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Genomic characterization of Brazilian hepatitis C virus genotypes 1a and 1b

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Abstract

Parts of 5' non-coding (5' NC) and of E1 envelope regions of the hepatitis C virus (HCV) genome were amplified from sera of 26 Brazilian anti-HCV antibody-positive patients using the reverse transcription-polymerase chain reaction (RT-PCR). Fourteen samples were PCR positive with primers from the 5' NC region and 8 of them were also positive with primers from the E1 region. A genomic segment of 176 bp from the E1 region of 7 isolates was directly sequenced from PCR products. The sequences were compared with those of HCV strains isolated in other countries and the Brazilian isolates were classified by phylogenetic analysis into genotypes 1a and 1b. This could have a clinical importance since it has been shown that individuals infected with type 1 viruses are less likely to respond to treatment with interferon than individuals infected with types 2 and 3 viruses. Two quasispecies isolated from the same patient with an interval of 13 months differed by two base substitutions (1.1%). The sequence of another isolate presented a three-nucleotide deletion at codon 329.

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Introduction

Hepatitis C virus (HCV), the major causative agent of parenterally transmitted non-A, non-B hepatitis (1), belongs to the Flaviviridae family. The genome of HCV is a single-stranded RNA with positive polarity of about 9,400 bases, which has a long open reading frame encoding structural proteins (core, E1, E2) and nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) with untranslated regions at both the 5' and 3' ends. Considerable genetic heterogeneity has been reported among isolates (2,3). Comparison of reported sequences has shown that some parts of the genome are more variable than others (4-6), the most conserved part being the 5' non-coding (5' NC) region. Six major genotypes (1 to 6) have been identified, some of which contain several subtypes referred to as a, b, c, etc. (7-11). New genotypes 7 to 10 have been recently proposed (12,13).

Infection with different genotypes may produce clinically relevant differences in the liver disease caused by HCV and furthermore the sensitivity of the virus to interferon treatment is not identical for all genotypes. Genotyping of patient isolates may thus be of great clinical importance. Genotyping requires the amplification of the viral genome by reverse transcription-polymerase chain reaction (RT-PCR). Once the genetic material is amplified, a number of approaches can

be used such as restriction fragment length polymorphism (14,15), hybridization assays such as the line probe assay (16), DNA enzyme immunoassay (17), and type-specific primer nested PCR (18,19). However, all these methods suffer from the same problems: i) unusual mutations at critical sites may occasionally produce erroneous results and ii) some samples are unclassifiable. Nucleotide sequencing followed by the analysis of the viral genome is the definitive method for identifying various HCV genotypes.

Few data on HCV genotypes circulating in South America are available. In the present study, we demonstrate the presence of HCV genotypes 1a and 1b in Brazilian patients by direct sequencing of PCR products and show the phylogenetic localization of the corresponding isolates.

Material and Methods

Serology

Samples were obtained from sera referred to the National Reference Center for Viral Hepatitis for serological analysis between 1994 to 1995. Sera were from patients living in the city of Rio de Janeiro, Brazil, and being followed at several health institutions. Twenty-six anti-HCV antibody-positive sera were selected by in-house 2nd-generation enzyme-linked immunosorbent assay (ELISA) with controlled specificity and sensitivity according to available commercial kits, and using recombinant antigens from the core, NS3, and NS5 regions obtained from the Research Foundation for Microbial Disease of Osaka University, Japan. Sera were also tested by ELISA for the presence of hepatitis B surface antigen (HBsAg) using the Hepanostika HBsAg Uni-form II system (Organon Teknika, Boxtel, The Netherlands).

Table 1 - Sequence and genomic location of the oligonucleotides used as PCR primers.

