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Hepatitis C Virus Isolates from Argentina Disclose a Novel Genotype 1-Associated Restriction Pattern

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Hepatitis C virus isolates which disclosed a novel genotype 1-associated restriction pattern by restriction fragment length polymorphism analysis were characterized. Except for a mother and child pair, the patients were unrelated. Sequence analysis showed a G→A substitution leading to a new *Rsa*I recognition site. Phylogenetic analysis revealed that these isolates constitute a novel genetic lineage within the main cluster of genotype 1 strains.

Hepatitis C virus (HCV) is an enveloped virus which belongs to the genus *Hepacivirus* in the family *Flaviviridae* (15). It is the most important cause of posttransfusion non-A, non-B hepatitis worldwide. The genome of HCV consists of a single strand of positive RNA (≈9.5 kb), which codes for at least 10 viral proteins and is flanked by 5′- and 3′-end noncoding regions.

The 5′-end untranslated region (5′UTR) of HCV is the most highly conserved portion of the viral genome and has been used to develop sensitive assays for RNA detection as well as for genotyping. According to current recommendations, HCV isolates are classified into six major clades, 1 to 6, whose nucleotide and inferred amino acid sequences differ by 35% (15, 18). Clade assignment is achieved by sequencing and aligning the HCV core, E1, or NS5B sequences with those of the prototypical strains of each clade (15). Although exact and reliable, this method cannot be easily implemented in conventional diagnostic laboratories. Consequently, several other methods to determine HCV genotype have been developed, including HCV RNA amplification followed by either reamplification with genotype-specific primers in the core region (11), hybridization with type-specific probes in the 5′UTR (16), or digestion of PCR products with restriction endonucleases that recognize genotype- and even subtype-specific sequence polymorphisms in the 5′UTR of the HCV (restriction fragment length polymorphism [RFLP]) (3, 9).

Using the RFLP method described by Davidson et al. (3), a widely used technique in our region, we reported a high prevalence of genotype 1a/c in children and infants in Argentina (6). Although rapid and simple, RFLP turned out to be unsatisfactory for the identification of HCV genotypes in isolates for five of our cases. Four of them were children and represented 14% of our pediatric population under study. Our aim was to characterize these isolates and to evaluate their diversity by

means of nucleic acid sequencing and subsequent phylogenetic analysis.

Plasma samples from five patients with chronic HCV infection (four children and one adult) were analyzed. Viral RNA was reverse transcribed, and the 5′UTR was amplified as previously described (5), with Kwok and Higuchi's recommendations (8). Amplicons were digested with restriction enzymes, followed by 15% or 12% polyacrylamide gel electrophoresis to evaluate the HCV genotype or subtype, as described by Davidson et al. (3). In addition, fragments were purified and sequenced with the Big Dye terminator cycle sequencing kit, version 1.1, and the 3100 genetic analyzer (Applied Biosystems).

DNA digestion of the five samples with *Hin*fI and *Mva*I gave a genotype 1- or 6-associated pattern (Fig. 1A), whereas they were untypeable by *Rsa*I and *Hae*III digestion (Fig. 1B). On the other hand, digestion with *Bst*UI gave the genotype 1a/c-associated pattern (data not shown). Thus, our strains exhibit a restriction map more similar to that of genotype 1 than to any of the other genotypes. Sequence alignment showed a G→A substitution at position −235 of the 5′UTR in all untypeable isolates tested compared to a prototypical genotype 1 strain (Fig. 2). This point mutation resulted in the generation of a new recognition site for *Rsa*I, modifying the typical pattern for HCV genotype 1. The analysis also included an Argentine isolate for which the genotype had been clearly determined as 1a/c by the same technique. This isolate does not display the above-mentioned substitution, confirming that the abnormal pattern was a consequence of this nucleotide substitution.

The mutation described above was not related to either the patient's age or a history of blood transfusions. This is supported by the fact that it was present in samples from children and an adult who were infected by different routes at different times. Except for one case of mother-to-child transmission, in which samples from both the mother and child were studied, the patients were unrelated to each other. This indicates that our observations depict a general phenomenon which should be taken into account when determining viral genotypes. The detection of the same mutation in the mother-and-child

