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Rapid Communication

Virus evolution during a severe dengue epidemic in Cuba, 1997☆

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Abstract

Full-length genomic sequences from six DENV-2 isolates sampled at different times during a dengue outbreak that occurred in Cuba in 1997 were determined. Phylogenetic analysis indicated that these isolates fall into the "American/Asian" genotype. Genome analysis revealed strong conservation of the structural proteins and the non-coding regions (5′ NCR and 3′ NCR). Nucleotide substitutions were observed in non-structural genes and most notably in the NS5 gene. There was a clear pattern of virus evolution during the epidemic; the earliest isolates sampled differed from those sampled later by amino acid replacements in the NS1 and NS5 proteins, although there was no evidence that these represented escape mutants. Further studies are therefore required to define the functional role of amino acid replacements observed and their possible relation to disease severity.

Keywords: Cuba; Virus evolution; Dengue

Introduction

Dengue viruses cause the most important arthropod-borne viral disease of humans with an estimated 50–100 million infections in 110 countries, resulting in 300,000–500,000 hospitalizations and case fatality rates of 1–5% (Guzman and Kouri, 2003). The dengue virus (DENV) consists of four antigenically distinct serotypes (DENV-1 to 4) assigned to the *Flavivirus* genus (family *Flaviviridae*). The genomes of flaviviruses comprise a single strand of RNA encoding three structural proteins, the capsid (C), premembrane/membrane (PrM/M), and envelope (E), and seven non-structural (NS) proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Rice et al., 1985). Like many other RNA viruses, dengue shows considerable genetic diversity. However, the overall base substitution rate in dengue is less than that observed in other RNA viruses. Some studies have suggested that evolutionary trade-offs inherent in the vector–host association are the most likely explanation for the reduced positive selection in vector-borne RNA viruses (Woelk and Holmes, 2002). Specifically for DENV-2, the stronger positive selection pressure has been observed in the most widely distributed genotype, which may correlate with its dispersal ability. Additionally, some studies indicate that viral genotypes might differ in fitness. In the evolutionary genetics of DENVs, most attention has been directed towards mutations, natural selection, and genetic drift (Holmes and Twiddy, 2003). The dengue epidemiology in the Caribbean island of Cuba has been characterized by well-defined epidemics with the circulation of only one serotype. In 1977, Cuba suffered a DF epidemic caused by DENV-1 that sensitized approximately 44.5% of the population. Four years later, a severe DHF epidemic caused by DENV-2 (of Asiatic origin)

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affected Cuba (Kouri et al., 1989). Afterwards, by 1997, dengue re-emerged in the country, affecting specifically the Municipality of Santiago de Cuba (Guzman et al., 2000a, 2000b; Kouri et al., 1998). This resulted in a severe epidemic of 3012 cases of dengue fever and 205 cases of DHF/DSS, including 12 deaths. Accordingly, Cuba presents a unique epidemiological setting to demonstrate that heterotypic antibodies are able to produce antibody-dependent enhancement 20 years after primary infection. Three key observations characterize the epidemiological history of dengue in Cuba: (i) the association of DHF/DSS with secondary infection in the sequence DENV-1 then DENV-2 (that is, with the presence of heterologous DENV-1 antibodies) (Guzman et al., 1990, 1999, 2000a, 2000b), (ii) the presence of DENV-2 strains with the potential to produce DHF/DSS, particularly those imported from Asia (Guzman et al., 1995; Kouri et al., 1998), and (iii) increased clinical severity through time during the epidemics expressed by a higher ratio of DHF to DF towards the end of the epidemic (Guzman et al., 2002). Because heterotypic antibodies could selectively favor genotypic changes in the virus, resulting in the release of escape mutants and in turn the observation of increased severity through time (Guzman et al., 2000a), of particular importance was the study of the envelope gene sequence from DENV-2 isolates obtained at different time points in the 1997 epidemic. However, contrary to our expectation, no changes were observed in this gene in twenty studied isolates (Rodriguez-Roche et al., in press). Taking into account this result, we decided to analyze the complete genome sequence of six of these isolates. In particular, we wished to determine whether or not the increased severity observed in the epidemic could be correlated with particular changes in the genetic make-up of the virus.

