

Molecular Diagnosis and Epidemiology of Dengue Virus Infection

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Abstract

Early diagnosis of dengue fever contributes towards appropriate management of the disease and its potentially severe complications. Better and more rapid molecular procedures are increasingly available for detecting dengue viral RNA, antibodies and antigens. Using consensus primers based on the conserved non-structural-3 gene, the reverse transcription-polymerase chain reaction (RT-PCR) technique can amplify all four dengue virus types as well as certain flaviviruses. Consensus primers used together with four type-specific downstream primers in single-step or semi-nested RT-PCR formats can discriminate the specific dengue virus type by virtue of the diagnostic size of the RT-PCR target fragment on agarose gel electrophoresis. Alternatively, RT-PCR products may be labelled with digoxigenin and allowed to hybridise with individual biotinylated type-specific PCR primers which act as capture probes immobilised on solid phase via streptavidin-coated tubes. With automated instrumentation for enzyme-linked immunosorbent assay (ELISA), the hybridised RT-PCR products can be quantitated spectrophotometrically via anti-digoxigenin antibodies conjugated with an enzyme which reacts with colourimetric substrate. While RT-PCR is highly sensitive, specific and successfully identifies the dengue virus type in clinical serum samples and adult Aedes mosquitoes, it generally yields positive results in viraemic sera collected within 2 to 5 days of pyrexia. Sera obtained after the period of viraemia are more likely to be positive by serological tests such as IgM capture ELISA or the commercial Dengue Blot kit. The RT-PCR primers can also be utilised for direct cycle dideoxy DNA sequencing to monitor the molecular epidemiology and evolution of geographically and temporally separated virus strains. To exemplify this, nucleotide and amino acid sequence data as well as phylogenetic trees of several strains of dengue 1 and 2 viruses from patients and field-caught Aedes mosquitoes are presented.

Ann Acad Med Singapore 1997; 26:820-6

Key words: Cycle sequencing, Polymerase chain reaction, Reverse transcription, Viral evolution, Virus typing

Introduction

Belonging to the family *Flaviviridae* and comprising four serotypes, the first dengue viruses were isolated some 50 years ago. *Aedes aegypti* and *Aedes albopictus* are the most important *Aedes* mosquito vectors which transmit dengue viruses. Dengue virus infection may be clinically inapparent or cause a wide spectrum of disease manifestations ranging from mild undifferentiated fever to more severe haemorrhagic phenomena which may progress to shock syndrome and even death.¹

Dengue is a major public health problem particularly in tropical and subtropical regions throughout the world. Worldwide, it is estimated that 2.5 billion persons are at risk, with 60 million cases and 30 000 fatalities occurring yearly.² Dengue haemorrhagic fever is one of the principal causes of childhood morbidity and mortality in Southeast Asia. From 1990 to 1996, Singapore has witnessed a rising trend in the incidence of dengue with 1733, 2179, 2878, 946, 1239, 2008 and 3128 cases, respectively. The predominant human and environmental factors influencing the dengue ecosystem and contributing to the re-emergence and spread of dengue include un-

controlled urbanisation and migration, modern transportation, international travel and trade, as well as changes in global climatic conditions. Thus, endemo-epidemic situations arise as a consequence of population growth outstripping infrastructure leading to improper water storage, the deterioration of cities, the proliferation of mosquito breeding sites and inadequate mosquito control.³⁻⁶

Rapid Dengue Diagnosis by Nucleic Acid Amplification

The early diagnosis of patients with suspected dengue fever contributes towards the appropriate clinical management of the disease and its potentially severe and occasionally fatal complications such as the dengue shock syndrome.⁷ Conventional methods for dengue diagnosis, such as virus isolation by cell culture and the haemagglutination inhibition test, are cumbersome and time-consuming. Molecular strategies are leading to better and more rapid techniques for detecting dengue viral RNA, antigens and antibodies. For example, nucleic acid hybridisation using suitable gene probes is being exploited for the detection of RNA genomes of

