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ORIGINAL ARTICLE

Molecular epidemiology and putative origin of hepatitis C virus in random volunteers from Argentina

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Abstract

AIM: To study the subtype prevalence and the phylogenetic relatedness of hepatitis C virus (HCV) sequences obtained from the Argentine general population, a large cohort of individuals was analyzed.

METHODS: Healthy Argentinian volunteers (n = 6251) from 12 provinces representing all geographical regions of the country were studied. All parents or legal guardians of individuals younger than 18 years provided informed written consent for participation. The corresponding written permission from all municipal authorities was obtained from each city or town where subjects were to be included. HCV RNA reverse transcription-polymerase chain reaction products were sequenced and phylogenetically analyzed. The 5′ untranslated region (5′UTR) was used for RNA detection and initial genotype classification. The NS5B polymerase region, encompassing nt 8262-8610, was used for subtyping.

RESULTS: An unexpectedly low prevalence of HCV infection in the general population (0.32%) was observed. Our data contrasted with previous studies that reported rates ranging from 1.5% to 2.5%, mainly performed in selected populations of blood donors or vulnerable groups. The latter values are in keeping with the prevalence reported by the 2007 Argentinian HCV Consensus (approximately 2%). HCV subtypes were

distributed as follows: 1a (25%), 1b (25%), 2c (25%), 3a (5%), and 2j (5%). Two isolates ascribed either to genotype 1 (5%) or to genotype 3 (5%) by 5'UTR phylogenetic analysis could not be subtyped. Subtype 1a sequences comprised a highly homogeneous population and clustered with United States sequences. Genotype 1b sequences represented a heterogeneous population, suggesting that this genotype might have been introduced from different sources. Most subtype 2c sequences clustered close to the 2c reported from Italy and Southern France.

CONCLUSION: HCV has a low prevalence of 0.32% in the studied general population of Argentina. The pattern of HCV introduction and transmission in Argentina appears to be a consequence of multiple events and different for each subtype.

Key words: Hepatitis C virus NS5B subtyping; Molecular epidemiology; Hepatitis C virus; Argentina; Hepatitis C virus 5' untranslated region

Core tip: This study reports an unexpectedly low prevalence of hepatitis C virus (HCV) (0.32%) in the general population of Argentina, with a subgenotype distribution of 1a (25%), 1b (25%), 2c (25%), 3a (5%), and 2j (5%) while two isolates ascribed either to genotype 1 (5%) or to genotype 3 (5%) could not be subtyped. Phylogenetic analysis of the NS5B region has enabled us to define the pattern of HCV introduction and transmission in Argentina as a consequence of multiple events that differed for each (sub)genotype studied. Furthermore, this report discusses the putative sources of HCV subgenotype introduction in Argentina.

del Pino N, Oubiña JR, Rodríguez-Frías F, Esteban JI, Buti M, Otero T, Gregori J, García-Cehic D, Camos S, Cubero M, Casillas R, Guàrdia J, Esteban R, Quer J. Molecular epidemiology and putative origin of hepatitis C virus in random volunteers from Argentina. *World J Gastroenterol* 2013; 19(35): 5813-5827 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i35/5813.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i35.5813

INTRODUCTION

The analysis of extensive sets of sequences from hepatitis C virus (HCV) isolates throughout the world has revealed the existence of six major genetic groups or genotypes (named 1 to 6), and a large number of subtypes within these groups (designated a, b, c, *etc.*)[1]. Overall sequence divergence ranges from 31% to 33% among genotypes and from 20% to 25% among subtypes[1,2]; in a single patient, cloned E1/E2 sequences may differ by up to 10%. The reason for this great variation is a high mutation rate and high level of viral replication through an error prone RNA polymerase without proofreading capacity. Consequently, in the infected individual, the virus circulates as a complex viral quasispecies[3] whose composition is subject to continuous changes due to competitive selection[4] and interactions among variants of with different levels of fitness[5]. The calculated average rate of fixation of mutations has consistently been found to range between 1.1 and 1.5 × 10^{-3} mutations per site and per year[6,7]. The rate of fixation of mutations is not evenly distributed throughout the genome, which has highly variable regions within the envelope coding genes and well conserved regions, such as the 5' untranslated region (5'UTR). The practical consequence of the high conservation at 5'UTR for HCV genotyping is that this region contains insufficient variation to solve HCV classification at the level of viral subtypes[8]. Furthermore, genotype 6 variants other than 6a and 6b show 5'UTR sequences identical or similar to those of type 1 and, therefore, cannot be differentiated[9-11]. It has been reported that sequence analysis of the highly conserved region in NS5B known as the "Okamoto region" (nt 8282 to 8610 in the H77 reference genome)[8] provides the best concordance with the full length genome phylogeny for precise genotype identification. The same primers can amplify genotypes 1 to 5, and they are less efficient for genotype 6 isolates, but they facilitate analysis and classification of the amplified sequences[11].