Results

The most significant observation of the full-length viral genomes from the Cuban DENV-2 strains was the remarkable conservation of the structural proteins and of the non-coding regions (5′ NCR and 3′ NCR); no mutations were observed during the sampling period. In contrast, it was possible to define a clear pattern of sequence evolution in the non-structural genes. In particular, the two strains from the beginning of the 1997 epidemic (13/97 and 58a/97) were genetically different from the four strains collected 4 or more months later; substitutions at NS1 (position 2911), NS2a (position 3894), and NS5 (positions 7947, 8868, 9471) reflect the divergence between initial and final isolates (Table 1). The nonsynonymous substitution at NS1 (position 2911) generated a conserved amino acid change Thr/Ser, making it only one of two amino acid changes seen during the epidemic. The second amino acid change occurred in the last isolate sampled (205/97), in which an Asn/Ser amino acid replacement was observed in the NS5 gene (position

Table 1
Summary of mutations among the complete genome of Cuban DENV-2 isolates relative to the strain Martinique/98

Isolates	Date of sample collection	Position																	
		C	NS1			NS2A	NS2B		NS3		NS4A	NS4B	NS5						
		480	2911	3123	3130	3894	4356	4407	5211	5958	6612	7356	7947	8430	8556	8868	9471	9696	9758	9822	9942
Mart/98	–	U	A	U	U	U	A	A	A	C	G	C	G	G	A	G	C	U	A	G	U
13/97	2	–	–	–	–	C	–	–	–	–	–	–	U	–	–	–	–	–	–	–	–
58a/97	5	–	–	–	–	C	–	–	–	–	A	U	U	–	–	–	–	–	–	–	–
89/97	23	–	U[a]	–	–	–	–	–	–	G	A	U	–	–	–	A	U	–	–	–	–
115/97	26	–	U[a]	–	–	–	G	G	G	–	–	–	–	–	–	A	U	–	–	–	–
165/97	29	–	U[a]	C	C	–	–	–	–	–	–	–	–	–	U	A	U	C	–	–	–
205/97	34	C	U[a]	–	–	–	–	–	–	–	–	–	–	A	–	A	U	–	G[a]	A	C

The date of sample collection is defined as epidemiological weeks.
[a] Mutations resulting in amino acid changes. Positions are numbered sequentially from the first position in the 5′NCR.

9758). Indeed, it is notable that approximately 50% of the nucleotide substitutions were found in the NS5 gene.

The ML phylogenetic analysis for 47 DENV-2 sequences revealed that the six Cuban isolates formed a tight cluster with strong bootstrap support (Fig. 1). Moreover, there was a clear division within the Cuban strains, with the isolates sampled later in the epidemic forming a separate phylogenetic group (82% bootstrap support), indicating that viral evolution had occurred during the epidemic period. Moreover, the Cuban isolates fell closer to another strain isolated from the Caribbean at a similar time–Martinique/98 (all of which cluster within the Asian/American genotype, as defined by Twiddy et al., 2002)–and some distance from the DENV-2 strains that are associated with DF in mainland South America (including I348600 from Colombia) that are members of the American genotype. Hence, the strain of DENV-2 that affected Cuba in 1997 was one that was circulating more widely in the Caribbean region in the preceding years, with its ultimate origins in Asia (Twiddy et al., 2002).