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dengue viruses.^{8,9}

However, the advent of nucleic acid amplification has completely revolutionised nucleic acid detection in view of its attributes of exquisite sensitivity and high specificity. The ability to amplify miniscule quantities of DNA is achieved by the polymerase chain reaction (PCR) consisting of repetitive cycles of *in vitro* replication of DNA involving denaturation of DNA template, annealing of synthetic oligonucleotide primers, and primer elongation mediated by thermostable DNA polymerase in the presence of bases or "DNA building blocks". Even RNA can be subjected to PCR by first converting RNA into complementary DNA (cDNA) catalysed by retroviral reverse transcriptase, a process known as reverse transcription (RT).¹⁰⁻¹³ Interestingly, certain DNA polymerases (e.g. *Tth* DNA polymerase derived from *Thermus thermophilus*) are able to catalyse both reverse transcription and DNA polymerisation, thus obviating the requirement for two separate enzymes when amplifying RNA templates.¹⁴

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Using Non-Structural-3 Gene Primers

Each of the four dengue virus types contains a single, positive-stranded genome of 11 kilobases which encodes structural proteins (capsid, membrane and envelope) as well as non-structural proteins (e.g. NS5 with putative RNA-dependent RNA polymerase activity). An RT-PCR procedure using consensus primers within the conserved non-structural-3 (NS3) gene has been developed.¹⁵ The DV1 upstream consensus primer was designed on the basis of the conserved glycine-threonine-serine-glycine-serine-proline sequence in the serine protease domain spanning the amino terminus of the NS3 protein, while the DV3 downstream primer was based on the methionine-aspartic acid-glutamic acid-alanine-histidine-phenylalanine motif at the NS3 carboxy1 terminus, corresponding to segment II of the RNA helicase domain. This NS3-based RT-PCR technique can amplify approximately 470 basepair (bp) target fragments from all four dengue virus types as well as related flaviviruses, i.e. Japanese encephalitis, Kunjin and yellow fever viruses. According to computer-assisted sequence homology, these consensus primers have the potential to be employed to amplify other flaviviruses such as Murray Valley encephalitis and West Nile viruses. Given their strong sequence conservation, these consensus primers may also be used to possibly amplify novel members of the Flavivirus family.

Four dengue virus type-specific primers (DSP1, DSP2, DSP3 and DSP4) have also been designed for use in conjunction with the consensus primers. The DV1 upstream consensus primer can be utilised with the four downstream type-specific primers in a rapid, single-

tube RT-PCR format.¹⁶ A more sensitive option is to adopt a semi-nested RT-PCR approach involving a first round of RT-PCR with both consensus primers, followed by a second round of semi-nested PCR using DV1 and DSP1 to DSP4 primers.¹⁷ Both procedures can discriminate the specific dengue type according to the size of the RT-PCR product on ethidium bromide-stained agarose gel electrophoresis. Thus, diagnostic fragments of 169, 362, 265 and 426 bp can be amplified from dengue virus types 1, 2, 3 and 4, respectively (Fig. 1), whose authenticities can be verified by direct cycle dideoxy DNA sequencing of RT-PCR products. A high sensitivity of one plaque-forming unit of dengue virus is achievable, while the specificity is assured by the absence of target fragments after RT-PCR of RNAs from Japanese encephalitis, Kunjin, yellow fever viruses or uninfected C6/36 *Aedes albopictus* cells. Other researchers have also reported PCR amplification of dengue and other flaviviruses using consensus and/or type-specific primers.¹⁸⁻²⁷

Alternatively, RT-PCR products may be labelled with digoxigenin and allowed to hybridise with individual