Region	Polarity	Position	Sequence (5′ to 3′)
5′ NC	+	-305	CACTCCCCTGTGAGGAACTACTGTC
	+	-279	TTCACGCAGAAAGCGTCTAGCC
	-	-26	GGGCACTCGCAAGCACCCTATCAGG
	-	2	ATGGTGCACGGTCTACGAGACCTCC
E1	+	478	GACGGCGTGAACTATGCAACAGGG
	+	812	TCTGTTCCGCCATGTACGTGGGGGA
	-	1037	TCCACGACGGCTTGTGGGATCCGGA
	-	1112	ACCTTAGCCCAGTTCCCCACCATGG

RNA extraction and RT-PCR

HCV RNA was extracted from 100 µl serum by the acid guanidinium isothiocyanate phenol-chloroform method (20) and finally resuspended in diethylpyrocarbonate-treated water. Reverse transcription was carried out with random primers and 200 U of Moloney murine leukemia virus reverse transcriptase (Gibco-BRL) in a final volume of 20 µl at 37°C for 1 h. One third of the cDNA was amplified by PCR with primers designed from the 5' NC and E1 regions. In some cases, nested PCR was done with internal primers. The oligonucleotides used as primers are shown in Table 1. PCR assays were carried out in a volume of 50 µl in the presence of 0.2 mM of each dNTP, 3 mM $MgCl_2$, and 1 unit of *Taq* DNA polymerase (Gibco-BRL). After an initial denaturation for 2 min at 94°C, DNA was amplified for 35 cycles at 94°C for 15 s, 50°C for 45 s, and 72°C for 1 min followed by a final elongation of 7 min at 72°C. When required, 1 µl of the product was subjected to nested PCR for 30 cycles under the same conditions (only increasing the $MgCl_2$ concentration to 5 mM). To avoid contamination, RNA extraction and reverse transcription, pre-PCR reagent preparation, DNA amplification, and gel electrophoresis of PCR products were performed in four separate rooms. In each series of experiments, five samples together with a negative and a positive control were tested. None of the 7 nucleotide sequences determined were identical to each other, which permits us to

eliminate the possibility of cross-contamination.

Nucleotide sequencing

Nested PCR products were made single-stranded and sequenced directly by the dideoxynucleotide chain termination method (21). Briefly, one of the internal primers used in the nested PCR was phosphorylated at the 5' end: 150 pmol of primer was incubated in the presence of 10 mM ATP and 15 units of T4 polynucleotide kinase at 37°C for 30 min and the reaction was stopped by heating at 65°C for 10 min. After PCR, DNA fragments were purified and the 5' phosphorylated strand was degraded by lambda exonuclease digestion (PCR template prep for SS DNA sequencing, Pharmacia), leaving a single-stranded DNA to be sequenced (T7 sequencing kit, Pharmacia). Primers used for sequencing were the internal PCR primers of the E1 region.

Phylogenetic analysis

Alignment of multiple nucleic acid sequences was performed with the University of Wisconsin Genetic Computer Group (GCG) PileUp program. This program uses the unweighted pair-group method with arithmetic average (UPGMA) procedure resulting in a clustered order of the sequences based on the degree of similarity that is represented as a dendrogram (22). For easier identification, DNA databank sequences were handled using their accession number.

Results and Discussion

PCR is becoming a universal detection method for a number of DNA and RNA viruses present in the serum of patients. HCV is one of the viruses most extensively investigated by PCR analysis. Two segments of the HCV genome were amplified by RT-PCR from the sera of 26 anti-HCV antibody-positive patients. Table 2 shows sex, age, and clinical data of the patients. Only one patient was HBsAg positive. Fourteen (54%) samples were PCR positive when using primers of the 5' NC region (12 after the first round of PCR and 2 after nested PCR). Of these 14 positive samples, 8 were also positive with primers of the E1 envelope region (4 after one-round PCR and 4 after nested PCR). The fact that a larger number of samples were positive when using primers derived from the 5' NC region may be due to the elevated conservation of this region. The primers from this region showed one or no mismatch when compared with published sequences of the most common genotypes 1, 2, and 3, while sequences of the selected E1 primers were from a much more variable region.

