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The use of direct sequencing of dengue virus cDNA from individual field-collected *Aedes aegypti* for surveillance and epidemiological studies

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Abstract. The relative efficiencies of four methods to extract viral RNA from individual dengue-2 virus (D-2V)-infected mosquitoes, *Aedes aegypti* (L.) (Diptera: Culicidae), were compared. The most efficient of these methods was then used to extract viral RNA for the preparation of cDNA from the abdomens of six engorged D-2V-infected mosquitoes and sera from three dengue fever (DF) patients collected in an isolated rural town in Colombia. Comparisons of viral envelope (E) gene sequences from each of these strongly suggested that the D-2V population which circulated in this study area was a homogeneous genotype which was unrelated to any of the D-2 viruses isolated from elsewhere in the world. When coupled with our rapid method to identify viruses in individual mosquitoes (Romero-Vivas *et al*. (1998) *Medical and Veterinary Entomology*, **12**, 101–105), the methodology we describe should be useful for epidemiological and surveillance studies of dengue viruses and other arboviruses.

Key words. *Aedes aegypti*, cDNA, dengue virus, immunofluorescence assay, polymerase chain reaction, sequencing, Colombia.

Introduction

All four dengue virus serotypes are now present in most countries of the Americas, which has lead to an increased frequency of epidemic dengue fever (DF) and the emergence of dengue haemorrhagic fever and dengue shock syndrome (DHF/DSS) (Gubler & Trent, 1994; PAHO, 1994). Secondary infections with heterologous dengue virus serotypes are usually associated with DHF/DSS (Halstead, 1997), but some viral strains have greater pathogenic capacity (Rosen, 1989; Gubler, 1997). Potential 'virulence markers' have been found in a number of regions of the dengue virus genome (Leitmeyer *et al*., 1999). In particular, the envelope (E) protein contains the sites required for viral cell-binding and membrane-fusion, as well as neutralizing epitopes and therefore most sequence analysis of the flaviviruses have been performed on this gene. Such analyses on the E gene have been used to classify different genotypes of each dengue virus serotype (Chu *et al*., 1989; Lewis *et al*., 1993; Lanciotti *et al*., 1994; Lanciotti *et al*., 1997) and to follow precisely the evolution and dispersal of flaviviruses (Zanotto *et al*., 1996; McGuire *et al*., 1998). Comparisons of the E gene sequences are therefore essential for surveillance and epidemiological studies. Because of the rapid mutations which occur in these RNA viruses, particularly in the E gene, during passage in animals or *in vitro* cell-culture (Lee *et al*., 1997), direct sequencing of viral cDNA from infected human samples (e.g. sera) or vector species (e.g. *Aedes aegypti*) would be highly desirable. We recently described a technique to screen for flavivirus infections amongst large numbers of *Ae. aegypti* by immunofluorescence assays (IFAs) on head squashes of individual mosquitoes, and then to identify the dengue virus serotypes in these flavivirus-positive individuals by IFAs using serotype-specific monoclonal antibodies on portions of their thoraxes (Romero-Vivas *et al*., 1998). All of these dengue virus-infected mosquitoes contained a bloodmeal (Romero-Vivas *et al*., 1998), which is known to affect PCR and cDNA sequencing reactions.

In this study we compared four methods for extraction of viral RNA from the abdomens which remained after the IFA screening of the heads and thoraxes of these engorged mosquitoes. The E gene nucleotide sequences were derived from products amplified by reverse transcriptase-polymerase

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chain reaction (RT-PCR), and these sequences were compared among six dengue-2 virus (D-2V)-infected mosquitoes and three DF patients from the same town and its immediate surrounding area.

Materials and Methods

Puerto Triunfo (Antioquia) is a port situated on the Magdalena River in central Colombia which, during this study period, had a population of 2370 who lived in 413 houses. Dengue type-1 virus (D-1V) and type-2 virus (D-2V) were identified in the adult *Ae. aegypti* population collected from 120 randomly chosen premises, located throughout the town, over a 6-month period (August 1996 to January 1997) (Romero-Vivas *et al*., 1998). Cases of DF-like illness were reported throughout this study period. Thirty-one single blood samples were obtained from suspected DF patients who consulted the outpatients' clinic of the local hospital and the sera were stored at – 70°C. After verbal consent, the name, age, sex, address and date of onset of symptoms (range: day 1–10) of each patient was registered (S#1 to S#31 in order of date).

