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BRIEF REPORT

Complete nucleotide sequence analysis of a Dengue-1 virus isolated on Easter Island, Chile

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Abstract Dengue-1 viruses responsible for the dengue fever outbreak in Easter Island in 2002 were isolated from acute-phase sera of dengue fever patients. In order to analyze the complete genome sequence, we designed primers to amplify contiguous segments across the entire sequence of the viral genome. RT-PCR products obtained were cloned, and complete nucleotide and deduced amino acid sequences were determined. This report constitutes the first complete genetic characterization of a DENV-1 isolate from Chile. Phylogenetic analysis shows that an Easter Island isolate is most closely related to Pacific DENV-1 genotype IV viruses.

Dengue viruses are responsible for the most important arthropod-borne viral diseases in humans in terms of morbidity and mortality. Dengue is caused by four antigenically distinct viruses designated as dengue virus type 1-4 (DEN 1–4), belonging to the genus *Flavivirus* of the family *Flaviviridae*. It is mainly transmitted by *Aedes aegypti* mosquitoes, which are present in most tropical and subtropical countries of the world [3, 4]. The disease can vary from asymptomatic to febrile disease, classic dengue fever, or complications such as dengue hemorrhagic fever or dengue shock syndrome. The genome of dengue virus consists of a single strand of non segmented, positive-sense ribonucleic acid (RNA) of approximately 10.7 kb in length that contains a single open reading frame [1]. The viral genome encodes three structural (C, capsid; PrM/M precursor of membrane; E, envelope) and seven non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) proteins [9]. The ORF is flanked by 5′ and 3′ nontranslated regions (NTRs) [9, 13–15].

In 2002, an outbreak of dengue fever was detected on Easter Island, which is located in the Pacific Ocean 3,800 km off the coast of Chile, and the isolates of dengue virus were identified as belonging to the DENV-1 serotype [12]. This paper reports the first complete genetic characterization of RNA from a DENV-1 isolate in our country.

Dengue virus was isolated from the acute-phase sera of five DF patients using Vero cells cultured with medium 199 supplemented with 2% heat-inactivated fetal bovine serum (FBS) [12]. After adsorption for 1 h, the infected cell culture was incubated at 37°C for 10 days and observed once a day for cytopathic effects. Virus RNA was extracted from the supernatant of cells when CPE became apparent, using guanidinium thiocyanate–phenol–chloroform [2]. The serotype was determined by multiplex RT-PCR as described previously, which can distinguish the four serotypes by the sizes of their products [7].

Twelve synthetic oligonucleotide primer pairs were designed to amplify overlapping fragments of approximately 800 bp spanning the complete DEN-1 genome (GenBank accession no. ABI178040). The primer sequences are available from the authors upon request. Reverse transcription was performed at 50–56°C for 60 min, directly by 35 cycles of amplification consisting of 95°C for 1 min, annealing at 50°–65°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 5 min. Amplification was conducted using a Model 2700 thermal

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Table 1 Dengue virus (DENV) strains used in the study

Virus	Strain	Origin	Year isolated	Genbank accession no.
DENV-1	NIID04-27	Yap Island	2004	AB204803
DENV-1	FGA/89	French Guiana	1989	AF226687
DENV-1	BR/90	Brazil	1990	AF226685
DENV-1	BR/97-111	Brazil	1997	AF311956
DENV-1	Abidjan	Côte d'Ivoire	1998	AF298807
DENV-1	Mochizuki	Japan	1943	AB074760
DENV-1	16007	Thailand	1964	AF180817
DENV-1	GZ/80	China	1980	AF350498
DENV-1	A88	Indonesia	1988	AB074761
DENV-1	Cambodia	Cambodia	1998	AF309641
DENV-1	Djibouti	Ethiopia	1998	AF298808
DENV-1	West Pac 74	Nauru	1974	U88535
DENV-1	98901530	Indonesia	1998	AB189121
DENV-1	98901518	Indonesia	1998	AB189120
DENV-1	259par00	Paraguay	2000	AF514883
DENV-1	295arg00	Argentina	2000	AF514885
DENV-1	ARG9920	Argentina	1999	AY277664
DENV-1	FP1104	French Polynesia	2001	DQ672559
DENV-1	FP0705	French Polynesia	2001	DQ672557
DENV-1	FP0203	French Polynesia	2001	DQ672556
DENV-1	HawM2540	Hawaii	2001	DQ672562
DENV-1	HawM2516	Hawaii	2001	DQ672560
DENV-1	HawO3758	Hawaii	2001	DQ672567
DENV-1	CHI3336-02	Eastern Island (Chile)	2002	EU863650
DENV-1	CHI3325-02	Eastern Island (Chile)	2002	EU863646
DENV-1	CHI3329-02	Eastern Island (Chile)	2002	EU863647
DENV-1	CHI3366-02	Eastern Island (Chile)	2002	EU863649
DENV-1	CHI3364-02	Eastern Island (Chile)	2002	EU863648
DENV-3	DENtype3-TB55i	Indonesia	2004	AY858048