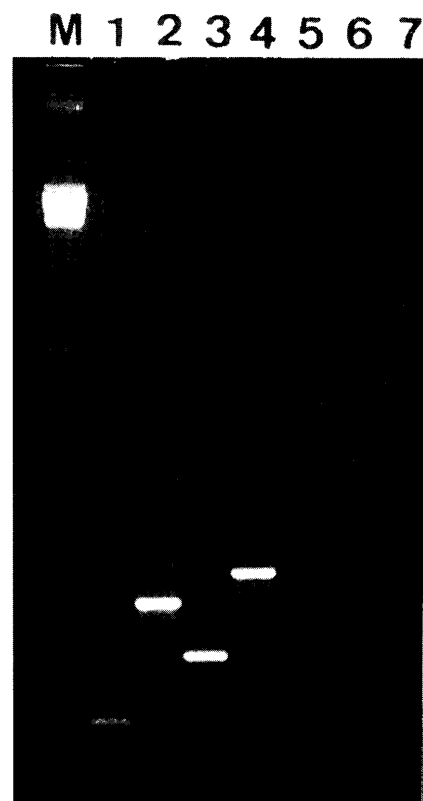


Fig. 1. Semi-nested RT-PCR assay using consensus and type-specific NS3 gene primers for amplifying dengue viruses in virus-inoculated adult *Aedes aegypti* mosquitoes. Agarose gel electrophoresis of RT-PCR products exhibiting diagnostic target fragments of 169, 362, 265 and 426 bp for dengue virus types 1, 2, 3 and 4, respectively (lanes 1-4), but not for Japanese encephalitis, Kunjin and yellow fever viruses (lanes 5-7, respectively). M indicates 123 bp DNA ladder markers.

biotinylated DSP1, DSP2, DSP3, DSP4 type-specific PCR primers which serve as capture probes immobilised on solid phase via streptavidin-coated tubes. With an automated enzyme-linked immunosorbent assay (ELISA) apparatus, the hybridised RT-PCR products are then quantitated colourimetrically by means of anti-digoxigenin antibodies conjugated with an enzyme (e.g. peroxidase) which reacts with substrate-chromogen (e.g. ABTS).²⁸ Computer-automated instrumentation renders this non-radioactive ELISA technology amenable for high throughput analysis of clinical samples.

Owing to the great sensitivity of PCR, it is imperative to practise strict measures to avoid contamination from "carryover" DNA and other sources. The institution of quality control standards (e.g. mandatory inclusion of stringent negative and positive controls in every batch of reactions) and efforts to simplify nucleic acid extraction protocols will accelerate the routine use of PCR in medical laboratories.^{13,29}

Practical Applications of RT-PCR Technology

The dengue type-specific RT-PCR assay is highly sensitive and specific, and could correctly identify the dengue virus type in retrospective clinical human serum samples which were positive by virus isolation.^{16,17} The semi-nested RT-PCR technique could confirm the presence of dengue virus in several sera collected from victims of the first documented outbreak of dengue haemorrhagic fever in Pakistan in 1994.³⁰

Semi-nested RT-PCR is able to type dengue virus in the excised heads of *Aedes aegypti* mosquitoes artificially inoculated intra-thoracically with virus-infected tissue culture fluid. This technique can detect one virus-infected mosquito head in a pool of up to 59 uninfected heads, with virus being detectable up to 42 days post-inoculation.³¹ The assay can successfully type dengue virus in field-caught adult *Aedes* mosquitoes, thus holding promise for improving sentinel supervision of dengue transmission foci and identification of circulating dengue virus types. This application of PCR for vector surveillance could enhance the rapid implementation of epidemic vector control measures to avert further spread of mosquito-borne infection.

RT-PCR generally yields positive results in dengue viraemic sera collected within 2 to 5 days of the fever.³²⁻³⁴ Sera obtained after the period of viraemia are more likely to be positive by serological assays such as IgM capture ELISA (with a positivity rate of up to about 80%), or the commercial Dengue Blot kit (which detects antibodies reactive with dengue antigens precoated onto membranes and is useful for acute secondary infections). It is therefore likely that RT-PCR will serve as a useful adjunct to robust serological assays rather than superseding them.³²

Molecular Epidemiology of Dengue

The NS3 PCR primers can also be used for direct cycle dideoxy DNA sequencing to provide readable DNA sequence ladders (Fig. 2) for the determination of the viral cDNA and deduced amino acid sequences in order to monitor the molecular epidemiology of dengue and to

