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Original article

Dengue-3 virus genomic differences that correlate with *in vitro* phenotype on a human cell line but not with disease severity

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Abstract

We compared the full genome sequence of nine clinical isolates of dengue virus obtained during an epidemic of dengue-3 in French Polynesia in 1989, from patients with various presentations of disease. The isolates, all belonging to Genotype I, had 25 amino acid substitutions. There was no association with disease severity. When cultured in the K562 human erythroleukemia cell line, the isolates induced a range of cell growth inhibitions that was not associated with the degree of disease severity. By contrast, some substitutions – charge changes in NS1 and NS5, side-chain differences in NS1, loss of the E-153 potential glycosylation site, and 11 nucleotide insertions in the 3′UTR – that have been suggested to result in an increase or attenuation of dengue infection, appeared to be associated with the level of inhibition. These data represent the first documented study of an association between differences in the full dengue-3 genome of clinical isolates and the *in vitro* phenotype of these isolates on a human cell line.

Keywords: Dengue; Dengue hemorrhagic fever; Severity; Pathogenicity; Molecular sequencing data; Viral genome; French Polynesia

1. Introduction

Dengue viruses (family *Flaviviridae*, genus *Flavivirus*) are positive-strand RNA viruses that are mainly transmitted by the mosquito *Stegomyia* (=*Aedes*) *aegypti*. There are four genetically and antigenically distinct serotypes: DEN-1, DEN-2, DEN-3, and DEN-4. The single-stranded RNA genome of about 10,700 nucleotides encodes a single polyprotein arranged in the order, 5′-C-prM(M)-E-NS1-NS2a-NS2b-NS3-NS4a-NS4b-NS5-3′. Flaviviruses enter cells by receptor-mediated endocytosis and low pH-induced fusion. The dengue virus envelope glycoprotein binds to target cells by a specific receptor–ligand mediated interaction independent of immunoglobulin Fc determinants. This envelope protein also mediates viral entry by membrane fusion initiated by the low-pH conformational change leading to formation of fusion-competent trimers [1].

Most symptomatic dengue infections are self-limiting, with abrupt onset of fever, headache, retro-ocular pain, muscle and joint pain, nausea, vomiting, and a rash that may be associated with moderate hemorrhagic manifestations. However, infections can be life threatening in a condition with a range of manifestations that include shock syndrome [2,3], neurological disorders [4,5], severe hepatic involvement [6–8] and severe haemorrhage [2,7,9,10]. In most cases, the shock syndrome results from a plasma leakage phenomenon, caused by an increase of vascular permeability, associated with a circulatory failure.

The factors that lead to severe manifestations are probably complex, involving the interaction of different factors related

Abbreviations: DF, dengue fever; DHF, dengue hemorrhagic fever; DSS, dengue shock syndrome; FCS, foetal calf serum; RT-PCR, reverse transcriptase polymerase chain reaction; $TCID_{50}$, 50% tissue culture infectious doses.

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to the viral strain, to the viral load, to the host, and to the vector [11–13]. Previous studies suggested that differences in virulence between dengue isolates could play a role in disease severity, especially in the occurrence of the plasma leakage phenomenon. We have previously shown that clinical isolates of dengue-3 virus have an *in vitro* inhibitory effect on human haematopoietic progenitor cell growth from fresh cord blood mononuclear cells, that was not related to the level of virus replication but was dependent upon the clinical isolate tested; e.g. clinical isolate from patients with severe manifestations had a stronger inhibitory effect compared to those with non-severe dengue [12,14]. We also found that the inhibitory effect induced by the different isolates was dependent upon the cord blood sample used (unpublished data) suggesting the influence of host factors. To avoid the variation of susceptibility to virus infection from donor to donor, we tested the inhibitory effects of nine clinical isolates of DEN-3 on the human K562 erythroleukemia cell line that supports dengue virus infection [15]. Finally we sequenced the entire genome of the nine DEN-3 isolates to explore the possible relationship between their sequences and the degree of disease severity as well as their *in vitro* phenotypes in the K562 cell line.