cycler (Applied Biosystems, Foster city, CA). Complete nucleotide sequencing of the RNA of isolate CHI3336/02 and of the E/NS1 region of isolates CHI3366-02, CHI3364-02, CHI3329-02, CHI3325-02 was performed. To sequence the DEN-1 virus genome, cDNA fragments amplified by PCR were cloned into pGEM-T (Promega Corporation) according to the manufacturer's protocol. The sequence between genome positions 1–250 and 10,270–10,735 was determined by direct sequencing of the PCR product. The E/NS1 region was amplified with primers originally described by Goncalvez et al. [5].

Sequencing was performed with fluorescence-labeled dideoxynucleotide terminators by using an ABI PRISM big Dye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA) and 5 pmol of T7 forward and M13 reverse primers combined with 0.2 ng of plasmid DNA. Nucleotide sequences were analysed using an ABI PRISM 310 genetic analyzer (Applied Biosystems). The sequence data generated were assembled and edited electronically with the ALIGN, EDITSEQ, and MEGALIGN programs (DNASTAR, Madison, Wis). Recombination was tested with Simplot (version 3.2; distributed by the authors, Stuart C. Ray, Division of Infectious Diseases, Johns Hopkins University School of Medicine. The RNA secondary structure of the 3′NCR was predicted with RNAdraw v1.01 [10]. The phylogenetic tree was reconstructed for aligned nucleotide sequences by using the neighbour-joining method with a Kimura 2-parameter model using MEGA3 software version 3.1 [6]. Bootstrap analysis of 500 replicates was used to estimate the reliability of the predicted tree. A representative sequence from DENV serotype 3 was used as an outgroup to root the tree.

The full-length RNA genome of CHI3336-02 was 10,735 nt. No deletions, insertions or premature polyprotein stop codons were found. The single ORF was located at positions 95–10,270, coding for a polyprotein of 3,392 amino acids.

Table 2 Replacement changes in the structural and non-structural proteins of dengue virus type 1 strains

ORF	Protein	Position	Indonesia 98901518	Easter Island CHI-3336-02	French Polynesia FP1104	Yap Island NIID27
367	E	88	A	T	T	T
530		230	A	V	V	V
585		305	S	**L**	S	P
625		344	V	**A**	V	V
1009	NS1	234	N	**H**	N	N
1248	NS2A	121	E	D	D	E
1467	NS2B	123	Y	H	H	Y
2117	NS4A	23	N	**K**	N	T
2183		89	M	T	T	M
2245	NS4B	1	N	**T**	N	N
2519	NS5	26	N	**K**	N	N
2596		103	Y	**S**	Y	Y
2914		421	A	**P**	A	A
2928		435	R	**H**	R	H
3122		629	S	**L**	S	S
3128		635	T	**P**	T	T
3136		643	L	**P**	L	L
3179		686	I	V	V	V

Replacement amino acid changes identified in the dengue virus type 1 Chilean isolate CHI3336-02 in relation to the IV genotype, represented by Indonesia 98901518 and the French Polynesia FP1104 and Yap Island NIID27-04 strains of the same genotype, according to their positions within the genes as indicated. Bold, differences between the Chilean CHI3336-02 and French Polynesia FP1104 isolates