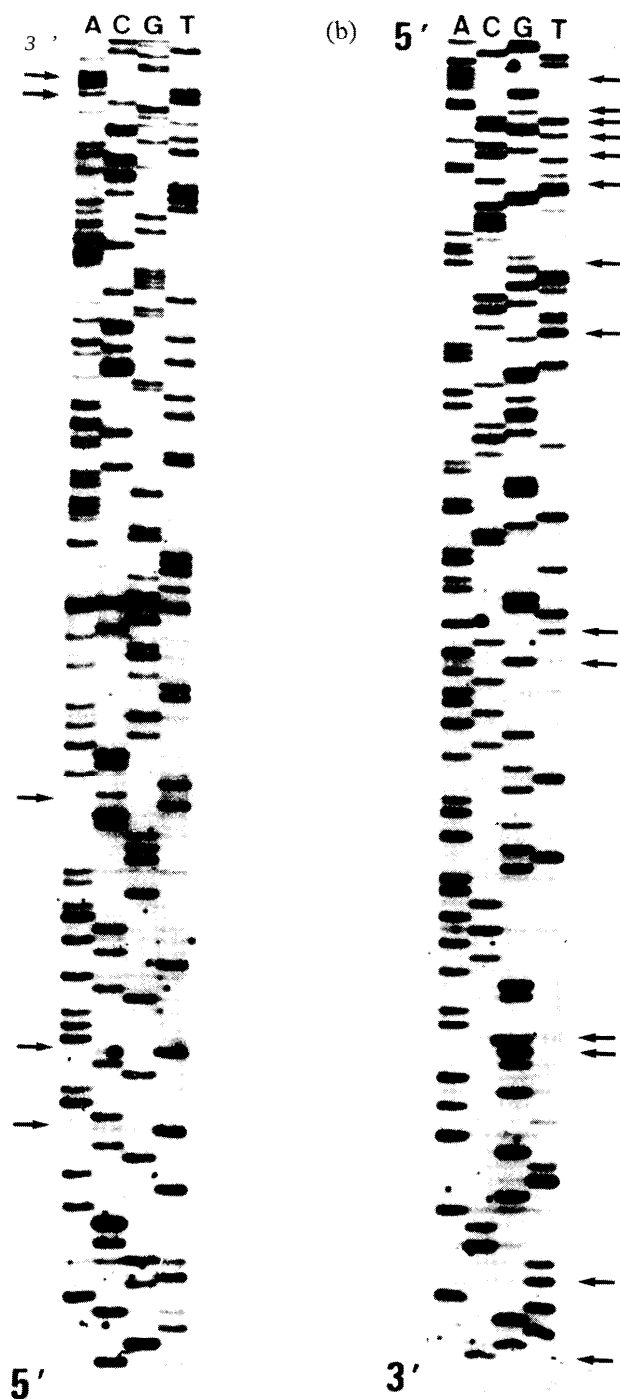


Fig. 2. Representative autoradiogram of direct cycle sequencing of amplified NS3 fragment of dengue virus type 1 (Hawaii strain) using (a) DV1 consensus primer and (b) DV3 consensus primer as sequencing primers. The arrows depict the nucleotides which differ from the published sequence of the Singapore S275/90 strain.

study the evolution of geographic strains of dengue and other flaviviruses.

Hence, NS3 consensus primers could be used for amplifying as well as for directly sequencing RT-PCR products from reference strains of all four dengue types, Japanese encephalitis and Kunjin viruses. The sequence data revealed that many of the nucleotide disparities between different viral strains were phenotypically silent transitions, culminating predominantly in conservative amino acid substitutions. In addition, these mutations occur outside of the tightly conserved active motifs of the serine protease and RNA helicase (segments I and Ia) domains, emphasising the role of these regions of the NS3 protein in flaviviral replication.³⁷ Interestingly enough, the NS3 protein was found to be immunodominant in the CD4-positive T-cell response of a donor who experienced dengue illness following receipt of a live experimental dengue virus type 3 vaccine.³⁵

Figure 3 illustrates the correlation between dengue viral NS3 sequences from patients and field-caught *Aedes* mosquito vectors in Singapore. Not unexpectedly, certain mosquito-derived viral NS3 sequences were identical to those from patients. However, significant fractions of mosquito-related viral NS3 sequences showed disparities, albeit limited, in comparison with patient-related sequences. While the dengue 1 viral nucleotide variations were phenotypically silent, the dengue 2 viral sequences displayed both nucleotide and amino acid sequence heterogeneity.

