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Homology of complete genome sequences for dengue virus type-1, from dengue-fever- and dengue-haemorrhagic-fever-associated epidemics in Hawaii and French Polynesia

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Abstract

Dengue epidemic virulence is thought to be conferred by various factors, including the genotype of the virus involved. Increased or decreased epidemic virulence has been associated not only with the introduction of type-2 (DENV-2) strains into the South Pacific, the Caribbean and South America, but also with newly emergent DENV-3 genotypes in Sri Lanka, and the year-to-year variation in the DENV-4 strains circulating in Puerto Rico. These observations indicate that there are inherent differences among viral genotypes in their capacity to induce severe disease, that is, their virulence potential. The present study involved a comparison of the complete genome sequences of DENV-1 viruses that had been isolated from cases of dengue fever (DF) or dengue haemorrhagic fever (DHF) that occurred in French Polynesia or Hawaii in 2001, when a virulent DHF-associated dengue epidemic was occurring throughout the Pacific region. Previous studies have identified putative virulence-associated motifs and substitutions in the DENV-2 genome, and the main aim of the present study was to identify similar changes in DENV-1 that may be associated with viral virulence. As no virulence determinants were seen, however, in any gene or untranslated region, it appears that genotype is not the sole determinant of virulence in DENV-1. Further studies, to compare DF- and DHF-associated strains of DENV-1 isolated from epidemics of variable virulence, in the same eco–biological context, are needed.

Dengue is a mosquito-borne illness found in more than 100 tropical and sub-tropical countries, predominantly in urban and semi-urban areas. The disease is caused by any one of four dengue viruses (dengue virus serotypes 1–4), which are single-stranded RNA viruses of the family Flaviviridae. Approximately 50 million human infections occur annually (WHO, 2002), the majority of which are asymptomatic. Most individuals with symptomatic dengue infection experience dengue fever (DF), an acute febrile illness typically lasting 3–7 days and characterised by non-specific symptoms including headache, fever, maculo–papular rash, retro-orbital pain, and body aches. A small percentage of DF cases develop severe

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disease characterised by vascular permeability, manifested around the time of defervescence, when haemoconcentration and pleural effusion or cardiovascular hypotension are present. This severe disease, known as dengue haemorrhagic fever (DHF), can progress to the most severe form of DHF, dengue shock syndrome (DSS), which can be fatal in the absence of aggressive fluid-replacement therapy. Most deaths occur in children, and no vaccine is currently available.

The dengue genome is a single-stranded positive-sense RNA molecule of approximately 10,700 nucleotides. The genome encodes an uninterrupted open reading frame that is flanked by 5′ and 3′ untranslated regions (UTR) of approximately 94 and 388–462 nucleotides, respectively (Chambers *et al*., 1990). The capsid (C), membrane (M), and envelope (E) structural genes are located at the 5′ end of the genome, and seven non-structural genes (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) are located at the 3′ end. In common with other RNA viruses, the dengue virus lacks a 3′ to 5′ exonuclease proofreading mechanism, and, in consequence, viral replication generates a swarm of diverse but closely related viruses. Adaptive evolution of selected viral lineages over time (Bennett *et al*., 2003) has partly contributed to the predominance of specific genotypes within each serotype (Holmes and Twiddy, 2003), and certain of these genotypes are thought to be associated with greater or lesser epidemic virulence (Rico-Hesse, 2003).

Infection with dengue induces a long-lasting humoral immune response (Fujita and Yoshida, 1979; Okuno *et al*., 1983; Innis, 1997; Imrie *et al*., 2007b) and probably life-long protective immunity to the same serotype. Secondary infections with heterologous serotypes increase the likelihood of severe disease developing (Thein *et al*., 1997). Various mechanisms have been postulated to explain this epidemiological observation, including pre-existing enhancing antibodies (Halstead, 1982) and cross-reactive dengue-specific T cells (Rothman and Ennis, 1999). Infection with dengue virus induces long-lasting, dengue-specific, memory T cells, which may be serotype cross-reactive (Sierra *et al*., 2002; Zivna *et al*., 2002; Imrie *et al*., 2007*a*). In secondary infections, these T cells may be differentially activated, by heterologous dengue-virus epitope peptides, to secrete pro-inflammatory cytokines and other vasodilatory molecules, which mediate the development of vascular leakage. Thus, infection with certain dengue-virus (DENV) strains can stimulate memory T cells to secrete immunopathogenic molecules and thus enhance the likelihood of the development of severe disease.