Discussion

Previous studies from the Santiago de Cuba epidemic confirmed that secondary infection was present in 98% of

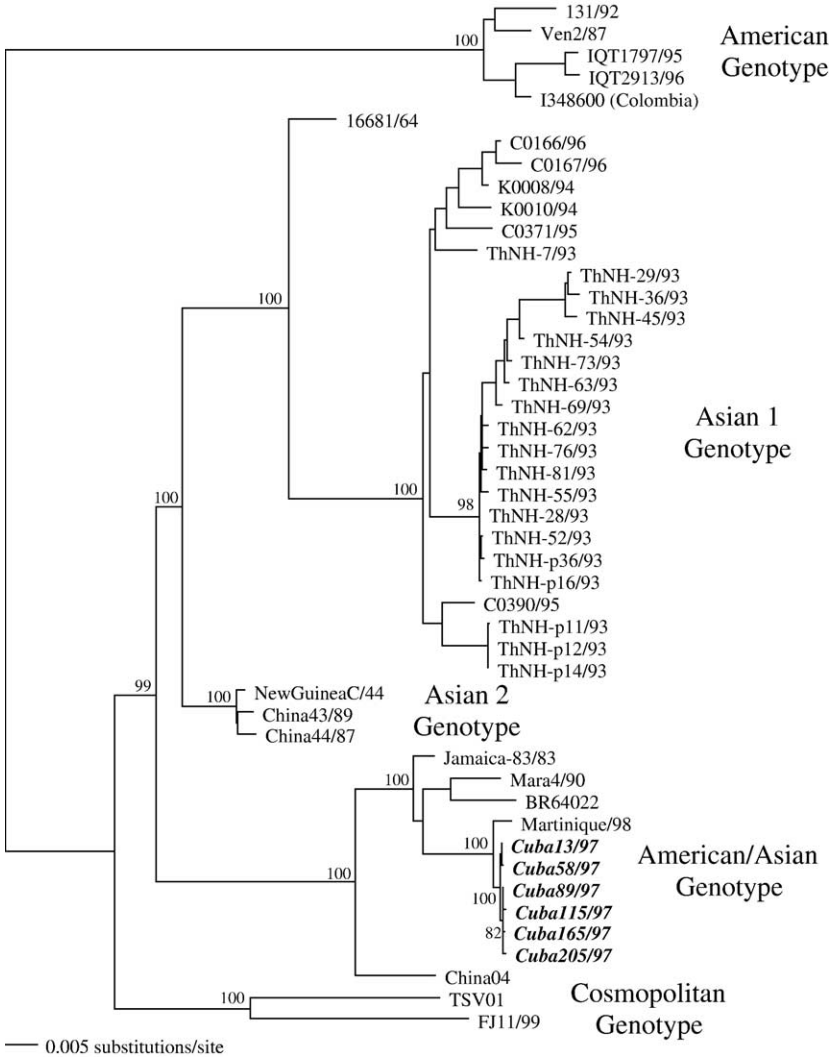

Fig. 1. Maximum likelihood (ML) phylogenetic tree showing the evolutionary relationships among the complete (coding) genome sequences of 47 strains of DENV-2. Bootstrap support values are shown for key nodes only and the genotype designations (according to Twiddy et al., 2002) are given. The tree was mid-pointed rooted for purposes of clarity only and all horizontal branch lengths are drawn to scale. GenBank accession numbers of the other DENV-2 strains used in this analysis are as follows: BR64022 (AF489932), China 04 (AF119661), China 43 (AF204178), China 44 (AF204177), C0166/96 (AF100463), C0167/97 (AF100464), C0371/95 (AF100461), C0390/95 (AF100462), F11/99 (AF359579), IQT1797/95 (AF100467), IQT2913/96 (AF100468), Jamaica 1409 (M20558), K0008/94 (AF100459), K0010/94 (AF100460), Mara4/90 (AF100466), Martinique/98 (AF208496), New Guinea C (M29095), ThNH81/93 (AF169688), ThNH76/93 (AF169687), ThNH73/93 (AF169686), ThNH69/93 (AF169685), ThNH63/93 (AF169684), ThNH62/93 (AF169683), ThNH54/93 (AF169682), ThNH55/93 (AF169681), ThNH45/93 (AF169680), ThNH36/93 (AF169679), ThNH29/93 (AF169678), ThNH-p36/93 (AF022441), ThNH-p16/93 (AF022440), ThNH-p14/93 (AF022439), ThNH-p12/93 (AF022438), ThNH-p11/93 (AF022437\), ThNH52/93 (F022436), ThNH28/93 (AF022435), ThNH7/93 (AF022434), TSV01 (AY037116), Ven2/87 (M32971), 131/92 (AF100469), 16681 (U87411).