2. Materials and methods

2.1. Clinical isolates and working stocks

Clinical isolates were obtained from nine children hospitalised with an acute dengue infection during a DEN-3 epidemic in French Polynesia in 1989 [16]. Table 1 lists the characteristics, clinical data and infection history of the isolates and their GenBank accession numbers.

We assessed the severity of disease as described by Murgue et al., 1999 [7]; using (i) the World Health Organization criteria [17] to characterize patients with evidence of plasma leakage (referred as dengue hemorrhagic fever: DHF) that leads on some occasions to a shock syndrome (referred as dengue hemorrhagic fever/dengue shock syndrome: DHF/DSS); and (ii) other clinical (haemorrhage, shock without plasma leakage, neurological manifestations, etc.) and biological (hepatic disorders, severe thrombocytopenia) criteria. Patients without evidence of plasma leakage were classed as dengue fever (DF) in accordance with the WHO definition. By these criteria, six isolates were obtained from patients with severe manifestations: three of these were classed as DHF/DSS (two fatal); one as DHF; two as DF (one fatal). Three isolates, all DF, were obtained from patients who presented non-severe or moderate disease. Six patients (two DHF/DSS, one DHF and three DF) had primary infections, two (one DHF/DSS and one DF) were secondary, and the history of one (DF) was uncertain.

For the working stocks, acute plasma samples were first inoculated into C6/36 *Aedes albopictus* cells and then passaged 2–6 times in this cell line or in *Aedes pseudoscutellaris* AP61 cells to generate working stocks. Virus stocks were prepared as follows: monolayers of C6/36 cells were grown in culture medium (RPMI 1640 medium [Sigma–Aldrich], 10% foetal calf serum [FCS], 1× non-essential amino acids, 50 μg/mL of gentamicin, and 2.5 μg/mL of amphotericin B), to 90% confluency in 25 cm^2 flasks, then inoculated with isolates, and incubated for 1 h at 28 °C in an atmosphere of 5% CO_2. The inoculum were discarded and flasks were supplemented with

Table 1
Characteristics, clinical data and infection history of DEN-3 cases

Strain	Passage history	Location	Year	Severity criteria	WHO classification/infection history	GenBank accession no.
PF89/27643	C6/36 5	Tahiti[a]	1989	Hepatic disorders, thrombocytopenia, haemorrhage, shock without plasma leakage, persistent high fever	DF[b]/I	AY744677
PF89/320219	C6/36 3	Tahiti	1989	Plasma leakage and shock	DHF/DSS/II	AY744678
PF90/3050	C6/36 1, AP61 2	Tahiti[a]	1990	Neurological symptoms	DF/I	AY744679
PF90/3056	C6/36 1, AP61 3	Tahiti[a]	1990	None	DF/–	AY744680
PF90/6056	C6/36 4, AP61 2	Tahiti[a]	1990	None	DF/I	AY744681
PF92/2956	C6/36 1, AP61 1	Tahiti[a]	1992	Plasma leakage and shock	DHF/DSS[b]/I	AY744682
PF92/2986	C6/36 1, AP61 1	Tahiti	1992	Hepatic disorders, thrombocytopenia	DF/II	AY744683
PF92/4190	C6/36 1, AP61 2	Tahiti[a]	1992	Plasma leakage and shock	DHF/DSS[b]/I	AY744684
PF94/136116	C6/36 4, AP61 1	Raiatea	1994	Haemorrhage	DHF/I	AY744685
H-87	–	Philippines	1956	–	Ref	M93130
80-2	–	China	1980	–	–	AF317645
1243	–	Martinique	1999	–	DF/–	AY099337
1266	–	Sri Lanka	2000	–	DF/–	AY099336

C6/36, *Ae. albopictus* cell line; AP61, *Ae. pseudocutellaris* cell line.
DF, dengue fever; DHF, dengue hemorrhagic fever; DHF/DSS, dengue hemorrhagic fever/dengue shock syndrome; Ref, reference strain; I, primary in fection; II, secondary infection; –, not determined.
[a] Patients hospitalised at the Centre Hospitalier Territorial de Tahiti.
[b] Deceased patients.