Severe disease may also occur during a primary infection. In the South Pacific, a virulent epidemic caused by dengue virus of type 2 (DENV-2) occurred in Niue Island in 1972, in a population that had not been exposed to dengue for at least 25 years (Barnes and Rosen, 1974; Rosen, 1977). Observations made during this epidemic and a subsequent mild DENV-1 epidemic in Tonga in 1975, in individuals with primary infection (Gubler *et al*., 1978), indicated that epidemic virulence may be conferred by viral determinants. This hypothesis was later strengthened when the introduction into Central America of the South–east Asian genotype of DENV-2 was associated with the first known DHF epidemic in the region, in Cuba in 1981 (Guzman *et al*., 1995; Rico-Hesse *et al*., 1997). Up until this time, DENV-1, DENV-3 and the American genotype of DENV-2 had co-circulated in Central and South America, and DHF cases had been uncommon (Neff *et al*., 1967; Pinheiro and Nelson, 1997). The DHF that subsequently emerged in South America was associated with the introduction of two distinct South–east Asian strains of DENV-2 into Brazil, Columbia, Mexico and Venezuela, in the late 1980s (Guzman *et al*., 1995; Rico-Hesse *et al*., 1997). In contrast, the American genotype of DENV-2 has only been associated with less virulent disease, and introduction of this genotype into Iquitos, Peru, in 1995, to a population that had been widely exposed to DENV-1 a few years previously, did not result in any cases of DHF, even though the incidence of secondary infection was high (Watts *et al*., 1999).

Associations between genotype and virulence have also been described for DENV-3; the appearance of DHF in Sri Lanka in 1989 corresponded to the emergence of a new DENV-3 virus of subtype III (Messer *et al*., 2003), and the DENV-3 strains circulating during a DHF epidemic in French Polynesia in 1989–1990 were South–east Asian in origin (Chungue *et al*., 1993).

Dengue is a major public-health problem in many Pacific Island nations (Singh *et al*., 2005). In contrast to the situation in hyper-endemic regions, where several serotypes often co-circulate, any dengue virus introduced into a Pacific Island nation generally replaces a serotype that may have previously circulated for several years. There have been six major region-wide outbreaks of dengue since the 1970s, each usually involving a single serotype. The introduction of a new dengue-virus serotype to a susceptible population can result in explosive epidemics associated with severe disease, followed by several years of low-level endemic transmission. In 2001, DENV-1 was re-introduced into French Polynesia, to a population that had not been exposed to this serotype since 1989. A virulent DHF epidemic lasting for about 10 months ensued (www.spc.int/phs/pphsn/Outbreak/Reports/Dengue_report2001-FrenchPolynesia.pdf), and the endemic transmission of DENV-1 in French Polynesia has continued to the present day. Infected travellers returning to Hawaii from French Polynesia in 2001 were among the earliest cases in what became the first dengue epidemic in Hawaii in almost 60 years (Effler *et al*., 2005). Analysis of the full-length E-gene sequences derived from locally transmitted, Hawaiian strains of DENV-1 confirmed the French-Polynesian origin of the dominant epidemic virus (Imrie *et al*., 2006). Although French Polynesia experienced a virulent epidemic, with many cases of severe disease and some deaths, no DHF was observed during the Hawaiian epidemic. In the present study, the complete genome sequences of DF-associated DENV-1 strains isolated during the Hawaii epidemic, and DF-and DHF-associated strains isolated in French Polynesia, were compared, in an attempt to identify the genomic differences or motifs that may be associated with the virulence of DENV-1.

MATERIALS AND METHODS

Ethics Statement

Ethical clearance for the research on human subjects was obtained from the University of Hawaii Institutional Review Board and the Ethics Committee of French Polynesia. Written informed consent was obtained from all study participants.