The dendrogram in Figure 4a reflects the genetic relationships between the recent dengue 1 isolates from

Singapore and Pakistan³⁶ with the Hawaii and Singapore S275/90 strains. As depicted by the phylogenetic tree in Figure 4b, the recent dengue 2 isolates from Singapore and Pakistan³⁶ are evolutionarily more closely related to the Puerto Rican strain compared with the other dengue 2 reference strains.

In 1992, a dengue epidemic with a record number of 2878 cases (with 4 deaths) was reported in Singapore, attributed mainly to dengue virus type 3. In order to postulate on the origin of this Singapore strain and to better understand the evolution of dengue 3 viruses in the Southeast Asian region, the NS3 genes of 21 dengue 3 strains isolated from five countries between 1956 and 1992 were sequenced and compared. Although few amino acid replacements were observed as expected, the presence of silent base mutations permitted the construction of phylogenetic trees.³⁷ In agreement with other studies,³⁸ consistent relationships were observed between clusters of strains from the Philippines, Indonesia, and those from Singapore and Malaysia of the 1970s and early 1980s, while the Thai cluster was relatively more distant. It is noteworthy that the more recent dengue 3 strains from Singapore and Malaysia of the late 1980s and early 1990s more closely resembled the Thai cluster, implying their evolution from the latter.

Applied to specimens from patients and mosquito vectors, such molecular epidemiological approaches reiterate the importance of geographical spread of viruses in view of ever-increasing travel between different parts of the world,⁵ and also serve to track the movement, exchange and evolution of these viruses.³⁶⁻⁴¹

					4969
D1S8393.SEQ	GTGAACAGAG	AGGGAAAAAT	AGTAGGTCTT	TATGGAAATG	GAGTGGTGAC
MS3.SEQ	*****	*****	*****	*****	*****
MS7.SEQ	*****	*****	*****	*****	*****
MS8.SEQ	*****	*****	*****	*****	*****
MS9.SEQ	*****	*****	*****	*****	*****
					5019
D1S8393.SEQ	AACAAGTGGA	ACCTACGTCA	GTGCCATCGC	TCAAGCTAAA	GCATCACAAG
MS3.SEQ	*****	*****	*****	*****	*****
MS7.SEQ	*****	*****	*****	C*****	*****
MS8.SEQ	*****	*****	*****A**	*****	**C*****
MS9.SEQ	*****	*****	*****A**	*****	*****
		5039			
D1S8393.SEQ	AAGGGCCTCT	ACCAGAGATT	G A G		
MS3.SEQ	*****	*****	**		
MS7.SEQ	*****	*****	**		
MS8.SEQ	*****	*****	**		
MS9.SEQ	*****	*****	**		

Fig. 3a. Comparison of nucleotide sequences of amplified NS3 gene fragments of dengue 1 viruses from Singapore (suffix "SEQ"). Strain D1S8393 was isolated from a patient with dengue in April 1995. Out of 20 representative dengue 1 virus PCR products amplified from field-caught *Aedes* mosquitoes collected in 1995, 5 showed sequences (represented by MS3) identical to the D1S8393 sequence, 13 showed sequences represented by MS7, while one each demonstrated sequences represented by MS8 and MS9. The phenotypically silent mutations did not give rise to any amino acid substitutions. Nucleotide positions are shown, and asterisks denote identity.