the 205 DHF/DSS cases (Guzman et al., 1999). According to one hypothesis, more virulent strains of DENV might appear as neutralization-escape mutants during the course of an epidemic (Guzman et al., 2000a). Noticeably, the nucleotide sequence of the six isolates obtained at different time points of the 1997 Cuban epidemic showed strong conservation in the structural genes. In this sense, similar results were obtained previously, studying the E gene in a collection of 20 DENV-2 isolates from the same epidemic (Rodriguez-Roche et al., in press). Therefore, at least in this study, antibody-driven selection of escape mutants in the structural genes cannot be the key selective force. In contrast, various nucleotide substitutions were found in the non-structural genes, although most were synonymous. These mutations generally correlate with the time of sampling; five substitutions allowed us to divide the isolates into two groups, representing the beginning (January–February) and the later part of the epidemic (June–July). One of these five nucleotide substitutions produced a conservative amino acid replacement (Thr/Ser (residue 164) in the NS1 protein, although its biological significance is unclear (Lindenbach and Rice, 1999). Additionally, the alignment of the NS1 protein sequence of these latest isolates with available sequences in GenBank revealed that the region of the NS1 protein containing this amino acid replacement is highly conserved. DENV-2 strains usually have Thr at this position with the exception of the Thailand strain PUO-280 that belongs to the Asian I genotype and which has Ser, like the latest Cuban isolates (Blok et al., 1991). Finally, it has been proposed that strains of DENV-2 may differ significantly in the structure of genomic viral RNA 3′NCR and that these structural differences correlate with virulence (Leitmeyer et al., 1999). However, the DENV strains sampled here were invariant in both the 3′NCR and 5′NCR despite the different clinical patterns observed.

Despite the low numbers of mutations observed here, it is important to note that the sequences compared represent a consensus of those observed within each patient, in other words, low frequency viruses including those that might affect virulence would not be sampled. Moreover, the methods used to isolate the viruses could also disturb the distribution of variants in the original clinical sample. This is particularly important given that studies of dengue virus populations sampled from within individual humans and mosquitoes have revealed large amounts of sequence variation (Wang et al., 2002). Unfortunately, original clinical samples were not available for this study. The most variable strain was 205/97, isolated from a fatal case at the end of the epidemic. This strain had accumulated ten changes, one resulting in the amino acid replacement Asn/Ser (residue 730) that is specifically located in the carboxyl half of the gene identified as the RNA-dependent RNA polymerase (RdRp) domain of NS5. However, this isolate was obtained from a spleen sample (not from serum). It is therefore possible that the viral population in this tissue differs from the isolates obtained from sera. Furthermore, it is noteworthy that Ser at this position in NS5 is unusual for DENV, although it is found in other flaviviruses such as St. Louis encephalitis, Ilheus, Stratford, and Kokobera (Kuno et al., 1998). Since isolates from patients with different clinical pictures were included and the amino acid replacement in the NS5 protein was observed only in the last isolate, no conclusions can be drawn about its contribution to disease severity.