The prevalence of HCV infection in Argentina has been reported at 1.5% when all age groups are considered, and up to 2.0%-2.5% in adults[12], a rate in keeping with the value reported by the Argentinian Consensus on Hepatitis C (approximately 2%) in 2007. A higher prevalence (4.9%-5.7%) has been described in small rural communities[13,14]. HCV genotype distribution in Argentina in groups at risk of HCV infection (*e.g.*, transfusion patients, hemodialysis patients, intravenous drug users, healthcare workers) has been reported at 53.5% for genotype 1, 23% for genotype 2 (mainly by subtype 2c [HCV-2c]), 8.6% for genotype 3, and 14.8% for mixed infections[15]. Similar results have been reported in studies on sequences deposited in GenBank (GB) and analyses by the Los Alamos database http://hcv.lanl.gov, with few genotype distribution changes (79.5% for genotype 1, 13.9% for genotype 2, 3.9% for genotype 3, 2.4% for genotype 4), but with some differences depending on the HCV subgroup at risk studied[16-18]. Phylogenetic characterization of genotype 4 isolates from Argentina has traced an independent origin of the three sequences studied[17]. Interestingly, HCV-2c was the most prevalent subtype (58%) in the city of Córdoba (Central geographical region of the country), followed by HCV-1b (33%), and to a lesser extent by HCV-1a (11%), HCV-3a (3%) and HCV-4a (3%)[19,20].

Here, we report an unexpectedly low prevalence of HCV infection (0.32%) as measured by anti-HCV antibodies detected by using both a second generation enzyme immune assay (EIA) and a confirmatory immunoblotting, and HCV RNA detected by reverse transcription - nested polymerase chain reaction (RT-nested PCR)

Table 1 Epidemiological profile of the population studied

Province/city of residence	Total number of individuals	Male/female number	Age[1] (yr), mean ± SE
Buenos Aires/C.A.B.A.	1461	685/776[a]	33.4 ± 0.3 (11.2, 30)[d]
Catamarca	648	267/381	39.3 ± 0.5 (13.0, 38)[d]
Córdoba	1061	393/668[b]	37.1 ± 0.4 (12.5, 35)
Chaco	353	175/178[b]	40.2 ± 0.8 (14.1, 40)[d]
Chubut	172	83/89	37.4 ± 0.9 (12.1, 37)
Entre Ríos	474	225/249	38.5 ± 0.5 (11.6, 38)
Jujuy	176	105/71[b]	35.2 ± 0.8 (10.3, 34)[d]
Río Negro	329	149/180	40.1 ± 0.7 (12.8, 39)[d]
Salta	561	230/331	41.3 ± 0.5 (12.8, 41)[d]
San Luis	195	52/143[b]	41.6 ± 1.2 (16.3, 40)[d]
Santiago del Estero	375	164/211[b]	38.0 ± 0.7 (13.2, 35)
Tucumán	446	209/237	37.7 ± 0.6 (12.0, 35)
Total	6251	2738/3513	37.5 ± 0.2 (12.7, 35)

[1]Data are expressed as mean ± SE (SD, median). [a]$P < 0.05$, [b]$P < 0.01$, regarding the gender distribution (male/female ratio) within the whole population studied; [d]$P < 0.01$ regarding the mean age ± SD from the whole population studied. C.A.B.A.: Ciudad Autónoma de Buenos Aires, the national capital city.

targetting the 5'UTR HCV RNA in a cohort of random Argentinian volunteers. The genotypes detected and the putative origin of the HCV sequences are discussed based based on both their phylogenetic clustering and on such clustering relative to other Argentinian and worldwide derived sequences deposited in GB, in an attempt to trace how HCV could have been introduced in the local community here represented by the cohort studied.

MATERIALS AND METHODS

Throughout the 2000-2007 period, a total of 6251 serum samples were collected from healthy volunteers from 12 Argentinian provinces, as well as from the Ciudad Autónoma de Buenos Aires (C.A.B.A. - the capital city of the country) as follows: Buenos Aires province and C.A.B.A., $n = 1461$; Catamarca, $n = 648$; Córdoba, $n = 1061$; Chaco, $n = 353$; Chubut, $n = 172$; Entre Ríos, $n = 474$; Jujuy, $n = 176$; Río Negro, $n = 329$; Salta, $n = 561$; San Luis, $n = 195$; Santiago del Estero, $n = 375$; and Tucumán, $n = 446$ (Table 1).