10 mL of maintenance medium (as culture medium but with 1% FCS) and maintained at 28 °C in an atmosphere of 5% CO_2. Infection was monitored 7 days post-infection by an indirect fluorescent antibody test and cell supernatants were harvested when more than 90% of the cells expressed dengue viral antigen. These supernatants were precipitated using 7% poly-ethylene-glycol 8000 (Sigma, St. Louis) and the precipitate was reconstituted with RPMI medium. The method of Reed and Muench [18] was used to calculate the tissue culture infectious doses/mL ($TCID_{50}$/mL) of the precipitate supernatants, expressed as the number of 50% infectious doses per millilitre of serum. Virus titres, determined on C636 cells ranged from 1.0×10^6 to 1.6×10^9 $TCID_{50}$/mL. Prototype DEN-3 (H-87), and passages of AP61 cell culture fluid from a dengue negative serum (virus negative control stock) were prepared by the same procedure.

Four additional complete sequences of DEN-3: Philippines 1956 (strain H-87), China 1980 (strain 80-2), Martinique 1999 (strain 1243), and Sri Lanka 2000 (strain 1266), were retrieved from GenBank.

2.2. K562 cell line assay

The continuous cell line K562 was kindly provided by Prof. Alice Dautry Varsat, Institut Pasteur Paris, France. Cells were maintained in RPMI 1640 medium (Sigma–Aldrich) with 10% FCS and grown in 100% humidity, 5% CO_2 at 37 °C.

DEN-3 viruses were incubated in K562 cells at multiplicity of infection of 10^{-1} $TCID_{50}$/cell in RPMI, for 2 h at 37 °C, 5% CO_2. The cells were then harvested and washed twice in RPMI 1640 medium. Thereafter, viable cells were counted in trypan blue under microscope in a Malassez counting chamber, and 5×10^4 cells were plated in duplicate in 35 mm dishes in 1 mL of RPMI 1640 medium supplemented with 10% FCS. Cultures were incubated at 37 °C, 5% CO_2 for 7 days, after which K562 cells were harvested. The percentage of inhibition induced by all isolates was determined by comparing the number of viable cells after 7 days culture with the number of preculture cells. Results were expressed as the mean ± standard error of the mean. Cell growth inhibition was assessed by analysis of variance (ANOVA) with the usual statistical significance at the 95% level ($p < 0.05$).

2.3. RNA extraction and RT-PCR

RNA was extracted from 140 μL of cell culture supernatant by using the QIAamp® Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

RT-PCR was performed using the QIAGEN® OneStep RT-PCR Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. For this, 10 μL of RNA was added to 40 μL of a mix containing 5× QIAGEN OneStep RT-PCR Buffer with 12.5 mM $MgCl_2$, 400 μM of each dNTP, 0.6 μM of primer sense, 0.6 μM of primer antisense, 10 units of rRNasin® Ribonuclease Inhibitor (Promega, Madison, USA) and QIAGEN OneStep RT-PCR Enzyme Mix (Qiagen, Hilden, Germany) included Omniscript and Sensiscript Reverse Transcriptase, and HotStarTaq DNA Polymerase. The RT was performed at 50 °C for 30 min followed by 15 min at 95 °C to activate HotStarTaq DNA Polymerase. PCR conditions were 30 cycles at 94 °C for 30 s, at 51–59 °C for 1 min according to primer pair characteristics, and at 72 °C for 1 min. A final extension was performed at 72 °C for 10 min.

Primer pairs generating 10 overlapping PCR fragments were required to elicit the complete genome. Their positions are given in Table 2.

PCR products were purified using either QIAquick PCR Purification Kit or QIAquick Gel extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The purified PCR products were used for direct sequencing.