D-1V (Nauru Island), D-2V (New Guinea-C), D-3V (H87) and D-4V (Dominica) were used to infect *Aedes alpopictus* C6/36 cell monolayers maintained in Leibovitz (L-15) medium containing 10% v/v tryptose phosphate broth and foetal calf serum (FCS) (Gibco, U.K.) as described elsewhere (Gould & Clegg, 1985). After incubation at 28°C for 7 days, the supernatants were collected and clarified by centrifugation. These viruses were then titrated on Vero (E6) cell monolayers, which were maintained in 48-well culture plates (Costar, U.K.) with M199 medium containing 3.5% v/v FCS (Gibco) and 2% w/v sodium carboxymethycellulose (25649: BDH, U.K.) (Gould & Clegg, 1985). After incubation for 7 days at 37°C in an atmosphere containing 5% CO_2, these cell monolayers were fixed with 8% w/v formaldehyde/PBS and then stained with 0.1% w/v crystal violet/PBS. After washing, the virus titres were calculated in plaque-forming units per millilitre (pfu/ml). As the titre of D-3V was considerably lower than those of the other virus serotypes, 100 ml of the D-3V cell-culture supernatant was concentrated by a factor of 20 by 8% w/v polyethlylene glycol 8000 (P2139: Sigma, U.K.) precipitation (Gould & Clegg, 1985).

Groups of laboratory-reared adult *Ae. aegypti* were inoculated intrathoracically with 0.3 µl of the cell-culture supernatants containing D-1V (258 pfu), D-2V (420 pfu), D-3V (780 pfu) or D-4V (660 pfu), as described previously (Gould & Clegg, 1985; Romero-Vivas *et al*., 1998). These infected mosquitoes were maintained at 28–30°C for 10 days to represent the natural extrinsic incubation period (Rhodain & Rosen, 1997). Viral infections were then confirmed in each of the infected mosquitoes by IFAs on head squashes as described (Romero-Vivas *et al*., 1998). These laboratory-reared mosquitoes were examined in pools of 10; each pool contained either two or 10 experimentally infected mosquitoes. Two pools (one of 2/10 and the other of 10/10 infected mosquitoes), infected with each of the four dengue viral serotypes, were examined using each of four RNA extraction methods (i.e. eight mosquito pools/method). Each mosquito pool was homogenized in 500 µl of Hank's BSS using 1 mm diameter glass beads in a mini bead-beater for 60 s prior to RNA extraction.

Four different RNA extraction methods were employed. Method 1, which employed silica to bind the viral RNA, was performed as described by Chungue *et al*. (1993). For this method, 10 µl of each mosquito homogenate was mixed with 90 µl of lysis buffer (4 M guanidine isothiocyanate/40 mM Tris/HCl pH 6.4/17 mM EDTA/1% v/v Triton X-100) and 4 µl of size fractionated silica. This mixture was then vortexed for 5 s, incubated for 10 min at room temperature and then centrifuged at 12 000 g for 15 s. The pellet was then washed twice with washing buffer (50% v/v ethanol/10 mM Tris-HCl pH 7.4/50 mM NaCl/1 mM EDTA) and once with 100 µl of RT buffer (10 mM Tris-HCl pH 8.3/50 mM KCl/2.5 mM $MgCl_2$/1 mM dithiothreitol).