Subjects included in this study [$n = 6251$; 2738 men; mean ± SE, 37.5 ± 0.2 years; mean ± SD = 37.5 ± 12.7; median age = 35 years (range 10-70 years)] were recruited as volunteers from the general population, local schools, and police stations, after being informed about the aim of the survey. All parents or legal guardians of individuals younger than 18 years provided informed written consent for participation. The corresponding written permission from all municipal authorities was obtained from each city or town where subjects were to be included.

Serological studies

The presence of anti-HCV antibodies was determined by using a second generation EIA test according to the manufacturer's recommendations (Abbott Diagnostics, North Chicago, IL, United States). Samples were further analyzed with a second generation recombinant immunoblot assay (RIBA 2.0: Chiron Corporation, Emeryville, CA, United States).

HCV-RNA detection and genotyping

Samples with serologically detectable anti-HCV antibodies were subjected to either RT-nested or RT-hemi-nested PCR amplification (see below). The 5'UTR region was used for RNA detection and initial genotype classification. The NS5B polymerase region, encompassing nt 8262-8610, was used for subtyping.

RNA extraction

RNA was extracted from 140 μL of serum by using the QIAamp Viral RNA Mini Kit (Qiagen Hilden, Germany). The measures to prevent contamination suggested by Kwok and Higuchi were strictly applied[21].

5'UTR RT-nested PCR amplification and sequencing

The 5'UTR RT-nested PCR was performed as follows. RT was carried out for 45 min at 42 ℃ (GeneAmp 2700 PCR system, Applied Biosystems, Foster City, CA, United States), using 50 U M-MLV reverse transcriptase, RNase H Minus, Point Mutant (200 U/μL Promega, Madison, WI, United States), 20 U RNase inhibitor (40 U/μL Promega, Madison, WI, United States), 10 mmol/L of each dNTP (Roche, Basel, Switzerland), 20 pmols of antisense PCR primer NR5 5'TGCTCATGGTGCACGGTCTACGAG3' and 1 × buffer from the high fidelity *Pfu* turbo DNA polymerase (Stratagene, San Diego, CA, United States) in a final volume of 20 μL. Then, 80 μL of PCR mix containing 1 × *Pfu* turbo buffer, 20 pmol of sense primer NF5 5'GTGAGGAACTACTGTCTTCACGCAG3' and 2.5 U *Pfu* turbo DNA polymerase were added to each tube. After an initial denaturation step of 2 min at 95 ℃, 5 initial cycles of 30 s at 94 ℃, 30 s at 55 ℃ and 2 min at 72 ℃ were carried out, followed by 35 cycles of 30 s at 94 ℃, 30 s at 60 ℃ and 2 min at 72 ℃, finishing with a single final step of 10 min at 72 ℃. Five microliters of the product were used for nested PCR, by using internal primers the internal primers, K80 5' AGCGTCTAGCCATGGCGT3' and K78 5'CACTCGCAAGCACCCTATCAGGCAGT3'. The nested PCR mix consisted of 1 × *Pfu* turbo buffer, 10 mmol/L of each dNTP, 20 pmol of internal primers, and 2.5 U of *Pfu* turbo DNA polymerase in a final volume of 100 μL. After a single denaturation step of 2 min at 95 ℃, we carried out 30 cycles of 30 s at 95 ℃, 30 s at 60 ℃, and 2 min at 72 ℃, and then, a final single step of 10 min at 72 ℃. The amplified products of 240 nucleotides length were analyzed by electrophoresis onto 2% agarose gels stained with ethidium bromide. PCR products were purified by using the QIAquick PCR purification kit for direct sequencing on an Abi Prism 310 Genetic analyser (Applied Biosystems).