2.4. Sequencing strategy

The nucleotide sequencing was performed by the ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Warrington, UK) as described in the manufacturer's protocol. For each sequencing reaction, approximately 60 ng of purified DNA from direct PCR product or sliced-gel band, were combined with 3.2 pmol of primer and the Terminator Ready Reaction Mix containing the four dye-labeled deoxynucleotide terminators. Sequencing conditions were 30 cycles at 95 °C for 30 s, at 50 °C for 15 s, and at 60 °C for 1 min. The reaction mixture was column purified with DyeEx™ (Qiagen) and the DNA was dried. The pellet was re-suspended in 20 μL of template suppression reagent,

Table 2
Oligonucleotide primers used for RT-PCR

Primer pairs	Nucleotidic position[a] of forward (f) primers	Nucleotidic position[a] of reverse (r) primers	PCR fragment length (bp)
CAP5′f-E/1184r	1–15	1169–1184	1184
E/1038f-NS1/2467r	1039–1055	2450–2467	1430
E/2088f-NS1/3087r	2088–2104	3070–3087	1000
NS1/2891f-NS2A/4074r	2891–2908	4057–4074	1184
NS2A/3892f-NS3/5497r	3892–3910	5480–5497	1606
NS3/5431f-NS4A/6472r	5431–5450	6452–6472	1042
NS3/6415f-NS5/7834r	6415–6432	7817–7834	1420
NS5/7714f-NS5/9097r	7714–7732	9077–9097	1384
NS5/8189f-NS5/9619r	8189–8209	9602–9619	1431
NS5/9524f-TERM3′r	9524–9541	10676–10696	1184[b]

[a] Genome positions are given according to the published sequence of H-87 reference strain.
[b] The length corresponds to PF strains, including the insertion of 11 nucleotides.

heated for 2 min at 95 °C, chilled and kept at −20 °C until it was loaded on the automated sequencer, an ABI Prism 310 (Perkin-Elmer, Applied Biosystems).

2.5. Sequence and phylogenetic analyses

Overlapping nucleic acid sequences were assembled to generate a consensus sequence translated into amino acid sequences. The nucleotide and amino acid sequence alignments were generated using the multiple sequence alignment ClustalW 1.83.XP software. Phylogenetic analyses were conducted using MEGA version 2.1 software [19], the Jukes–Cantor algorithm for pairwise distance determination and the neighbor-joining method for tree drawing. The robustness of the resulting groupings was tested by 500 bootstrap replications.

3. Results

3.1. Effects of DEN-3 isolates on the growth of K562 cell line

The inhibition induced by French Polynesian isolates of DEN-3 and by the prototype H-87 strain on K562 cell growth ranged from 3% to 32% (Fig. 1). This enabled us to distinguish three groups of isolates with significant differences in the mean value of inhibition cell growth among them ($p < 0.00001$): (i) a group of isolates inducing a high (>25%) inhibitory effect; (ii) a group inducing a moderate (>10% and <15%) effect; and (iii) a group inducing a low (<10%) effect. The first group was significantly different from the second one ($p < 0.0001$), itself significantly different from the third one ($p = 0.0019$). The group inducing a low inhibitory effect was significantly different from the dengue negative control ($p = 0.0003$) but not from the prototype strain H-87.