HCV strains have been classified into genotypes, subtypes, isolates, and quasispecies with polyprotein amino acid sequence identities of 68% to 75% between genotypes, 83% to 85% between subtypes of a given genotype, and 92% to 98% between isolates (23). Virus quasispecies are defined as a variable mixture of a master nucleotide sequence and a spectrum of mutant molecules isolated from the same individual (24). Genotyping of HCV isolates has been shown to be of clinical importance since certain genotypes may be more pathogenic (25), more responsive to interferon treatment (26), and associated with cirrhosis and hepatocellular carcinoma (27). For example, it has been established that individuals infected with type 1 viruses are much less likely to respond to treatment with interferon than individuals infected with types 2 and 3 viruses (28). Within type 1, the primary interferon response was the lowest in patients infected with subtype 1b (29). The most definitive classification method of HCV is the determination of its complete nucleotide sequence. However, nucleotide sequencing of a selected part of the genome has been used to determine the genotype of HCV

Table 2 - Clinical and serological data of the patients and detection of HCV in the serum by RT-PCR.

The HCV genome was detected after one-round PCR (1) or only after nested PCR (2). n.d., Not determined. HBsAg, Hepatitis B surface antigen.

Patient	Sex	Age (years)	Clinical data	HBsAg	PCR (5' NC)	PCR (E1)
Braz01	F	46	Hepatomegaly, transfusion	-	+ (1)	+ (1)
Braz02	M	49	Cirrhosis, transfusion, hepatomegaly	-	+ (1)	+ (2)
Braz04	F	56	Transfusion, asymptomatic	n.d.	+ (1)	+ (2)
Braz05	F	30	Transfusion	-	-	n.d.
Braz06	M	56	Hemodialysis	-	-	n.d.
Braz07	F	33	Hemodialysis	-	+ (1)	+ (1)
Braz08	M	54	Hepatitis	-	+ (1)	+ (2)
Braz09	F	62	Splenomegaly, transfusion	-	-	n.d.
Braz10	M	46	Cirrhosis	-	+ (1)	-
Braz11	M	67	Hemodialysis	-	-	n.d.
Braz12	M	Unknown	Chronic hepatitis	n.d.	-	n.d.
Braz13	F	65	Transfusion	-	-	n.d.
Braz14	M	45	Asymptomatic	-	-	n.d.
Braz15	F	50	Transfusion	-	-	n.d.
Braz16	F	40	Asymptomatic	-	-	n.d.
Braz17	M	57	Hemophiliacs	-	-	n.d.
Braz18	M	47	Transfusion	-	-	n.d.
Braz19	F	34	Hepatitis	n.d.	+ (1)	-
Braz20	F	1	Hemodialysis	-	-	n.d.
Braz21	M	40	Transfusion	+	+ (1)	+ (1)
Braz22	F	Unknown	Transfusion	-	+ (1)	-
Braz23	M	43	Hemodialysis	-	+ (1)	+ (1)
Braz24	M	53	Hemodialysis	-	+ (2)	-
Braz35	M	59	Hemodialysis	-	+ (1)	+ (2)
Braz36	M	80	Hemodialysis	-	+ (1)	-
Braz37	M	70	Hemodialysis	-	+ (2)	-

isolates. The E1 region has been shown to be one of the most appropriate regions for this purpose (30) while the 5' NC and core regions, also frequently used, are less effective in distinguishing between genotypes, subtypes, and isolates (23).

The nucleotide (nt) sequence of a 176-bp DNA fragment of the E1 region from nt 837 to nt 1012 was determined for 7 samples and the sequences are shown in Figure 1. Sequences of our samples were compared by computer analysis with those of 15 HCV isolates from different parts of the world representing all the six major genotypes currently known. A phylogenetic analysis was carried out and is presented as a dendrogram in Figure 2. Samples Braz04-1, Braz04-2, and Braz08 were assigned to genotype 1a whereas samples Braz01, Braz02, Braz07, and Braz35 were assigned to genotype 1b. Our results demonstrating the infection of South American patients with types 1a and 1b viruses corroborate previous sequence data showing the presence of types 1b in Peru (7) and 1a (31) and 1b (32) in Argentina. The data also agree with the characterization of Brazilian HCV isolates from genotype 1 by hybridization line probe assay (16). Furthermore, genotype 1 has been shown to be the predominant type among the blood donors of São Paulo State (33).