Viruses

Virus isolates were obtained from acute-phase blood samples collected during epidemiological investigations at the time of the DENV-1 epidemics in French Polynesia and Hawaii in 2000–2002, as previously described (Laille and Roche, 2004; Imrie *et al.*, 2006). Serum or plasma was inoculated onto C6/36 cells. Nine low-passage isolates were obtained from individuals diagnosed with DF or DHF (see Table 1). Sample aliquots were identified as DENV-1 either in IFAT with type-specific monoclonal antibodies, or by PCR and sequencing. Viral RNA was extracted using the QIAmp viral RNA mini kit (QIAGEN, Hilden, Germany)

Primer Design and PCR

PCR primers were designed using Primer3 software (Whitehead Institute for Biomedical Research, MA) based on the Nauru Island strain of DENV-1 (GenBank accession U88535). Primer sequences are available upon request. Synthesis of complementary DNA and subsequent PCR were performed using the QIAgen OneStep RT-PCR kit (QIAGEN).

Complete Genome Sequencing

The products of the OneStep reverse-transcriptase PCR were purified with the QIAquick PCR purification kit (QIAGEN) followed by direct sequencing in an ABI Prism 377 DNA sequencer (Applied Biosystems, Foster city, CA) using forward and reverse primers (available from A.I. upon request) and standard dye-labelling reactions. The complete genome of each isolate was constructed by assembling overlapping fragments of nucleotide sequences, with the Lasergene DNASTAR V5.2 software package (DNAStar, Madison, WI). All the sequence data have been deposited in GenBank.

Phylogenetic Analysis

Maximum likelihood (ML) phylogenetic trees were estimated using PAUP 4.0b10 (Swofford, 2002) under the GTR+G+I model of nucleotide substitution, with the GTR substitution matrix, base composition, gamma distribution of among-site rate variation (G) and proportion of invariant sites (I) all estimated from the data. (The parameter values are available from S.N.B. upon request.) Using the SplitsTree 3.2 software package (University of Tübingen, Tübingen, Germany), recombinant sequences were ruled out based on the absence of networks in a split decomposition analysis for identifying conflicts in the phylogenetic signal (Huson, 1998). To assess the support for the ML tree topologies, bootstrap values based on 1000 resampling events were generated and used to produce neighbour-joining (NJ) trees (under the ML substitution model described above). In addition, 100 ML replicates were generated, using RA×ML BlackBox (Stamatakis *et al.*, 2008). The consensus NJ topology was concordant with the ML topology.

RESULTS

Complete genome sequences were successfully determined for all nine DENV-1 isolates (see Figure) and deposited in GenBank, as accessions DQ672556, DQ 672557, DQ672558, DQ 672559, DQ672560, DQ-672561, DQ672562, DQ672563 and DQ-672564. Five of the isolates came from people who presented with the symptoms of DF during the 2001 DENV-1 epidemic in Hawaii, and the other four from individuals who experienced DF or DHF in French Polynesia during the DENV-1 epidemic that began there in late 2000 (Table 1).

Nucleotide-sequence similarities for the complete genomes, including the 5′ and 3′ UTR, ranged from 95.3%–100%, while amino-acid-sequence similarities for the translated regions ranged from 98.2%–100%. Nucleotide similarities between eight of the nine isolates analysed ranged from 99.9%–100%, reflecting the strong epidemiological linkages among the Hawaiian and Tahitian isolates studied. An earlier French Polynesian virus isolated from a DF case during the low-virulence DENV-1 epidemic in 1989, D1/PF/FP5103/1989 (AY63048), was distinct from the 2001 isolates (Figure), and fell into DENV-1 genotype V.

Strain D1/US/HawO3663/2001 differed significantly from the other eight isolates analysed in the present study, with nucleotide similarities of 95.3%–95.4%. This isolate, which came from a subject who became acutely ill with DF upon returning to Hawaii from Samoa in 2001, represented a minor strain of DENV-1 introduced into Hawaii during the epidemic; the other eight isolates investigated in the present study were either epidemiologically linked to French Polynesia or came from individuals living in French Polynesian.

No DHF-specific changes or motifs were identified for the DHF-associated strains D1/PF/FP0203/2001 or D1/PF/FP1104/2001. Strain D1/PF/FP0203/2001 differed from the consensus sequence at two nucleotide positions, with synonymous C→T substitutions at C_{337} and $NS5_{49}$. The sequence of the other DHF strain, D1/PF/FP1104/2001, was identical to the consensus at all positions, including the 5′ and 3′ UTR.