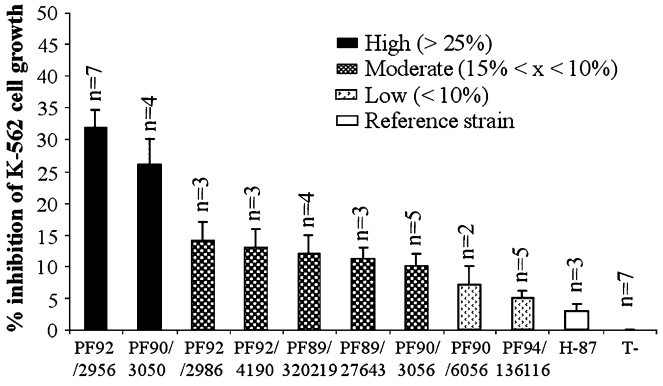

Fig. 1. Inhibition of the growth of K562 cells after infection with DEN-3 isolates and H-87. K562 cells were not infected (negative control) or infected with isolates of DEN-3 or with the prototype strain H-87 (as reference) at multiplicity of infection of 10^{-1} $TCID_{50}$/cell. See Tables 1 and 2 for characteristics of the isolates and of the dengue cases. The percentage of inhibition induced by DEN-3 French Polynesian isolates on K562 cell growth ranged from 5% to 32%. The three groups of isolates; (i) black = high inhibitory effect (>25%); (ii) grey = moderate inhibitory effect ($15\% < x < 10\%$); (iii) picted white = low inhibitory effect (<10%), had significant differences ($p < 0.00001$) in their inhibitory effect. The inhibitory effect induced by the prototype strain H-87 is not significantly different from the group with a low effect. Error bars = SE and T− = negative control.

The group of isolates with a high inhibitory effect comprised a primary, fatal DHF/DSS isolate (PF92/2956), and a primary, non-severe DF isolate (PF90/3050). Of the isolates that induced moderate inhibition, there was one primary, fatal DHF/DSS (PF92/4190) case; one primary, fatal DF (PF89/27643); one secondary, DHF/DSS (PF89/320219); one secondary, severe DF (PF92/2986); and one non-severe DF case of unknown infection history (PF90/3056). Finally, within the group inducing low inhibition, there was one primary DHF (PF94/136116), one primary, non-severe DF (PF90/6056) case and the prototype H-87.

3.2. Nucleotide and amino acid sequence homology

The complete sequence of the nine French Polynesian DEN-3 isolates was compared with the four complete sequences available in GenBank. Distance data for nucleotides and amino acid sequences are presented in Table 3.

The nucleotide sequences of the French Polynesian isolates were very similar, with less than 0.7% of divergence between the furthest strains, PF94/136116 and PF90/3050, and 0.09% of divergence for the closest, PF89/27643 and PF90/3056. The amino acid sequences were much closer, from 0.03% of divergence between PF90/3056 and PF89/27643, PF92/2986, PF92/4190 and PF89/320219, to 0.44% between FP90/3050 and PF94/136116. More than 75% of the nucleotide substitutions occurred at the third codon position, and less than 17% of the changes resulted in an amino acid substitution in the E gene (24%) and in the NS5 (36%), NS1 (20%), NS2a (16%) and NS3 (4%) genes. The number of amino acid differences per gene was: E, 6/493 (1.22%); NS1, 5/352 (1.42%); NS2a, 4/218 (1.83%); NS3, 1/619 (0.16%); and NS5, 9/900 (1%).

Compared to the nucleotide sequences retrieved from GenBank, PF89/27643 was the closest isolate to the strains 80-2 (4.22%), H-87 (4.28%), 1243 (6.31%), and 1266 (6.32%); whereas PF94/136116 was the furthest from the strains 80-2 (4.57%), H-87 (4.63%), 1243 and 1266 (6.57%). All the French Polynesian isolates, as well as 1243, showed an insertion of 11 nucleotides (AGTGAAAAAGA) in the 3′UTR region, between positions 10275 and 10276, bringing their total genome length to 10,707 bases as opposed to 10,696 for the reference strain H-87 or the strain 80-2. The strain 1266 also had an 11-nucleotide insertion, but with a different sequence: AGTG*G*AAAAGA. At the amino acid level, PF90/3056 was the nearest to the strains 80-2 (1.42%), H-87 (1.62%), 1243 (2.04%), and 1266 (2.15%); whereas PF94/136116 was the most distant to the strains 80-2 (1.62%), H-87 (1.83%), 1243 (2.18%), and 1266 (2.36%).