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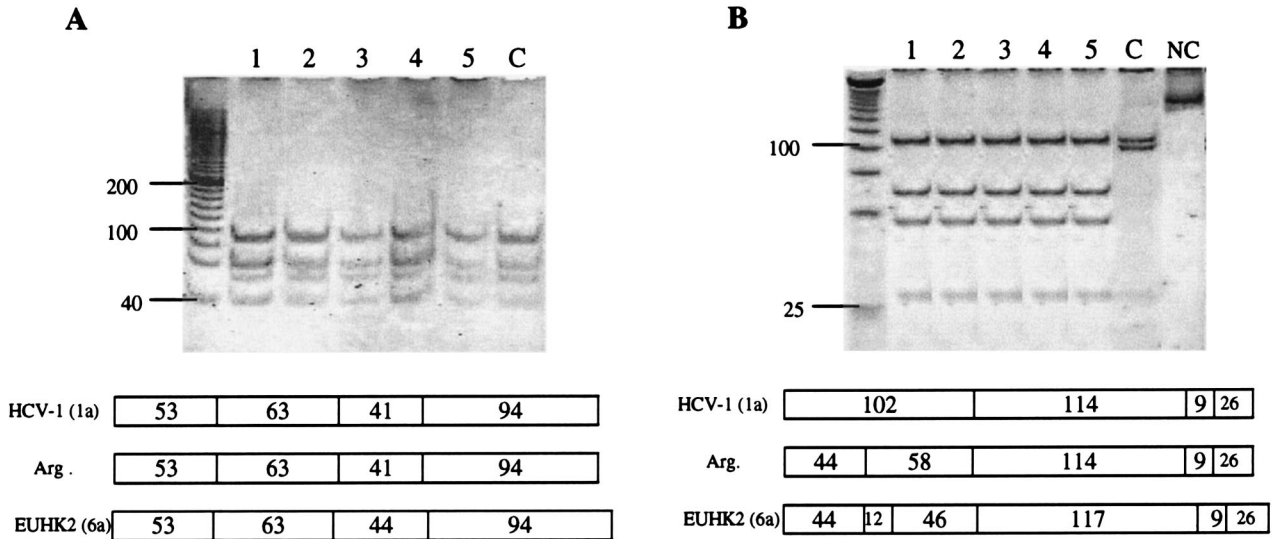
FIG. 1. RFLP analysis of Argentine HCV isolates. Amplified DNA fragments were digested with *Hin*fI and *Mva*I (A) or *Rsa*I and *Hae*III (B). (Top) Polyacrylamide gel electrophoresis. Lanes 1 to 5, untypeable isolates; lane C, Argentine isolate corresponding to genotype 1a/c; lane NC, negative control (251 bp). (Bottom) Restriction map of the isolates tested and prototypic HCV strains, as predicted from the DNA sequences (Webcutter program version 2.0). The numbers indicate the lengths of the restriction fragments (in base pairs). Genotypes are indicated in parentheses. HCV-1 (1a), prototypic HCV genotype 1 strain; Arg, untypeable Argentine isolates; EUHK2, prototypic HCV genotype 6 strain. The numbers on the left indicate the sizes of the standards (in base pairs).

paired samples strongly supports the idea that this variant is adapted to the host environment. Therefore, the mutation is unlikely an intrahost substitution due to natural quasispecies dynamics.

To elucidate the genetic heterogeneity of our isolates and to determine their phylogenetic relatedness to prototypic HCV strains, we applied phylogenetic analysis with the programs of the PHYLIP package (DNAdist, Neighbor, Seqboot and Consense) (4). The five untypeable isolates clustered together inside the main cluster of the genotype 1 strains, and they were segregated from HCV prototypes corresponding to subtypes a, b, and c (Table 1; Fig. 3A). We also evaluated the genetic variability of our isolates in comparison to other genotype 1 HCV isolates recently described in our region as well as in the rest of the world (Table 1; Fig. 3B). The two HCV isolates from Montevideo, Uruguay, displayed the same point mutation at position −235 and clustered with our mutated isolates. The nonmutated Argentine isolate used as a genotype 1 control and other HCV isolates from Brazil clustered apart from the untypeable isolates which have been described here.
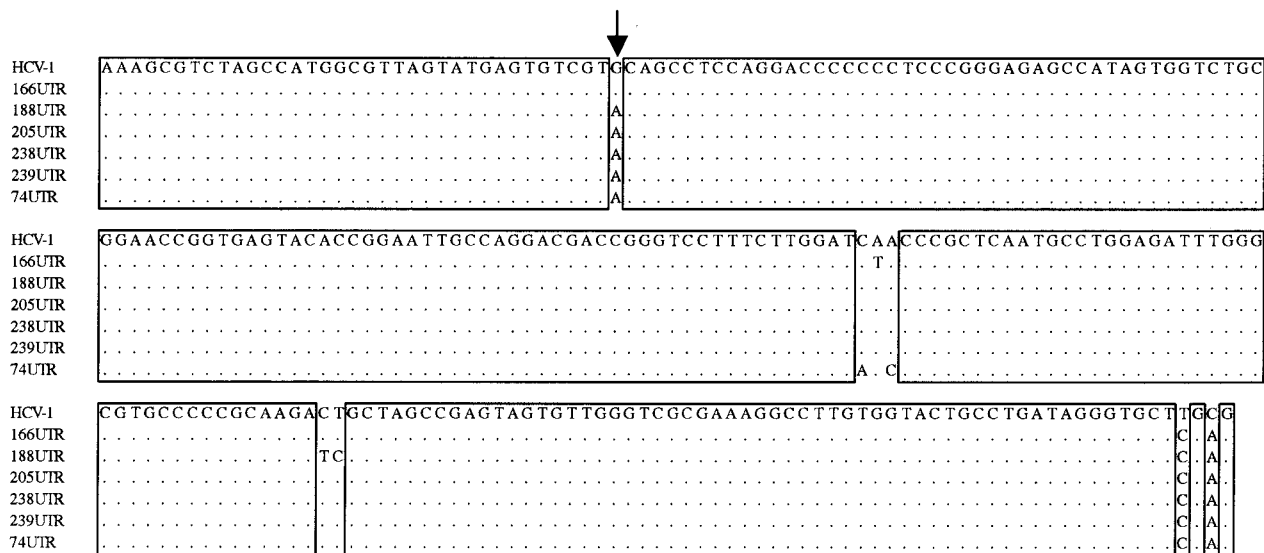