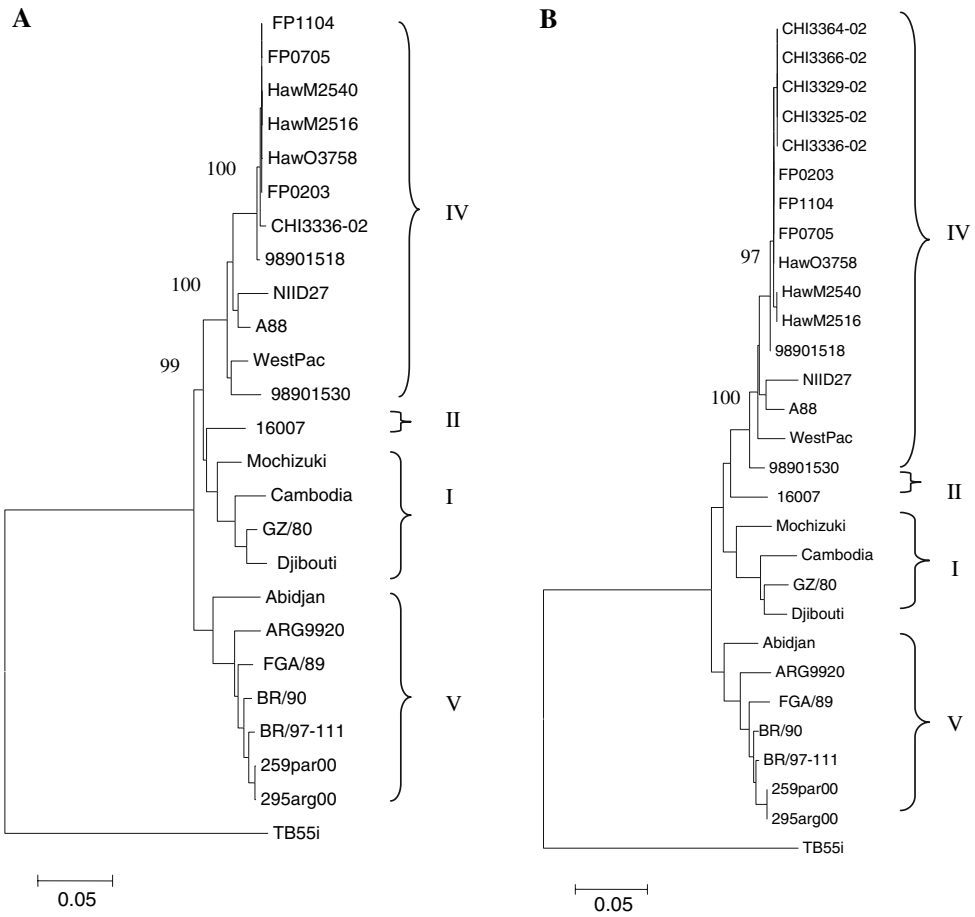

Fig. 1 Phylogenetic analysis of dengue virus type1 (DENV-1) genomes. **a** Full-length genomes. **b** E/NS1 region. The nucleotide sequences of 23 representative DENV-1 strains were analyzed using the neighbor-joining method. The percentage of successful bootstrap replicates is indicated at the nodes. Representative strain DENV-3 was used to root the tree. Genotypes I, II, III, IV, and V correspond to DENV-1 genotype as defined by Goncalvez et al. [5]

To characterize the molecular structure of the genome, the complete CHI3336-02 nucleotide sequence was compared with genotype I–V DENV-1 strains available from the NCBI database (Table 1). The Easter Island isolate was most closely related to genotype IV (95.3–99.4% nt, 98.2–99.6% aa similarity, respectively) followed by genotype III (94.5% nt, 97.8% aa similarity) and finally genotypes I and V (91.4–93.7% nt, 97.1–97.3% aa similarity, respectively). The nucleotide changes were distributed throughout the E and NS5 genes, with most of them located at the third nucleotide of a codon, resulting in no amino acid changes. The Easter Island isolate was most closely related to (99.5% nt, 99.6% aa similarity) the Hawaii and French Polynesia strains.

Pairwise comparisons of the sequences showed that the similarity between CHI3336/02 and the Indonesia 98901518 strain, representing genotype IV, was above 99.1%, and the same percentage of similarity was observed for the genes E, NS1, NS2, M, NS5, and NS3. Of 89 substitutions, 18 were non-synonymous and 71 were synonymous. Replacement changes were mostly found in E (4/16) and NS5 (8/16), as shown in Table 2. Within the E glycoprotein, the two amino acid substitutions at positions 305 and 344 (domain III) are non-conservative. Domain III (amino acids 302–404) is thought to play an important role in cell attachment and tropism. The impact of these changes on biological properties is not known. Six of the non-silent changes in CHI3336-02 were conservative in the French Polynesia FP1104 strain. On the other hand, the E (2/4) and NS5 (2/8) mutations were identical to those found in a Yap Island isolate from 2004 [11]. Nucleotide changes were generally conservative, leading to few amino acid changes. The impact of these amino acid changes on biological and pathogenic properties is not known. When we compared the E/NS1 region of the isolate CHI3336-02 with the sequences obtained from the other Chilean isolates, 100% nucleotide and amino acid similarity were found.