NS5B RT-heminested-PCR amplification and sequencing

Extracted RNA was reverse transcribed using the de-

generate primer NS5B8704 5'GADGAGCADGATGTWATBAGCTC3' (nucleotide positions 8682-8704), where D = G + A + T, W = A + T and B = G + T + C, following the same conditions as for 5'UTR (see before). PCR was carried out by using the primer NS5B8256 5' TAYGAYACCMGNTGYTTTGACTC3' (nucleotide positions 8256-8278), where Y = C + T, M = A + C, and N = A + T + G + C, with an initial denaturation step of 2 min at 95 ℃, five initial cycles of 30 s at 95 ℃, 30 s at 43 ℃, and 2 min at 72 ℃, followed by 35 cycles of 30 s at 95 ℃, 30 s at 46 ℃ and 2 min at 72 ℃, and completed with a single final step of 10 min at 72 ℃. Heminested PCR was performed with an initial denaturation step of 2 min at 95 ℃, 30 cycles of 30 s at 95 ℃, 30 s at 48 ℃, and 2 min at 72 ℃, completed with a single final step of 10 min at 72 ℃ using primers NS5B8256 and NS5B8641 5'GARTAYCTGGTCATAGCNTCCGT3' (nucleotide positions 8641-8619), where R = A + G, to obtain a final product of 386 nucleotides. Purification and sequencing were performed as mentioned above.

Genotyping and subtyping-GB sequences

A GB query to Nucleotide collection (nr/nt), using the Megablast programme (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) was performed for each of the NS5B sequences obtained in this study. The 100 GB sequences with the highest sequence similarity to each of our samples were selected. A tree was constructed by using either the Neighbor-joining or Fast Minimum Evolution algorithm from a given matrix of distances using the Jukes-Cantor method to calculate the distances. For every query, we obtained a tree that situates each sequence with the most closely related reference from the GB. The most similar sequences were downloaded for further analysis, the genotype was assigned, and a putative origin of local isolates was inferred. GB was accessed to download already published sequences of Argentine origin by searching with the words "HCV" and "Argentina" on the website: http://www.ncbi.nlm.nih.gov/sites/gquery. Published sequences obtained by several authors[15,17,22,23] were downloaded in Fasta format, and their length was then adjusted using the GeneDoc program[24].

Phylogenetic analysis

Nucleotide sequences were resized by using GeneDoc and aligned by the CLUSTALW program[25]. Sequence similarity with other sequences from Argentina and with other GB sequences was ascertained by both distance and parsimony methods. Statistical support for each node in the trees drawn by both methods was obtained by performing 100 or 1000 bootstrap replicates of the original nucleotide sequence alignment. Phylogenetic analysis was carried out with the PHYLIP package[26]. Trees were drawn by using the Treeview program, v. 1.6.5[27].

Genetic divergence analysis

Genetic divergences were calculated by using the DNASP program (version 3.53)[28] in three populations: our sequences grouped as a population, the closest GB sequences as another, and other Argentine sequences as the third one. Pairwise distances were calculated by using the MEGA3.1 program.

Statistical analysis

They were performed by using either the GraphPath Prism (version 5.0 for Windows) or the SigmaStat software. The non-paired Student's *t* test was used to analyze the statistical differences between the mean age ± SD of the whole population of the country regarding those values recorded from each Province (therefore, examining a data set from two groups), as well as for such comparison with previous studies. When such study was performed among three or more groups, the one-way analysis of variance (ANOVA) was applied. Pairwise distances were statistically compared by using the χ^2 test with Yates' correction (SigmaStat software). *P* values lower than 0.05 were considered statistically significant.

RESULTS

HCV prevalence in the general population of Argentina

A total of 6251 serum samples from a cohort of random volunteers was studied. Initially, 25 samples (0.40%) tested anti-HCV antibody positive as determined by EIA. However, 5 of the 25 samples failed to exhibit specific anti-HCV antibodies by immunoblotting analysis and also tested negative by RT-nested PCR to detect HCV RNA; hence, they were discarded for further studies.

HCV RNA was detected in serum samples from 7 out of 12 provinces. The prevalence of ongoing HCV infection as determined by RT-nested PCR from 12 of 23 provinces of Argentina, representing 73% of the country's total population, was 0.32%. The highest prevalence was detected in Buenos Aires province (0.62%; 9/1461), a geographical area inhabited by 40.52% of the total population of Argentina. In the remaining provinces studied, the prevalence ranged from 0% in Chaco, Chubut, Entre Ríos, Jujuy, and San Luis, to up to 0.53% in Santiago del Estero. Intermediate values were observed in Salta (0.18%) and Córdoba (0.19%), Río Negro (0.30%), as well as in Catamarca (0.46%) and Tucumán (0.45%).

Genotype 1 was the most prevalent, accounting for 55% (11/20) of infected individuals, 25% were subtype 1a (5/20) and 25% subtype 1b (5/20). The second in prevalence was genotype 2 accounting for 35% (7/20); most sequences were ascribed to subtype 2c (n = 5), except one that clustered much closer to subtype 2j reference sequences. Lastly, genotype 3 accounted for the remaining 10% (2/20; Table 2). One genotype 1, one genotype 2 , as well as one genotype 3 isolates (according to their initial 5'UTR genotype assignment) were excluded from further analysis because of their respective NS5B RT-heminested PCR amplification failure.

