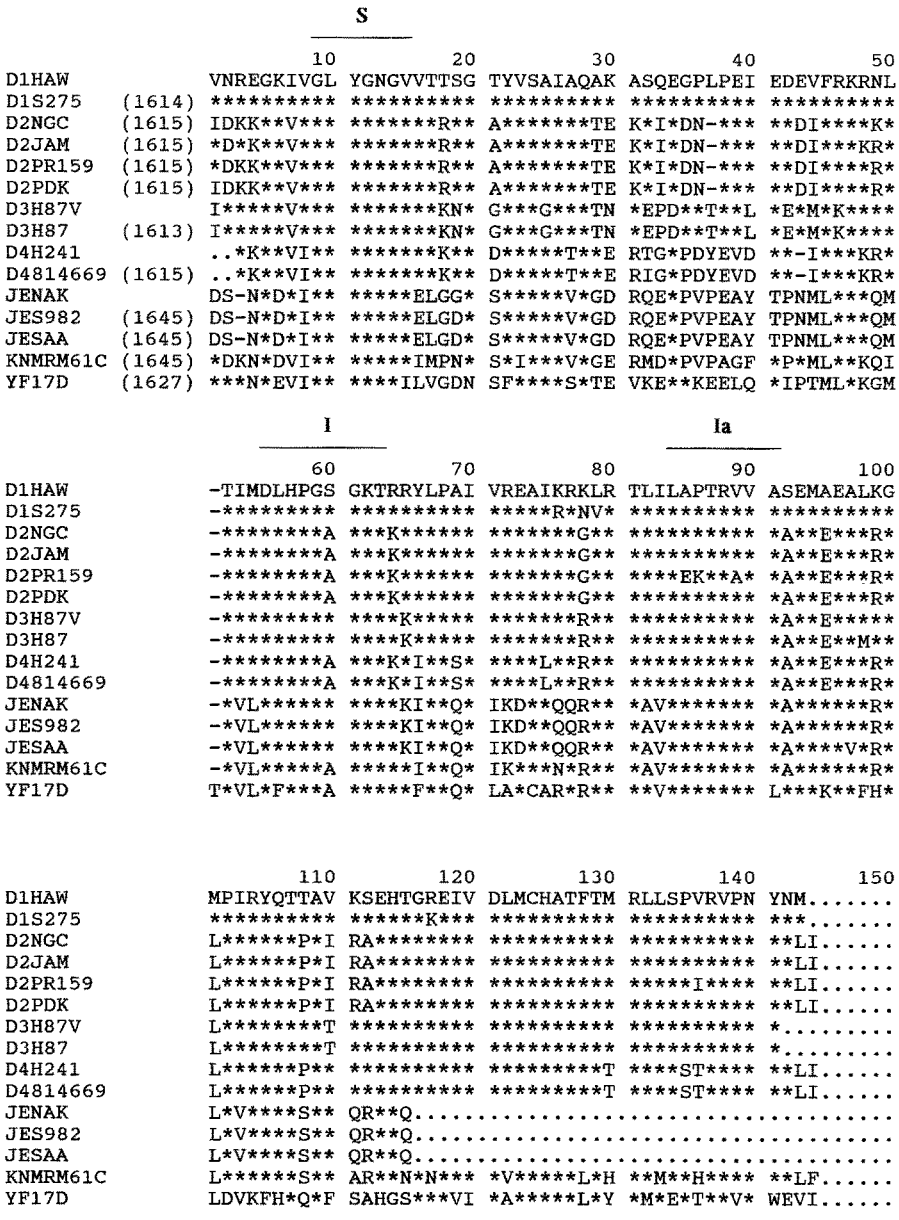

Fig. 4. Comparative analysis of deduced amino acid residues corresponding to nucleotide sequences in Fig. 4. Known sequences of dengue 2 NGC (*D2NGC*), JAM (*D2JAM*), PR159 (*D2PR159*), and PDK (*D2PDK*), Japanese encephalitis SAA (*JESAA*), Kunjin MRM61C (*KNMRM61C*) and yellow fever 17D (*YF17D*) strains are also included. Numbers within parentheses refer to published locations. Asterisks represent residues identical to dengue 1 Hawaii strain and dashes permit optimal alignment. *S* Conserved serine protease motif; *I*, *Ia* RNA helicase segment I and Ia motifs

viruses, partially attributable to random polymerase-mediated base misincorporations [30].

We found 2 nucleotide differences between our NS 3 sequence and the published one for dengue 3 (strain H 87). This discrepancy may be partly explained by differences in passage history of this viral strain. Our H 87 strain was passaged 114 times in suckling mouse brain followed by 5 times in C 6/36 cells.

Whilst comparative sequence analysis of strains of dengue 1, dengue 4, and Japanese encephalitis viruses revealed numerous nucleotide differences (21, 16, and 11 respectively), these were not commensurate with the amino acid residue differences (only 4, 1, and 1 respectively). All these differences occur outside of the tightly conserved putative active motifs of the enzymatic domains within NS 3, and many mutations are silent or result predominantly in conservative amino acid substitutions. Moreover, the ability of our consensus primers to amplify this NS 3 fragment of dengue and other flaviviruses further demonstrates the highly conserved nature of these motifs. Furthermore, the apparent absence of differences at conserved motifs of the serine protease and RNA helicase domains is consistently observed even for geographically distinct and temporally unrelated strains (Fig. 4). These phenomena reiterate the slow rate of evolution of NS 3 genes in flaviviruses, especially the catalytic sites, reinforcing the notion of their indispensibility in flaviviral processing and replication. In contrast, higher molecular evolutionary rates have been estimated for the envelope/NS 1 gene junctions of dengue virus types 1 and 2 [38], the pre-M gene of Japanese encephalitis virus [8], the VP 1 and 2 A genes of poliovirus type 1 [39], the NP and M genes of paramyxoviruses [33], and for the regulatory sequences of human papillomavirus type 16 DNA [7, 23].

Our data offer some insight into the structure-function analysis of NS 3 and also demonstrate the feasibility of the strategy of coupling this RNA-PCR consensus amplimer technique with direct DNA sequencing of PCR products in the molecular diagnosis, epidemiology and vector surveillance for infections with dengue and other flaviviral strains. In order to type dengue viruses, internal type-specific primers for all 4 dengue virus types in a nested PCR format are currently being investigated.

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