Two sequences (Braz04-1 and Braz04-2) were derived from viruses from the same asymptomatic patient, whose blood was collected two times with an interval of 13 months. These two sequences differed by two base substitutions: in sample Braz04-2, T was replaced by C at position 858 and C

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                837
Braz01     1b   TCTCTGCGGATCTGTTCTTCTCTTCTCCCAGCTGTTCACCTTCTCGCCTCGCCATCATC
Braz02     1b   ---------------C--C--TA-----------------------------GG---G
Braz07     1b   ---------C------T-C--TG-G--T---------GT---T--A-------GGT--G
Braz35     1b   ---------------CT-C--TG-T--T--------TGT---T--A-------GGT---

Braz04-1   1a   CT-G-----G-----CT----TG--GGT-----------------T-----G-GC--CT
Braz04-2   1a   CT-G-----G-----CT-----G--GGT-----------------T---A-G-GC--CT
Braz08     1a   CT-G-----G-----CT----TG--GGT-----------T-----T--CA-G--C--CT



                937
Braz01     1b   ATAACAGGTCATCGTATGGCTTGGGATATGATGATGAACTGGTCACCTACGACAGCCC
Braz02     1b   G-G--------C--C-------------------------------A--AG-T----
Braz07     1b   G--T----------C-----G----------------------------AG------
Braz35     1b   G--T----A-----C-----G----------------------------A-------

Braz04-1   1a   -----G-----C--C-----G----------------------C--------G--AT
Braz04-2   1a   -----G-----C--C-----G----------------------C--------G--AT
Braz08     1a   -----G-----C--C-----G----------------------C---   --G--GG
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Figure 1 - Partial nucleotide sequence of the E1 region from nt 837 to nt 1012. Samples Braz01, Braz02, Braz07, and Braz35 were assigned to genotype 1b, and the others to genotype 1a. Samples Braz04-1 and Braz04-2 were from the same patient, whose blood was collected two times with an interval of 13 months. The sequence of patient Braz08 presented a one-codon deletion (nt 985-987).

was replaced by A at position 886. These changes did not modify the amino acid sequence and therefore may not have originated from random misincorporation during PCR. The mutation rate was about 1 x 10^{-2} base substitutions per genome site per year, a value similar to that found for the NS5-coding region (34) and higher than that of 1-2 x 10^{-3} reported for the entire genome (2,35).

Isolate Braz08 presented a three-nucleotide deletion, from nt 985 to nt 987, which would result in the lack of one amino acid residue, located at position 329 of the polyprotein and at the C-terminal side of the E1 envelope protein. We examined 278 HCV E1 region sequences currently available in DNA databanks. Since we found 8 insertions and 3 deletions, all varying from 1 to 4 codons, we may conclude that small insertions and deletions are not rare events in the E1 region. However, no deletion has been reported at codon 329. Further studies are necessary to understand the biological significance of these genome modifications.

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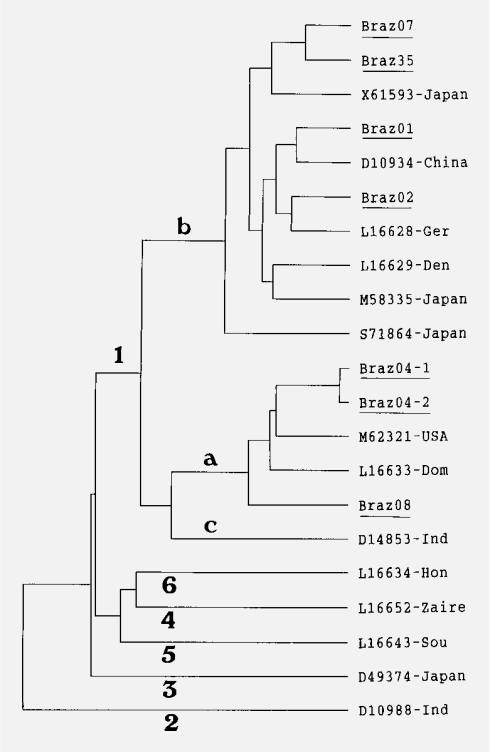

Figure 2 - Phylogenetic analysis of partial E1 sequences of the HCV genome. HCV genotypes are indicated by numerals. The Brazilian isolates studied here are underlined, and the other isolates are reported as their Genbank accession number and country of origin. Den, Denmark; Dom, Dominican Republic; Ger, Germany; Hon, Hong-Kong; Ind, Indonesia; Sou, South Africa.

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