Method 2 was performed following the manufacturer's protocol. Briefly, 375 µl of Trizol (15596–026: Gibco Life Technologies, U.K.), a mono-phasic solution of phenol and sodium citrate/guanidine isothiocyanate (1 : 3), was mixed with 125 µl of each mosquito homogenate and then centrifuged at 12 000 **g** for 15 min at 4°C. The supernatants were incubated at 15–30°C for 5 min before 100 µl of chloroform was added. After incubation at 4°C for 15 min, these samples were centrifuged again, the upper phase was collected and the RNA was precipitated by the addition of 500 µl of isopropyl alcohol. After incubation at room temperature for 10 min, the RNA was pelleted by centrifugation, washed once with 75% ethanol, air dried and resuspended in 10 µl of RNase-free water.

Method 3 was performed as described by Vodkin *et al*. (1994). For this method, 100 µl of each mosquito homogenate was mixed with an equal volume of 0.2 M Tris/HCl pH 8.0, which contained 150 mM NaCl, 1% w/v bovine serum albumin and 0.001% w/v phenol red as a pH indicator. A 10 µl aliquot was then mixed with 90 µl of 50 mM Tris/HCl pH 7.4, which contained 5 mM EDTA and 1% w/v sodium dodecyl sulphate (SDS). These samples were then heated at 95°C for 5 min and aliquots were diluted by 1 : 10 in RNase-free water.

Method 4 was performed as described by Pierre *et al*. (1994). For this method, 100 µl of each mosquito homogenate was added to 100 µl of lysis buffer which contained 25 mM sodium citrate pH 7.0, 4 M guanidine isothiocyanate (G-9277: Sigma), 0.5% w/v Sarkosyl (L-9150: Sigma) and 100 mM 2-mercaptoethanol (M-3148: Sigma). Twenty microlitres of 2 M sodium acetate pH 4.0 was then added prior to 615 µl of a mixture of water saturated phenol/chloroform (7 : 3 v/v). This mixture was then placed on ice for 15 min, centrifuged at 3000 r.p.m. at 4°C and the supernatant was removed. The RNA in the supernatant was then precipitated over a period of 60 min at – 20°C after the addition of an equal volume of isopropanol. This mixture was then centrifuged at 10 000 r.p.m. for 20 min at 4°C, the RNA pellet was washed with 75% v/v ethanol, air dried and then resuspended in 10 µl of RNase-free water.

The two-step dengue serotype-specific reverse transcriptase polymerase chain reaction (RT-PCR) was performed as described (Lanciotti *et al*., 1992). Briefly, 10 µl of the products obtained using each RNA extraction method were used in this assay. Ten microlitres of the second-round PCR products and a

100-bp ladder (G2101: Promega, U.K.) were subjected to electrophoresis for 75 min at 100 V on a 2% w/v agarose gel in TAE buffer (40 mM tris-acetate/1 mM EDTA) pH 8.0. These gels were then photographed under ultraviolet light transillumination after staining in a 0.5 μg/ml ethidium bromide solution.

Viral RNA was extracted using method 4 (Pierre *et al*., 1994) from the abdomens of six engorged field-collected adult *Ae. aegypti* (M#1–6), confirmed to be infected with D-2V by IFA (head and thorax) (Romero-Vivas *et al*., 1998), and three human serum samples (S#12, S#21, S#29), confirmed to contain D-2V by the RT-PCR. cDNA of the region which encoded the D-2V E protein was then synthesized from each sample using oligonucleotide primer C2496. The 3′ half of the E gene of each of these nine samples was amplified from this cDNA by PCR using either the S1699/C2496 (M#1 & M#2; 815 bp product) or S1699/C2434 (M#3–6, S#12 & S#29; 750 bp product) primer pairs as described (Sittisombut *et al*., 1997). The full-length envelope protein gene was amplified from serum sample S#21 using primer pair S838/C2434 (1611 bp product) as described (Sittisombut *et al*., 1997). These nine PCR products were then separated by agarose gel electrophoresis, excised and purified by electroelution. PCR products from M#1, M#2 and M#6 were first cloned into plasmid pGEM-T Easy (Promega) to facilitate sequencing. The PCR products from all nine samples were sequenced in each direction using the S1974 and C2260 primers, while the 1611 bp PCR product from sample S#21 was sequenced using the S838, S1151, S1380, S1699, S1974, C1204, C1530, C1763 and C2260 primers as described (Sittisombut *et al*., 1997) using a ThermoSequenase dye-terminator kit (Amersham Life Sciences, U.K.) and an automatic DNA sequencer (ABI 371: Perkin Elmer, U.S.A.).