					4988
D2SINR8.SEQ	GTCGACAAAA	AAGGAAAAAGT	TGTGGGCCTT	TATGGCAATG	GTGTCGTCAC
MS1.SEQ	*****	*****	*****	*****	*****
MS15.SEQ	*****	*****	*****	*****	*****
MS4-23.SEQ	*****	*****	*****	*****	*****
					5038
D2SINR8.SEQ	AAGGAGTGGA	ACATATGTGA	GTGCCATAGC	CCAGACTGAA	AAAAGCATTG
MS1.SEQ	*****	*****	*****	*****	*****
MS15.SEQ	*****	*****	*****	*****	G*****
MS4-23.SEQ	*** A *****	*****	*****	*****	*****
					5088
D2SINR8.SEQ	AAGACAATCC	AGAGATTGAA	GATGACATCT	TTCGAAAGAA	AAGATTGACC
MS1.SEQ	*****	*****	*****	*****	*****
MS15.SEQ	*****	*****	*****	*****	*****
MS4-23.SEQ	*****	*****	*****	C*****	*****
					5138
D2SINR8.SEQ	ATCATGGACC	TCCACCCAGG	AGCGGGAAAG	ACGAAGAGAT	ACCTCCCAGC
MS1.SEQ	*****	*****	*****	*****	*****
MS15.SEQ	*****	*****	*****	*****	***** T
MS4-23.SEQ	*****	* T*****	*****	*****	*****
					5188
D2SINR8.SEQ	AATAGTCAGA	GAAGCCATAA	AACGAGGCTT	GAGAACATTA	ATCCTGGCCC
MS1.SEQ	*****	*****	*****	*****	*****
MS15.SEQ	*****	*****	*****	*****	*****
MS4-23.SEQ	*****	*****	*****	*****	*****
					5238
D2SINR8.SEQ	CCACTAGAGT	TGTAGCGGCT	GAAATGGAGG	AAGCTCTTAG	AGGACTTCCA
MS1.SEQ	*****	*****	*****	*****	*****
MS15.SEQ	*****	*****	*****	*****	*****
MS4-23.SEQ	*****	*****	*****	*****	*****
		5258			
D2SINR8.SEQ	ATAAGATACC	AAACCCC . . .			
MS1.SEQ	*****	***** . .			
MS15.SEQ	*****	***** . . .			
MS4-23.SEQ	*****	***** . . .			
					1664
D2SINR8.AMI	VDKKGKVVL	YNGVVT RS G	TYVSAIAQTE	KSIEDNPEIE	DDIFRKKRLT
MS 1.AMI	*****	*****	*****	*****	*****
MS 15 .AMI	*****	*****	*****	E*****	*****
MS4-23.AMI	*****	*****	*****	*****	*** S*****
					1714
D2SINR8.AMI	IMDLHPGAGK	TKRYL PA I VR	EAI KRGLRTL	I LAPTRVVAA	EMEEALRGLP
MS 1.AMI	*****	*****	*****	*****	*****
MS15.AMI	*****	***** V**	*****	*****	*****
MS4-23.AMI	*****	*****	*****	*****	*****
D2SINR8.AMI	I R Y Q T . . .				
MS 1.AMI	*****				
MS 15 .AMI	*****				
MS4-23.AMI	*****				

Fig. 36.

Fig. 3c.

Figs. 3b and 3c. Comparative analysis of (b) nucleotide sequences of amplified NS3 gene fragments of dengue 2 viruses from Singapore (suffix "SEQ"), and (c) corresponding deduced amino acid residues (suffix "AMI"). Strain D2SINR8 was derived from a patient with dengue in 1993 to 1994. Out of 5 representative dengue 2 virus PCR products amplified from field-caught *Aedes* mosquitoes collected in 1995, 3 showed sequences (represented by MS1) identical to the D2SINR8 sequence, while one each demonstrated sequences represented by MS15 and MS4-23. Both nucleotide and amino acid disparities were noted for the respective MS15 and MS4-23 sequences. Nucleotide and amino acid positions are shown, while asterisks denote identity.