3.3. Sequence analysis of the French Polynesian DEN-3 isolates

Table 4 summarizes the 25 amino acid replacements observed for the French Polynesian isolates. A sequence identical to the strain PF90/3056, named PF consensus,

Table 3
Comparison of amino acid and nucleotide sequences of the full genome of DEN-3 viruses[a]

		% Amino acid divergence												
		1	2	3	4	5	6	7	8	9	10	11	12	13
1	PF89/27643		0.03	0.06	0.12	0.06	0.06	0.15	0.21	0.29	1.45	1.65	2.06	2.18
2	PF90/3056	0.09		0.03	0.09	0.03	0.03	0.12	0.18	0.27	1.42	1.62	2.04	2.15
3	PF89/320219	0.11	0.13		0.12	0.06	0.06	0.15	0.29	0.35	1.47	1.68	2.09	2.21
4	PF90/6056	0.15	0.17	0.19		0.12	0.12	0.21	0.27	0.35	1.50	1.71	2.12	2.24
5	PF92/2986	0.20	0.22	0.17	0.27		0.06	0.15	0.21	0.29	1.45	1.65	2.06	2.18
6	PF92/4190	0.22	0.24	0.20	0.30	0.14		0.15	0.21	0.29	1.45	1.65	2.06	2.18
7	PF92/2956	0.26	0.28	0.23	0.34	0.31	0.34		0.29	0.35	1.47	1.68	2.09	2.21
8	PF90/3050	0.25	0.27	0.29	0.33	0.37	0.40	0.44		0.44	1.45	1.65	2.06	2.18
9	PF94/136116	0.51	0.52	0.49	0.56	0.57	0.60	0.64	0.67		1.62	1.83	2.18	2.36
10	80-2	4.22	4.23	4.26	4.30	4.31	4.32	4.34	4.25	4.57		0.38	1.80	2.01
11	H-87	4.28	4.29	4.32	4.36	4.37	4.38	4.40	4.31	4.63	0.26		2.01	2.21
12	1243	6.31	6.32	6.32	6.35	6.35	6.42	6.42	6.35	6.57	5.37	5.41		1.18
13	1266	6.32	6.35	6.37	6.38	6.40	6.45	6.45	6.36	6.57	5.36	5.40	2.95	
		% Nucleotide divergence												

[a] Isolates listed in Table 1 were compared for sequence divergence by using ClustalW 1.83.XP software.

representing the consensual sequence of the French Polynesian strains, is used to underline these replacements. Each amino acid substitution affected one isolate, except for NS1-174, which involved two isolates that resulted in two changes: Met → Val and Met → Lys. Ten of these changes resulted in a side-chain difference (polar versus non-polar), four in a charge difference, and one in both side-chain and charge changes. Moreover, the amino acid substitutions at E-153 (Asn → Ser) for PF90/6056 and at E-155 (Thr → Met) for PF94/136116 led to the loss of an N-linked glycosylation site. All the cysteine residues were perfectly conserved and no deletions or insertions were detected within the coding region. Twenty-one of the 25 amino acid substitutions were observed in four of the isolates; PF90/6056, PF90/3050, PF92/2956 and PF94/136116.

3.4. Phylogenetic analysis

The phylogenetic analysis showed that the French Polynesian isolates formed a clearly distinct group, whereas the strains H-87 and 80-2 on one hand, and 1243 and 1266 on the other formed two different groups (Fig. 2). These results are in agreement with the phylogenetic analyses performed on the E gene by other authors [20–23], demonstrating that the DEN-3 French Polynesian strains belonged to genotype I, strains H-87 and 80-2 to genotype V, and strains 1243 and 1266 to genotype III. Within the French Polynesian isolates, although PF90/3050 and PF94/136116 were relatively distinct from the others, there was no segregation according to severity of the disease.

4. Discussion

It is widely assumed that the severity of dengue is determined by viral and host factors. Indirect evidence suggests that both the host's genetic or immune status and the infecting viral strain influence the severity of the disease, but their relative contributions cannot be directly measured. Phylogenetic studies, mostly on the E gene of the virus, have suggested a possible association between specific genotypes and disease severity [24]. In addition, complete genome sequences of DEN-2 isolates have revealed structural differences that may be involved in disease severity [25,26]. However, similar studies have not been reported for DEN-3 virus.