The DNA sequences were aligned using the CLUSTAL W program available at the Baylor College of Medicine URL: http://www.hgsc.bcm.tmc.edu/SearchLauncher/. The cDNA sequences of 27 D-2V E genes were obtained from GENBANK and phylogenetic relationships between these viruses and the full-length E gene sequence obtained from S#21 (PTCOL96: GENBANK Accession No. AF163096) were determined using DNAML 3.5 (maximum likelihood – J. Felsenstein, obtained from the PHYLIP URL: http://evolution.genetics.washington.edu/phylip.html), PUZZLE (maximum likelihood, quartet puzzling method, – K. Strimmer and A. von Haeseler, obtained from the Pasteur Institute URL: http://bioweb.pasteur.fr/seqanal/interfaces/Puzzle.html) and DNApars with bootstrapping (a parsimony method provided within the PIE suite of programs at the HGMP facility, MRC, U.K.: http://www.hgmp.mrc.ac.uk). Phylogenetic trees were generated using the TreeView software package (Page, 1996).

Results

Pools which contained either 2/10 or 10/10 *Ae. aegypti* infected with each dengue virus serotype were processed to compare four different RNA extraction methods using silica (method 1), Trizol (method 2), sodium dodecyl sulphate (SDS) (method 3) or sodium citrate/guanidine isothiocyanate with Sarcosyl (method 4) when coupled with a dengue virus serotype-specific (two-step) RT-PCR (Lanciotti *et al*., 1992). In this study, cDNA bands were identified in the nested RT-PCR in the mosquito pools containing either 10/10 and 2/10 mosquitoes infected with each dengue virus serotype after RNA extraction was performed using method 4 (Fig. 1A). When the nested RT-PCR was performed on the viral RNA extracted using method 2, weaker serotype-specific cDNA products were observed in most lanes, but in the pools which contained 2/10 mosquitoes infected with D-1V and D-4V, no products were identified. Serotype-specific cDNA bands were obtained only from the pool of 10/10 D-2V-infected mosquitoes using method 1, and only from the pools of 10/10 and 2/10 D-2V and D-3V-infected mosquitoes when the RNA was extracted using method 3. Method 4 was therefore used for the remaining studies.

Our methodology was also able to identify the D-2V serotype in single infected *Ae. aegypti* (Fig. 1B) and, while the addition of fresh human blood to mosquito homogenate did not affect the RT-PCR, the addition of freeze-thawed human blood reduced the intensity of the serotype-specific cDNA bands in this assay. This suggested that the bloodmeals present in the D-2V-infected *Ae. aegypti* collected in Puerto Triunfo could reduce the efficiency of the RT-PCR.

Using the RT-PCR, dengue viruses were detected in 4/31 (13%) serum samples obtained from patients with DF-like illness who lived in (S#12, S#21 & S#26), or immediately outside (S#29), Puerto Triunfo. Each of these RT-PCR positive samples was obtained soon (2 days) after the onset of symptoms and they contained either D-1V ($n = 1$: S#26) or D-2V ($n = 3$: S#12, S#21 and S#29).

With the exception of two silent base substitutions of nucleotide 843 G by A (S#12 & S#29) and nucleotide 1170 T by C (S#21, M#4, M#5 & M#6), the 705 bp which encoded the carboxy-half of the D-2V envelope (E) protein of the six infected mosquitoes (M#1–6) and three human serum samples (S#12, S#21 and S#29) were identical (Table 1). The E gene sequence of the D-2V from patient S#29, who lived 3 km outside the town, was therefore identical to that from patient S#12, who lived in the main urban area of Puerto Triunfo. The lack of sequence diversity in this region of the gene, which is known to exhibit variation between D-2V isolates (Lewis *et al*., 1993), suggested that the D-2 viruses which circulated in and around Puerto Triunfo during the study period were homogenous.