Three Hawaiian strains (DI/US/HawM2516/2001, D1/US/HawM3430/2001 and D1/US/HawM2540/2001) clustered together and differed from the consensus at E_{1446} (T→C) and $NS5_{2571}$ (C→T); both substitutions were synonymous (see Figure). These viruses were isolated from individuals whose acute illnesses began within a period of 10 days in the September of 2001, and who lived in the Hana area of Maui, where the focus of the Hawaii epidemic was located. The index case is thought to have returned to Maui from French Polynesia in May 2001, and the identity of these three isolates, obtained within a period of 5 days, some 4 months after the introduction of dengue into Maui, therefore reflects a founder effect.

Strain D1/US/HawO3758/2001, with 95.3%–100% nucleotide homology to the other isolates investigated (99.9%–100% when the Samoan strain D1/US/HawO3663/2001 was excluded from the analysis), was isolated from an individual living on the North Shore of the island of Oahu. No epidemiological linkages were identified between Maui and Oahu, and travellers returning from French Polynesia are thought to have introduced DENV-1 directly to Oahu. The 0.1% nucleotide divergence between D1/US/HawO3758/2001 and the three Maui strains supports this assertion, as does the former's distinct position in the phylogenetic analysis (Figure).

Nucleotide substitutions were present throughout the genome of each isolate; 22.1% of the 517 substitutions were in the structural genes, 75.0% were in the non-structural genes, and the remaining 2.9% were in the 3′ UTR. Most (80%) of the substitutions occurred at the third codon position and were silent. Synonymous substitutions in the C, prM, E, NS1, NS3, NS4B and NS5 genes were present in all of the DF-associated strains (see Table 2) and — although it is conceivable that these mutations are in sites that are important for the development of DHF — there were no consistent differences either among DF-associated strains or between the DF- and DHF-associated strains.

No deletions or insertions were noted. A total of 62 amino-acid changes were observed compared with the consensus sequence, occurring throughout the genome, in both structural and non-structural genes. Forty-seven (75.8%) of these changes occurred in the non-structural proteins, and 58 (93.6%) were identified in strain D1/US/HawO3663/2001, occurring throughout the genome. Substitutions were identified in three other strains: DI/US/HawO3758/2001 (prM S28P; E T145N), D1/US/FP0908/2001 (E V250A), and D1/US/FP0705/2001 (NS5 A341S). No DHF-associated changes were observed in any gene or UTR (see Figure).

DISCUSSION

In 2001 dengue was introduced into Hawaii after an absence of 57 years. Epidemiological investigations confirmed that infected travellers returning from French Polynesia, where a major DENV-1 epidemic was occurring, served as index cases on the Hawaiian islands of Maui, Oahu and Kauai. Although the epidemic in French Polynesia was characterised by high frequencies of severe disease, and some deaths, no DHF was identified in Hawaii. In the present study, complete genome sequences of DENV-1 isolated from individuals with DF and DHF in Hawaii and French Polynesia were determined and compared, with the aim of identifying motifs or substitutions that may act as determinants of virulence.

With the exception of the single DF-associated strain isolated from an individual infected in Samoa in 2001 (D1/US/HawO3663/2001), the investigated viruses exhibited a very high degree of similarity, and no DHF-specific changes were identified in any gene or UTR.

Although a small number of isolates was analysed in the present study, the DHF:DF ratio seen in the sample (2 : 7) is representative of the ratio observed in virulent DHF epidemics.

It seems very likely that, among DENV-1 strains of this French Polynesian/Hawaiian lineage, viral virulence is not determined purely by virus genotype but may depend on a complex series of factors, including variation between hosts in response to infection, the mosquito vector, history of previous infections, and the density of the human host population. The major vector in French Polynesia is *Aedes (Stegomyia) aegypti*, whereas the vector in Hawaii in 2001 was *Ae. (S.) albopictus*. In experimental, oral infections of these two mosquito species with DENV-1, the intensities of infection and levels of subsequent transmission have been found to be less for *Ae. albopictus* than for *Ae. aegypti* (Chen *et al.*, 1993). It is therefore possible that the transmission of DENV-1 in Hawaii was less efficient than that in French Polynesia, and that this had a direct impact on disease severity and, consequently, on epidemic virulence.