In summary, a clear pattern of viral evolution was documented over the course of the 1997 Cuban DENV-2 epidemic, although whether or not the observed changes were fixed by natural selection or genetic drift was uncertain. Additionally, it is important to note that cytotoxic T-lymphocytes (CTLs) play a crucial role in controlling infection in RNA viruses, including dengue (Mongkolsapaya et al., 2003). Variation in epitopes recognized by CTL is common and frequently offers potential escape routes for mutant virus. Hence, forthcoming studies should assess whether the mutations in NS1 and NS5 observed here fall into antibody or CTL epitopes. This is undoubtedly an area where future research would be highly fruitful.

Materials and methods

Four serum samples and two macerated spleen pieces (Table 2) were used to infect C6/36 HT continuous *Aedes albopictus* cells by rapid centrifugation assay as described by Rodriguez-Roche et al. (2000). All the isolates were obtained from the same municipality covering the whole epidemic period (from January to July). After virus

Table 2
Details of DENV-2 isolates analyzed in this study

Isolates	Date of fever onset	Source of isolation	Passage histories	Clinical classification	Type of infection[a]
13/97	30/1/1997	Serum	2P C6/36	DF	Primary
58a/97	5/2/1997	Serum	2P C6/36	DF	Primary
89/97	**1/6/1997**	**Spleen**	**2P C6/36**	**DHF/DSS**	**Secondary**
115/97	10/6/1997	Serum	2P C6/36	DF	Unknown
165/97	12/6/1997	Serum	1P C6/36	DHF	Secondary
205/97	**1/7/1997**	**Spleen**	**1P C6/36**	**DHF/DSS**	**Secondary**

[a] The type of infection was determined by plaque reduction neutralization test using convalescent sera. Fatal cases are in bold.

detection using an indirect immunofluorescence assay, viral RNA was extracted from 200 μl of supernatant medium of virus-infected cells using the RNAgents Total RNA Isolation system (Promega). For some samples, a second passage in C6/36 HT was needed. First strand cDNA synthesis was carried out according to the protocol described by Gritsun and Gould (1995). A sample of 3 μl of the cDNA from the RT reaction was used for PCR amplification by 30 cycles of denaturation at 94 °C (40 s), annealing at 55 °C (1 min), and extension at 72 °C (1 min). A final extension step was carried out at 72 °C for 10 min. Taq DNA polymerase (Sigma) was used. The primers used for PCR were designed on the basis of published DEN virus sequences. Five overlapping PCR fragments named I, II, III, IV, and V were obtained to sequence the complete genome of the viruses. The sequences of the primers are available from the authors on request. PCR products were directly sequenced after purification. Double-stranded sequencing was performed on an ABI sequencer using the manufacturer's protocol (Applied Biosystems, Foster City, USA). 31 forward and 31 reverse primers designed on the basis of Jamaica1409 DENV-2 strain were used for sequencing. Cycle sequencing was performed as follows: 25 cycles at 96 °C (30 s), 50 °C (60 s), and 60 °C (4 min). The sequencing reaction was purified using Centri-Sep Spin columns (Applied Biosystems, Foster City, USA).

To infer the evolutionary history of the 1997 DENV-2 viruses in Cuba, a phylogenetic analysis was conducted using polyprotein coding regions from six Cuban isolates and a single DENV-2 isolate sampled from Colombia during 1986 (strain I348600) sequenced in this study, as well as 40 complete DENV-2 genomes taken from GenBank. This produced a total data set of 47 viral isolates 10176 nucleotides in length. Maximum likelihood (ML) phylogenetic trees were estimated using the general time-reversible model (GTR) of nucleotide substitution, with the GTR substitution matrix, the base composition, the gamma distribution of among-site rate variation (Γ), and the proportion of invariant sites (I) all estimated from the data (Posada and Crandall, 1998; Rodriguez et al., 1990). To assess the robustness of particular phylogenetic groupings, a bootstrap analysis was undertaken using 1000 replicate neighbor-joining (NJ) trees using the ML substitution matrix described above. All analysis were performed using the PAUP* package (Swofford, 2003).

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