FIG. 2. Alignment of the amplified 5′UTR sequences of Argentine HCV isolates with the corresponding sequence of strain HCV-1 (genotype 1a). Isolates are indicated by name on the left side. Dots indicate nucleotide identity with strain HCV-1. The arrow marks position −235.

TABLE 1. HCV isolates used for phylogenetic analysis

Isolate	HCV genotype	Country of origin	Accession no.	Isolate	HCV genotype	Country of origin	Accession no.
HCV-1	1a	USA	M62321	Montevideo 2	1	Uruguay	AJ012832
HCV-J	1b	Japan	D90208	Minas 53	1	Brazil	AF077230
HC-G9	1c	Indonesia	D14853	Minas 70	1	Brazil	AF077232
K1R2	1b	Japan	D50482	Minas 77	1	Brazil	AF077235
LC	1	Taiwan	U89019	Minas 82	1	Brazil	AF077236
HD1	1	Germany	U45476	Valdivia 2	1	Chile	AJ291456
L2	1	Korea	U01214	Valdivia 3	1	Chile	AJ291457
JK1	1b	Japan	X61596	Valdivia 4	1	Chile	AJ291458
HCV-J1	1	Japan	D10749	CR 15	1	Costa Rica	AJ437146
H77	1a	USA	AF009606	CR 20	1	Costa Rica	AJ437148
JS	1	Japan	D85516	CR 26	1	Costa Rica	AJ437150
HC-J6	2a	Indonesia	D00944	CR 29	3	Costa Rica	AJ437144
HC-J8	2b	Indonesia	D10988	CR 30	1	Costa Rica	AJ437147
BEBE1	2c	Italy	D50409	CR 32	1b	Costa Rica	AJ437145
JK049	3	Indonesia	D63821	CR 33	1	Costa Rica	AJ437149
NZL1	3a	Japan	D17763	ARHG166	1a/c	Argentina	AY376832
HCV-Tr	3b	Thailand	E10839	ARHG188	UT[a]	Argentina	AY376833
ED43	4a	Egypt	Y11604	ARHG205	UT	Argentina	AY376834
EUH1480	5a	United Kingdom	Y13184	ARHG238	UT	Argentina	AY376835
EUHK2	6a	Hong Kong	Y12083	ARHG239	UT	Argentina	AY376836
Montevideo 1	1	Uruguay	AJ012831	ARHG74	UT	Argentina	AY376837

[a] UT, untypeable.