The other major difference between the French Polynesian and Hawaiian epidemiological contexts concerns the extent of the pre-existing immunity in each setting. The 2001 DENV-1 epidemic in Hawaii involved the first known autochthonous transmission of any dengue virus since 1943, whereas French Polynesia has experienced both mild and severe dengue epidemics in the last few decades, with consistently high incidences of secondary infection. Prior to the introduction of DENV-1, in 2001, DENV-2 had circulated in French Polynesia since 1996, following a 7-year period, beginning in 1989, when only DENV-3 was being transmitted (Deparis *et al.*, 1998). Although the 1996 DENV-2 epidemic in French Polynesia was associated with a high incidence of secondary infection, only a single fatality was recorded, in a 22-year-old man (Murgue *et al.*, 1999, 2000). Overall, this epidemic was not considered to be a virulent one, in contrast to the DENV-3 DHF epidemic of 1989 and the DENV-1 DHF epidemic of 2001 (Chungue *et al.*, 1992b, 1993; www.spc.int/phs/pphsn/Outbreak /Reports/Dengue_report2001-FrenchPolynesia.pdf). In addition, in contrast to the 2001 DENV-1 epidemic, no severe disease was reported during the DENV-1 epidemic of 1988–1989 (Chungue *et al.*, 1992a). What can account for the observed differences in epidemic virulence?

Increased or decreased epidemic virulence has been associated with the introduction of DENV-2 strains into the South Pacific (Gubler *et al*., 1978), the Caribbean (Chungue *et al*., 1992*a*; Rico-Hesse *et al*., 1997) and South America (Watts *et al*., 1999), with newly emergent DENV-3 genotypes in Sri Lanka (Messer *et al*., 2003), and amongst the DENV-4 strains circulating, in different years, in Puerto Rico (Bennett *et al*., 2003). Together, these observations indicate that there are inherent differences among viral genotypes in their capacity to induce severe disease, that is, their virulence potential. Comparison of the American and South–east Asian genotypes of DENV-2 identified virulence-associated substitutions and motifs in the E gene and the 5′ and 3′ UTR (Leitmeyer *et al*., 1999; Cologna and Rico-Hesse, 2003). These DENV-2 studies involved the comparison of the genomes of viruses belonging to different genotypes. Roche *et al*. (2007) compared DENV-3 genomes isolated from individuals who experienced DF or DHF in French Polynesia during the DENV-3 epidemic in 1988–1989, and the present analysis, of DENV-1, involved the comparison of DF-and DHF-associated viruses of one genotype (IV) isolated in different epidemic settings. No motifs or substitutions associated with severe disease were discovered in the investigation by Roche *et al*. (2007) or in the present study.

The putative virulence-associated motifs or substitutions in the E gene described for DENV-2 are not consistently seen in all isolates from individuals with severe disease, and it is very likely that pre-existing host factors ultimately determine clinical outcome. The results of recent research indicate that dengue virulence may be associated with the degree of cross-protective immunity conferred in sequential epidemics with particular serotypes (Adams *et al*., 2006). Given the homology between E-gene antigens, the cross-neutralizing antibodies conferred by prior infection with certain serotypes may diminish disease severity

and, ultimately, epidemic virulence (Kochel *et al*., 2002, 2005). An assessment of the envelope amino acids shared among the French Polynesian DENV-3, DENV-2 and DENV-1 strains may be very informative in this regard, in the context of the varying degree of virulence observed for each of the corresponding epidemics.

In summary, although genotype IV of DENV-1 was associated with severe disease in the French Polynesian epidemic of 2001, comparison of the complete genomes of DF- and DHF-associated isolates of this genotype failed to reveal virulence determinants. The identification of genomic motifs that confer virulence potential in DENV-1 may require a detailed comparison of the French Polynesian strains transmitted during the mild DENV-1 epidemic of 1988 with those transmitted, in the same eco–biological context, during the virulent DENV-1 epidemic of 2001. This work is ongoing.

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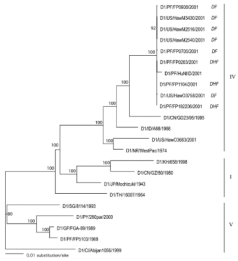

FIG.