The full-length D-2V E gene sequence (1485 bp) from S#21, selected as the representative genotype (PTCOL96), was then compared with the sequences from D-2 viruses isolated from elsewhere in the world. The PUZZLE maximum likelihood tool (Fig. 2) and two other phylogenetic tools (DNAML 3.5 and DNApars with bootstrapping) (data not shown) placed PTCOL96 in its own distinct outgroup with no closely related sequences among the other 27 D-2V isolates included in the analysis (NB no other full-length E gene sequences of Colombian D-2V isolates were available). A single D-2V sequence from Brazil, L10041Braz was also placed as a single outgroup (Fig. 2) but, as this isolate contained 22 nucleotide

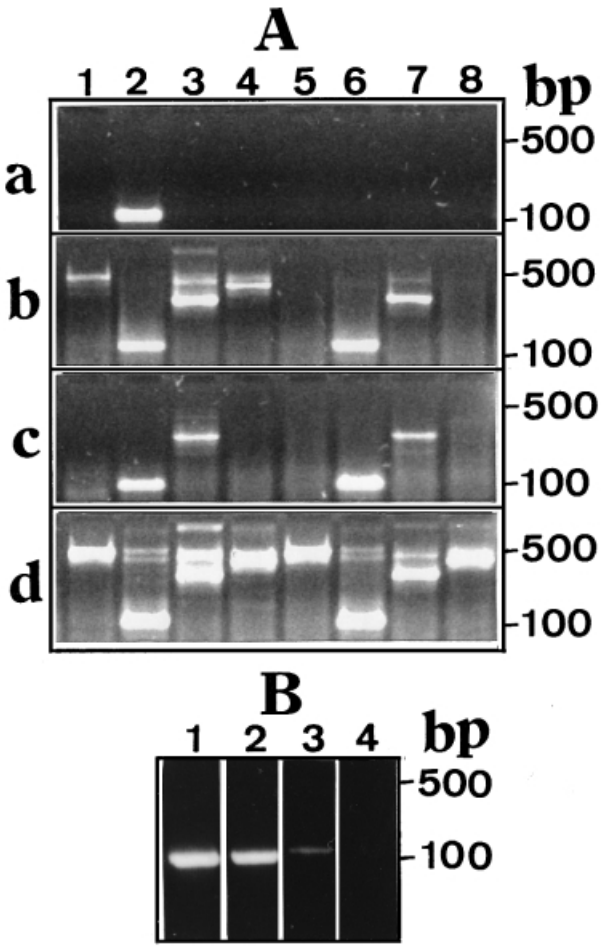

Fig. 1. Comparison of RT-PCR from dengue virus-infected *Aedes aegypti*. (A) Viral RNA was extracted from pools of 10/10 (lanes 1–4) or 2/10 (lanes 5–8) *Ae. aegypti* infected with D-1V (lanes 1 and 5), D-2V (lanes 2 and 6), D-3V (lanes 3 and 7) or D-4V (lanes 4 and 8) using (a) method 1, (b) method 2, (c) method 3 or (d) method 4 , and then the nested RT-PCR was performed. The second-round RT-PCR products were then compared with the expected cDNA products of 482 bp (D-1V), 119 bp (D-2V), 290 bp (D-3V) and 392 bp (D-4V). The location of the 100 and 500 bp markers are shown. (B) Viral RNA was extracted using method 4 from single D-2V-infected *Ae. aegypti* in the absence (lane 1) or presence of either fresh (lane 2) or freeze/thawed (lane 3) human blood, or from an uninfected *Ae. aegypti* (lane 4). The location of the 100 and 500 bp markers are shown.

and three amino-acid differences, it was not closely related to D-2V PTCOL96 (Sutherland *et al*., unpublished). PTCOL96 was clearly distinct from the other D-2 viruses isolated from the same geographical region [e.g. Jamaica (M15075Jmai & M20558Jmai), Trinidad (L10053TRIN), Puerto Rico (L10046PRic) and Peru (AF093674Pe)].