Interestingly, the HCV strains from Central and South America were more related to each other than to the others from the rest of the world. This further supports the idea of regional diversification of HCV. In fact, a geographic distribution of HCV genotypes has been documented; genotypes 1, 2, and 3 are the most commonly detected worldwide (18). HCV genotype 1 in particular has been extensively reported by other authors in Argentina (5, 14), Chile (10), Venezuela (12), Uruguay (2), and Brazil (7). In a recent study, Pybus et al. used a mathematical model to analyze the epidemic behavior of HCV
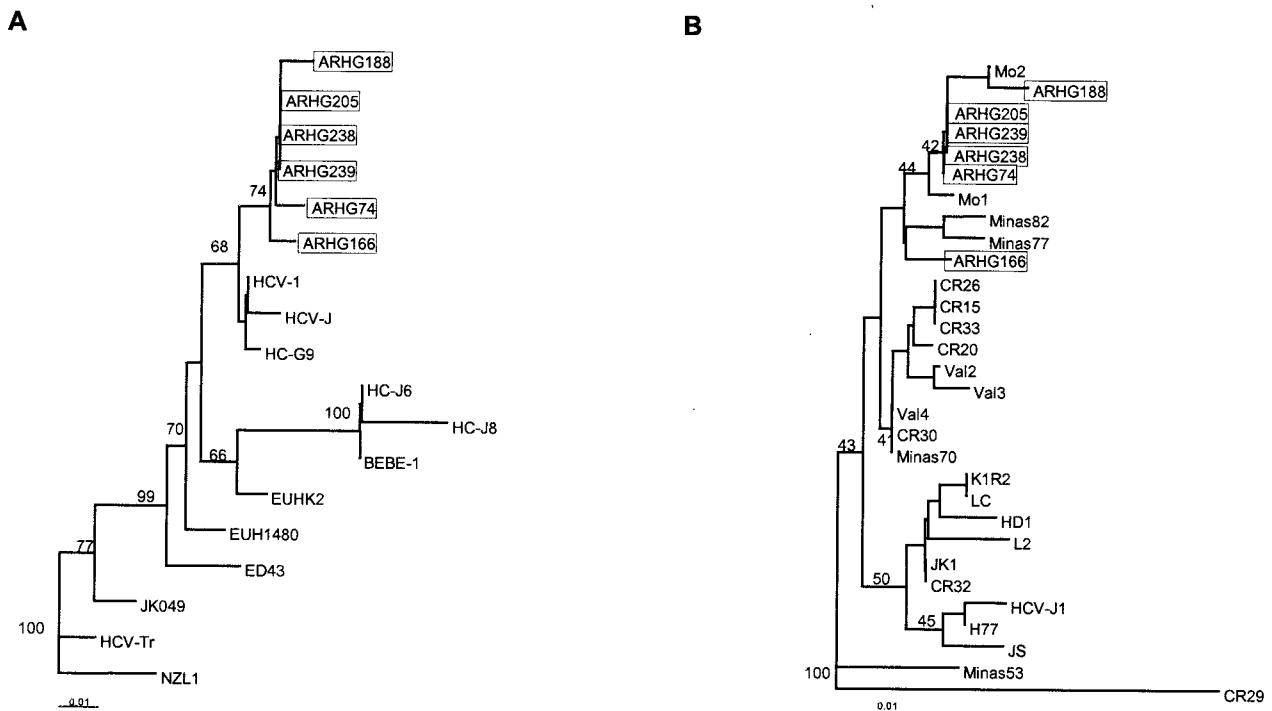

FIG. 3. Phylogenetic analysis of the amplified 5′UTR of untypeable isolates and corresponding sequences of other HCV strains. Isolates are shown by their names. The values obtained after 100 bootstrap resamplings are indicated. The horizontal branch lengths are proportional to the genetic distances. (A) Phylogenetic tree obtained with our untypeable isolates and prototypic HCV strains of all genotypes. (B) Phylogenetic tree of untypeable isolates and other genotype 1 isolates.

infection. Strikingly, it seems that HCV genotypes 1a and 1b originated about 100 years ago and are evolving at a faster rate than genotypes 4 and 6 (13). This dissimilar evolution rate between HCV genotypes may account for the mutated variant described in this paper that clustered into the novel genetic lineage reported by Vega et al. (Fig. 3B) (17).

The existence of HCV variants containing an A at position −235 may affect clade determination by RFLP in conventional diagnostic laboratories. Other authors have demonstrated that, as it is the most conserved region in the HCV genome, the 5′UTR may also be useful to identify many of the different genotypes by phylogenetic analysis (1). For genotype assignment purposes, our mutated isolates may be classified as genotype 1. Nevertheless, it should be borne in mind that although the untypeable strains disclosed a subtype a/c-associated pattern of bands when digested with *Bst*UI, phylogenetic analysis did not indicate an association between them and the prototypic genotype 1 subtype a and c strains. Thus, it may be advisable to classify the new HCV isolates showing this abnormal restriction pattern only as genotype 1, without a subtype label, until this novel genetic lineage is completely characterized.

Nucleotide sequence accession numbers. The GenBank accession numbers of the sequences reported in this work are AY376832 to AY376837.

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