We made 1011 virus isolations during the 1989 epidemic of DEN-3 in French Polynesia. For the nine DEN-3 isolates that were fully sequenced after a few passages in mosquito cell line, we were unable to demonstrate any association between the 25 amino acid substitutions that we detected, and either the disease severity, the infection history, or the chronology of the epidemic.

We also tested the inhibitory effect of the isolates on the growth of K562 cells. In the three groups that we defined by inhibitory effect, we did not observe any association between the level of inhibition and severity or infection history.

The level of inhibition was, however, associated with some differences in amino acids; the group of five isolates with a moderate level of inhibition had very similar sequences. These were obtained from patients with a range of severity: two DHF/DSS (one primary and fatal, one secondary and non-fatal), three DF (one primary and fatal, one secondary and severe and one non-severe with unknown infection history). In this group, we found only four amino acid substitutions of which the significance is unknown.

However, an NS5-676 (N → S) substitution for PF89/320219 that did not change the structure has been reported previously by Leitmeyer et al. [25] when they compared Thai and American DEN-2 isolates. In this study, all the Thai isolates and one DHF isolate obtained from Venezuela in 1990 had a Ser at this position.

In the four remaining isolates, there were 21 amino acid substitutions, the number of substitutions between each of these isolates ranged from 7 to 15. These isolates were all from primary dengue cases, and induced either a low (one non-severe DF and one DHF) or a high (one fatal DHF/DSS and one moderate DF) inhibition.

Table 4
Summary of amino acid changes between French Polynesian strains

Position /Gene	PF[a] consensus	PF94/136116	PF90/6056	PF90/3056	PF89/27643	PF89/320219	PF92/4190	PF92/2986	PF90/3050	PF92/2956
	-	5%	7%	10%	11%	12%	13%	14%	26%	32%
E										
64	K	K	R	K	K	K	K	K	K	K
129	A	A	A	A	A	A	A	A	V	A
140	I	I	I	I	I	I	I	I	I	T
153	N	N	S	N	N	N	N	N	N	N
155	T	M	T	T	T	T	T	T	T	T
471	T	A	T	T	T	T	T	T	T	T
NS1										
11	K	K	K	K	K	K	K	K	Q	K
88	I	I	I	I	I	I	I	I	V	I
147	S	L	S	S	S	S	S	S	S	S
174	M	K	M	M	M	M	M	M	M	V
293	T	I	T	T	T	T	T	T	T	T
NS2a										
5	A	T	A	A	A	A	A	A	A	A
34	A	A	A	A	A	A	A	A	A	S
150	V	I	V	V	V	V	V	V	V	V
159	F	F	F	F	F	F	S	F	F	F
NS3										
466	P	S	P	P	P	P	P	P	P	P
NS5										
16	K	K	K	K	R	K	K	K	K	K
275	V	V	V	V	V	V	V	V	I	V
399	I	I	I	I	I	I	I	I	M	I
482	Y	Y	Y	Y	Y	Y	Y	F	Y	Y
503	Y	H	Y	Y	Y	Y	Y	Y	Y	Y
562	Q	Q	R	Q	Q	Q	Q	Q	Q	Q
637	H	H	H	H	H	H	H	H	H	Y
676	N	N	N	N	N	S	N	N	N	N
831	T	T	T	T	T	T	T	T	S	T

[a] The PF consensus is the consensual sequence of French Polynesian strains and is identical to the strain PF90/3056

Shaded cells indicates mutated position by reference to PF consensus, side chain changes are in boldface, charge changes are underlined and significant changes are italicised.