The sequence of the 5′NTR of CHI3336-02 is identical to that described for DENV-1. The nucleotide sequence of the 3′NTR was most closely related to those of FP1104 (98.2% nt similarity) and 16,007 (98% nt similarity) of genotypes IV and II, respectively. A detailed analysis of the RNA secondary structures demonstrated that the 3′NTR has conserved areas that were previously predicted to be involved in flavivirus secondary structure [13, 14].

To understand the genetic relationships of DENV-1 strains, we performed phylogenetic analysis, using the neighbor-joining algorithm, of CHI3336-02 and full-length DENV-1 sequences retrieved from the NCBI database. The tree placed the Easter Island isolate in the Pacific group of DENV-1 genotype IV, represented here by the Indonesia A88, 98901518, NIID04-27, Hawaii, French Polynesia and Westpac74 strains (Fig. 1a). A similar phylogenetic tree was generated, using the E/NS1 region, with other Easter Island isolates (Fig. 1b). Finally, the CHI3336-02 isolate was phylogenetically linked to the Hawaii, French Polynesia and Indonesia (98901518) strains. These observations suggest that the outbreak that occurred on Easter Island in 2002 was probably due to the introduction of genotype IV DENV-1 viruses that were previously involved in outbreaks in Hawaii and French Polynesia in 2001 [8].

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References

1. Chambers TJ, Hahn CS, Galler R, Rice CM (1990) Flavivirus genome organization, expression, and replication. Annu Rev Microbiol 44:649–688
2. Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by guanidium thiocyanate–phenol–chloroform extraction. Anal Biochem 162:156–159
3. Domingo C, Palacios G, Niedrig M, Cabrerizo M, Jabado O, Reyes N, Lipkin WI, Tenorio A (2004) A new tool for the diagnosis and molecular surveillance of dengue infections in clinical samples. Dengue Bull 28:87–95
4. Henchal EA, Putnak (1990) The dengue viruses. Clin Microbiol Rev 3:376–396
5. Goncalvez AP, Escalante AA, Pujol FH, Ludert JE, Tovar D, Salas RA, Liprandi F (2002) Diversity and evolution of the envelope gene of dengue virus type 1. Virology 303:110–119
6. Kumar S, Tamura K, Nei M (2004) MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform 5:150–163
7. Lanciotti RS, Callisher CH, Gubler DJ, Chang GJ, Vorndam AV (1992) Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. J Clin Microbiol 30:545–551
8. Laille M, Roche C (2004) Comparison of dengue-1 virus envelope glycoprotein gene sequences from French Polynesia. Am J Trop Med Hyg 71:4787–484
9. Lindenbach BD, Rice CM (2003) Molecular biology of flaviviruses. Virus Res 59:23–61
10. Matzura O, Wennborg A (1996) RNAdraw: an integrated program for RNA secondary structure calculation and analysis under 32-bit Microsoft Windows. Comput Appl Biosci 12:247–249
11. Nukui Y, Tajima S, Kotaki A, Ito M, Takasaki T, Koike K, Kurane I (2006) Novel dengue virus type 1 from travelers to Yap State, Micronesia. Emerg Infect Dis 12:343–346
12. Perret C, Abarca K, Ovalle J, Ferrer P, Godoy P, Olea A, Aguilera X, Ferres M (2003) Dengue-1 virus isolation during first dengue fever outbreak on Easter Island, Chile. Emerg Infect Dis 9:1465–1467
13. Proutski V, Gould EA, Holmes EC (1997) Secondary structure of the 3′ unstranlated region of flaviviruses: similarities and differences. Nucl Acids Res 25:1194–1202
14. Shurtleff AC, Beasley DWC, Chen JJY, Ni H, Suderman MT, Wang H, Xu R, Wang E, Weaver SC, Watts DM, Russell KL, Barrett ADT (2001) Genetic variation in the 3′ non-coding region of dengue viruses. Virology 281:75–87
15. Tajima S, Nukui Y, Takasaki T, Kurane I (2007) Characterization of the variable region in the 3′ non-translated region of dengue type 1 virus. J Gen Virol 88:2214–2222