Discussion

An effective RNA extraction method requires nucleases to be immediately inactivated following cell lysis in order to prevent degradation. Guanidine isothiocyanate is the most widely used reagent for this purpose (Chomczynski & Sacchi, 1987). The RNA extraction methods which employed silica (40 min) or SDS (1 h) were rapidly performed, but both of these methods gave relatively poor results in our study. The SDS method may possibly have failed to inactivate the RNase that was found in mosquito homogenates (Lanciotti *et al*., 1992) due to the absence of guanidine isothiocyanate, which was present in each of the other three methods. The poor results obtained using silica (method 1) could therefore either be due to the poor binding of the viral RNA to these particles in this study or to the lower volumes of the mosquito homogenates (10 µl vs. 100–125 µl) used in this method.

Freeze-thawed human blood, but not fresh blood, was found to reduce the efficiency of this RT-PCR, possibly through the release of unidentified substances which inhibited the PCR (Siridewa *et al*., 1996). We collected large numbers of *Ae. aegypti* before screening each of them by IFA (Romero-Vivas *et al*., 1998), which necessitated storing them at – 70°C. As we found that all of the dengue virus-infected adult female *Ae. aegypti* collected in Puerto Triunfo contained a human bloodmeal (Romero-Vivas *et al*., 1998), the freeze/thaw cycles performed on them could therefore be expected to reduce the efficiency of the RT-PCR. Nevertheless, the cDNA sequence of the 3′ half of the E gene was obtained from the abdomens of all six of these D-2V-infected *Ae. aegypti*.

RNA extraction method 4 was successfully used to isolate viral RNA from individual D-2V-infected *Ae. aegypti* collected in this study area of Colombia where the virus population appeared to be homogeneous and only associated with mild disease. The initial analysis described in this study, as well as further analysis, showed that D-2V PTCOL96 was unique among the Old World and New World D-2V virus isolates (Sutherland *et al*., unpublished). Elsewhere, the rapid evolution and dispersal of dengue viruses throughout the world

Table 1. Substitutions in the 3′ half (705 nucleotides) of the envelope (E) gene of D-2 viruses present in DF patients' serum samples and *Aedes aegypti* collected in Puerto Triunfo.

	Nucleotide position[b]	
Sample[a]	843	1170
S#12	A	T
S#21[c]	G	C
S#29	A	T
M#1	G	T
M#2	G	T
M#3	G	T
M#4	G	C
M#5	G	C
M#6	G	C

[a]Serum samples (S#12, S#21 & S#29) or abdomens of *Aedes aegypti* (M#1–6).
[b]Nucleotides were numbered from the ATG initiation codon of the D-2V E gene and aligned using CLUSTAL W.
[c]Designated PTCOL96.