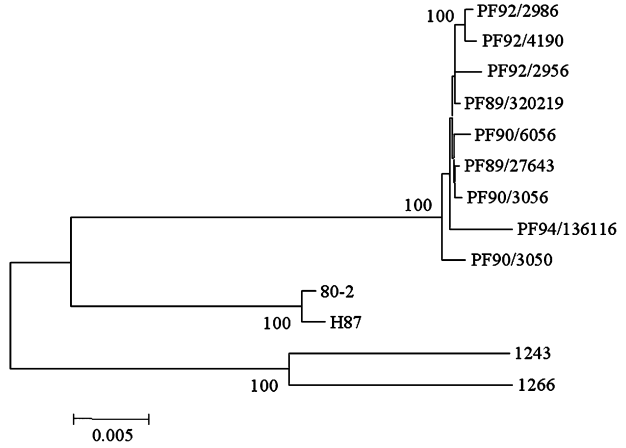

Fig. 2. Phylogenetic analysis of dengue-3 virus isolates based on the entire genome sequences. The phylogenetic tree was constructed by using the neighbor-joining method with the MEGA 2.1 software program. Bootstrap values are given as percentage equivalents and correspond to 500 replications.

Two amino acid substitutions in the E gene (Asn153Ser and Thr155Met) occurred in the isolates that induced low inhibition: PF94/136116 and PF90/3056. These substitutions resulted in the loss of an N-linked glycosylation site on the envelope gene; the importance of this site has been suggested in several studies. Indeed, reduced infectivity has been described for a St. Louis encephalitis isolate with an analogous non-glycosylated site [27]. Furthermore DEN-2 virus mutants that grow more slowly than the parent strain and have a deletion at the same site have been described. This deletion appeared to result in an altered fusion activity that affected the entry of the virus into cells [28]. It has been suggested that a disruption of virus entry, sufficient to delay viral replication may explain why a variant of Murray Valley encephalitis virus that is defective in fusion is unable to replicate *in vivo* in lymph node dendritic cells [29]. This delay could give sufficient time for the host's immune system to clear the infection resulting in reduced severity of the infection.

We also observed amino acid substitutions that lead to charge changes in the NS1 and NS5 genes. The isolates that induced low or a high inhibition of cell growth were associated exclusively with these substitutions. In those that induced low inhibition, PF90/6056 had an NS5-562 (Q → R) substitution and PF94/136116 had both an NS1-174 (M/V → K) and an NS5-503 (Y → H) substitution. These substitutions all led to changes from neutral to positive. By contrast, charge changes from positive to neutral were observed exclusively for the two isolates that induced high inhibition; PF90/3050 with a NS1-11 (K → Q) substitution, and PF92/2956 with an NS5-637 (H → Y) substitution. The relevance of such charge changes has not been elucidated, but it has been suggested that gain of neutral to positive charge amino acid substitutions on the E protein is involved in the virulence attenuation of Japanese encephalitis virus serotype flaviviruses [30].

Among other substitutions, we also observed that whereas eight isolates had a serine at position 147 in the NS1 gene, the PF94/136116 isolate had Leu at that position. The DEN-2 Asian genotype (considered to be associated with relatively severe disease) had a Ser at position 128, but the American genotype − mostly associated with moderate disease − had a Leu at this position. This Ser → Leu change is associated with a change of hydrophobicity and presumably of antigenicity [31]. The significance of the remaining substitutions for DEN-3 needs to be determined.

Finally, phylogenetic studies indicate an association between specific genotypes and the severity of the disease [24,25]. In our study, we found that the DEN-3 isolates all belonged to the genotype I, considered to be relatively virulent. These isolates had an insertion of 11 nucleotides at the 3′UTR position. The same insertion has been reported for genotype III, also considered virulent; by contrast, genotype V lacked this insertion. Deletions and modifications in the 3′UTR have been suggested to lead to changes in secondary structures of dengue viral RNA and to the attenuation of the virulence of the virus [25,26]. Additional studies are needed to investigate further the importance of such changes.

Studies that have approached the question of the virulence of dengue viruses have generally relied on clinical observations. In our study, we observed no association between amino acid substitutions and disease severity among our nine isolates of DEN-3. Six of which were from cases with severe manifestations and included both primary and secondary infections, and both DF and DHF. However, we did find an apparent association between amino acid substitutions − some of which are suggested to be associated with the degree of severity in DEN-2 infections − and the level of inhibition of cell growth induced by these isolates. This study should be extended with DEN-3 viruses from other geographic locations in order to investigate further the relevance of our observations.

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