A maximum-likelihood (ML) phylogenetic tree of the complete genome sequences from the nine isolates of dengue virus type-1 collected in French Polynesia and Hawaii in 2001. The numbers at nodes represent bootstrap support values. Labels include country, identifier and year of isolation, as well as, for the 2001 clade, whether the isolate was derived from a case of dengue fever (DF) or dengue haemorrhagic fever (DHF). Other isolates included in the tree (and their GenBank accession numbers) are D1/PF/HuNIID/2001 (AB111070), D1/PF/FP192206/2001 (AY630407), D1/CN/GD23/95/1995 (AY373427), D1/ID/A88/1988 (AB074761), D1/NR/WestPac/1974 (DVU88535), D1/KH/658/1998 (AF309641), D1/CN/GZ/80/1980 (AF350498), D1/JP/Mochizuki/1943 (AB074760), D1/TH/16007/1964 (AF180817), D1/SG/8114/1993 (AY762084), D1/PY/280par/2000 (AF514878), D1/GF/FGA-89/1989 (AF226687), D1/PF/FP5103/1989 (AY630408), and D1/CI/Abijan1056/1999 (AF298807).

TABLE 1

Origins and disease classifications of the nine investigated isolates, all of which were circulating in Hawaii or French Polynesia in 2001

Strain	Origin	Disease category	GenBank accession
DI/PF/FP0203/2001	French Polynesia	Dengue haemorrhagic fever	DQ672556
D1/PF/FP0705/2001	French Polynesia	Dengue fever	DQ672557
D1/PF/FP0908/2001	French Polynesia	Dengue fever	DQ672558
D1/PF/FP1104/2001	French Polynesia	Dengue haemorrhagic fever	DQ672559
D1/US/HawM2516/2001	Maui, Hawaii	Dengue fever	DQ672560
D1/US/HawM3430/2001	Maui, Hawaii	Dengue fever	DQ672561
D1/US/HawM2540/2001	Maui, Hawaii	Dengue fever	DQ672562
D1/US/HawO3758/2001	Oahu, Hawaii	Dengue fever	DQ672563
D1/US/HawO3663/2001	Oahu, Hawaii	Dengue fever	DQ672564

Am J Trop Med Hyg. Author manuscript; available in PMC 2013 April 28.

TABLE 2

Nucleotide and amino-acid changes detected between French Polynesian and Hawaiian isolates of dengue virus type 1

			Change seen in isolate from a case of:*							
			Dengue haemorrhagic fever		Dengue fever					
Gene and codon	**Nucleotide position**	**Consensus**	**D1/PF/FP0203**	**D1/PF/FP1104**	**D1/PF/FP0705**	**D1/PF/FP0908**	**D1/US/HawM2516**	**D1/US/HawM3430**	**D1/US/HawM2540**	**D1/US/HawO3758**
C 81L	243	A	A	A	A	A	A	A	A	**G *SYN***
C 113L	337	C	**T *SYN***	C	C	C	C	C	C	C
prM 28S	82	T	T	T	T	T	T	T	T	**C *NS S→P***
PrM 59T	177	G	G	G	G	**A *SYN***	G	G	G	G
E 145E	434	C	C	C	C	C	C	C	C	**A *NS T→N***
E 250V	749	T	T	T	T	**C *NS V→A***	T	T	T	T
E 313A	939	T	T	T	**C *SYN***	T	T	T	T	T
E 482V	1446	T	T	T	T	T	**C *SYN***	**C *SYN***	**C *SYN***	T
NS1 133S	699	C	C	C	**T *SYN***	C	C	C	C	C
NS1 286V	858	T	T	T	T	T	T	T	T	**C *SYN***
NS3 58L	174	C	C	C	C	**T *SYN***	C	C	C	C
NS3 353T	1059	T	T	T	T	T	T	T	T	**A *SYN***
NS4B 74A	222	T	T	T	T	T	**C *SYN***	T	T	T
NS4B 212K	636	A	A	A	**G *SYN***	A	A	A	A	A
NS5 1L	49	C	**T *SYN***	C	C	C	C	C	C	C
NS5 341A	1021	G	G	G	**T *NSA→S***	G	G	G	G	G
NS5 385S	1155	T	T	T	T	**C *SYN***	T	T	T	T
NS5 857T	2571	C	C	C	C	C	**T *SYN***	**T *SYN***	**T *SYN***	C

NS, Non-synonymous substitution; SYN, synonymous substitution.

*For clarity, the year of isolation (2001) has been omitted from each of the strain designations, and no details of strain D1/US/HawO3663/2001, which was quite distinct from all the other viruses, are shown.