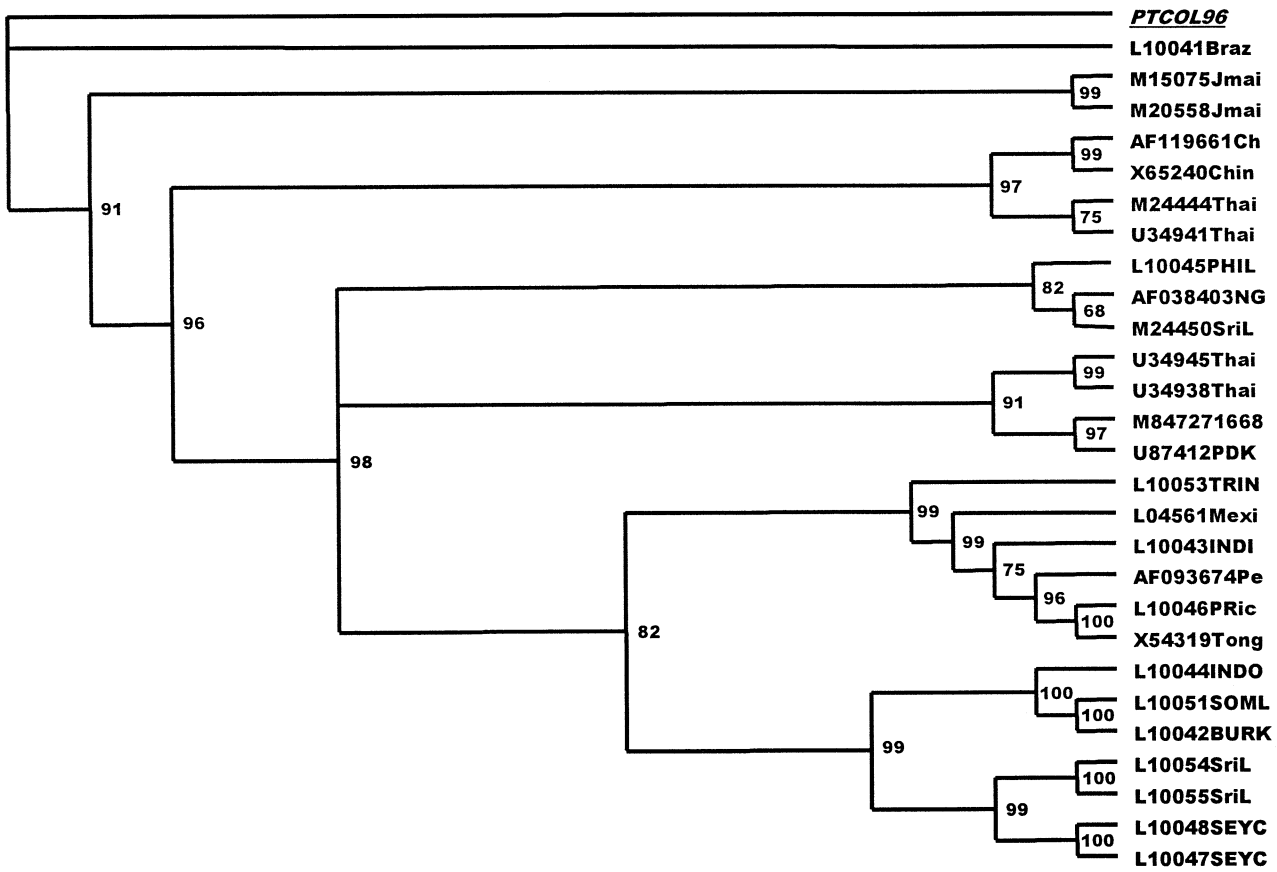

Fig. 2. Phylogenetic tree of the likely relationship between PTCOL96 and 27 D-2 viruses isolated from elsewhere in the world, based on comparisons of the full E gene sequence. Each sequence is designated by its full GENBANK accession number followed by an abbreviation of either its country of origin or strain number. These sequences were analysed using PUZZLE 4.0.1 and the percentage support for each internal branch is shown.

has been associated with a dramatically increased incidence of DHF/DSS, and an estimated 40% of the world's population are now at risk of infection (Gubler, 1998). Whilst flavivirus E gene sequences have been the most widely studied, sequence analyses of other genes or of portions of the E gene (Chungue *et al*., 1995), the E/NS1 junction (Rico-Hesse *et al*., 1997) or the NS3 gene (Chow *et al*., 1994) may, alternatively, be used to compare virus diversity or rapidly identify the origin of a virus in mosquito vectors. These and other regions of the genome (e.g. 3′ non-coding region) may also be used to identify potential virulent strains (Leitmeyer *et al*., 1999) or recombination in flavivirus populations (Worobey *et al*., 1999).

We believe that the single-mosquito RT-PCR method described here, together with our previously described technique for the rapid screening of large numbers of *Ae. aegypti* for flavivirus infections and the identification of dengue virus serotypes in portions of the thorax of positive mosquitoes (Romero-Vivas *et al*., 1998), are important tools for surveillance programmes and epidemiological studies of the dengue viruses and other arboviruses. We are therefore applying these methods to study the dengue viruses in the *Ae. aegypti* populations in southern Vietnam, where we have identified all four dengue virus serotypes associated with DSS during the same year (1998).

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