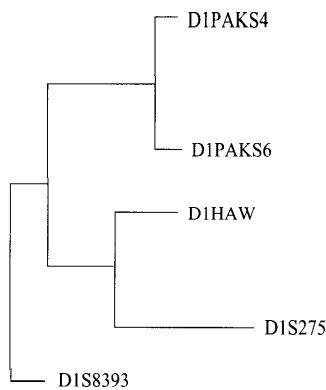


Fig. 4a.

Fig. 4. Phylogenetic relationships between dengue virus strains. Multiple sequence alignments of dengue 1 nucleotides 4920-5348 and dengue 2 nucleotides 4936-5364 were performed by the "PileUp" programme. Phylograms were then constructed using the "GrowTree" software.

(a) Recent dengue 1 strains from Singapore [D1S8393(1995)] and Pakistan [D1PAKS4 and D1PAKS6(1994)] are compared with the published Singapore S275/90 strain (1990) and Hawaii strain (1944), with the year of isolation shown within parentheses.

(b) Recent dengue 2 strains from Singapore [D2SINGR8 (1993-4)] and Pakistan [D2PAKS9 (1994)] are compared with reference strains-New Guinea C (1944), Thailand 16681 PDK (1964), Puerto Rico PR159 (1969) and Jamaica (1983), with the year of isolation within parentheses.

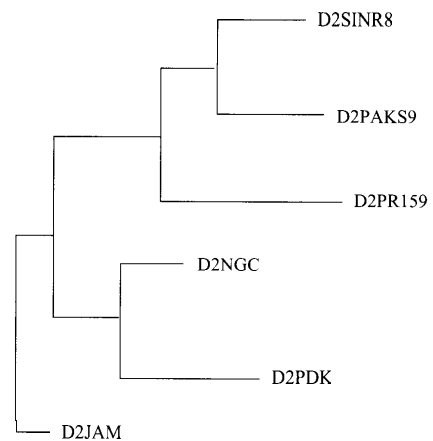


Fig. 4b.

Current and Future Directions

The "immune enhancement" or "secondary infection" hypothesis has been proposed to explain the greater severity of dengue in children with secondary infections.⁴² Notwithstanding this, it is also recognised that high rates of mutation affect the RNA genomes of dengue viruses culminating in their genetic variability and the appearance of certain viral strains with heightened virulence. For example, higher neurovirulence is observed in mice inoculated intracerebrally with the Mexican strain of dengue 2 virus which harbours a single substitution at nucleotide 1168 leading to an amino acid change in domain III of the envelope protein involved in viral binding.⁴³ The evolution of dengue virus serotypes has also led to the prediction that a fifth and even newer serotypes may emerge in the future, an event that would fundamentally alter the epidemiology of dengue and its impact on human health.^{1,41}

The lack of both specific antiviral therapy and routinely available vaccines against dengue viruses emphasise that the reduction of dengue incidence and prevention of epidemics must be dependent upon mosquito control measures which include public health education, community participation, competent vector control programmes and epidemiological surveillance.⁴⁴

However, the recent testing of a live, attenuated dengue 2 vaccine (strain 16681 PDK 53) in ten American volunteers revealed promising preliminary results. This trial showed that this candidate vaccine was safe and highly immunogenic, and that all vaccinees developed neutralising antibody which persisted for two years.⁴⁵

Another exciting prospect is the potential control of

dengue via genetic manipulation of mosquito vectors to disrupt viral transmission to human hosts. In an experimental model, a recombinant Sindbis virus was employed to transduce female *Aedes aegypti* with an antisense RNA targeted to the premembrane region of dengue 2 virus. The transduced mosquitoes could not support dengue 2 viral replication in their salivary glands and were thus unable to transmit the virus.⁴⁶

Acknowledgements

The author acknowledges the collaboration of Y C Chan, C L K Seah, RY Y Yong, S Doraisingham, and staff of the Vector Control and Research Department and Ministry of the Environment. This work was funded by research grants (RP910438 and RP930337) from the National University of Singapore.

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