Phylogenetic analysis and genetic divergence

NS5B phylogenetic trees and divergence analysis were

Table 2 Genotype and subtype assignment of the Argentinian isolates studied by using both 5'-untranslated region and NS5B sequences

HCV genotype	HCV[+] 5'UTR sequences	Relative	From total population (n = 6251)	HCV subtype	HCV[+] NS5B sequences	Relative	From total population (n = 6251)
1	11	55.00%	0.176%	1a	5	25.00%	0.080%
				1b	5	25.00%	0.080%
				1[1]		5.00%	0.016%
2	7	35.00%	0.112%	2c	5	25.00%	0.080%
				2j	1	5.00%	0.016%
				2[1]		5.00%	0.016%
3	2	10.00%	0.032%	3a	1	5.00%	0.016%
				3[1]		5.00%	0.016%
Total	20	100.00%	0.320%	-	17	100.00%	0.320%

[1]Untypeable at NS5B due to reverse transcription-heminested polymerase chain reaction amplification failure. HCV: Hepatitis C virus; 5'UTR: 5'-untranslated region.

Table 3 Genetic divergence (DNASP software)

	Argentine general population sequences	GB deposited sequences from Argentina	Closest GB deposited sequences
HCV-1a			
Argentine general population sequences	0.034		
GB deposited sequences from Argentina	0.042	0.040	
Closest GB deposited sequences	0.034	0.037	0.032
HCV-1b			
Argentine general population sequences	0.082		
GB deposited sequences from Argentina	0.074	0.046	
Closest GB deposited sequences	0.054	0.050	0.045
HCV-2c			
Argentine general population sequences	0.087		
GenBank deposited sequences from Argentina	0.092	0.098	
Closest GB deposited sequences	0.076	0.080	0.076

Hepatitis C virus (HCV)-1b (Argentine general population sequences): 0.082 *vs* 0.054, $P < 0.001$; 0.082 *vs* 0.074, $P > 0.05$; HCV-1b [GenBank (GB) deposited sequences from Argentina]: 0.046 *vs* 0.050, $P < 0.001$; HCV-2c (Argentine general population sequences): 0.087 *vs* 0.076, $P < 0.001$; 0.087 *vs* 0.092, $P > 0.05$; HCV-2c (GB deposited sequences from Argentina): 0.098 *vs* 0.08, $P < 0.001$. All P (HCV-1a) > 0.05. Genetic divergence (DNASP software), considering three separate HCV groups: our Argentine general population (sequenced in this study), Argentinian sequences already deposited in GB, and the closest GB worldwide sequences. All values obtained were statistically paired and compared by using the χ^2 test with Yates' correction.

performed by using the five HCV-1a, five HCV-1b, and five HCV-2c sequences. However, divergence could not be ruled out with those genotypes encompassing one single representative sequence (as occurred with 2j and 3a).

As expected, local NS5B sequences clustered together with their corresponding GB references (Figure 1), but the profile differed depending on the genotype. All of our HCV-1a Argentinian sequences clustered together with strong bootstrap support (92%; Figure 2A) and exhibited a low genetic divergence (0.034; Table 3). Furthermore, genetic divergence was not statistically different in comparison with other Argentinian sequences deposited in GB or with the closest non-Argentinian GB deposited sequences included in the study, suggesting a putative common source of infection/transmission.

In the case of Argentinian HCV-1b, we observed two clusters with very low bootstrapping support (19%-25%), and one sequence distantly located regarding such clusters (Figure 2B). Genetic divergence was higher (0.082) than that observed among GB deposited Argentinian 1b sequences but the differences were not statistically significant. Similarly, HCV-2c sequences were intermingled along the tree with no particular clustering (Figure 2C) and showed a high genetic divergence (0.087). Phylogenetic analysis of Argentinian HCV-1b and HCV-2c sequences suggested a different origin of infection/transmission when compared with HCV-1a sequences.

Origin of closest GB sequences

The closest GB sequences obtained by distance (UPGMA and NJ) and parsimony methods (DNAPARS) are represented in Table 4 (trees not shown). The geographic localization of the closest GB sequences is represented in Figure 3.

All local HCV-1a sequences grouped with GB United States sequences (St. Louis, Boston, or New York areas). The results suggest a narrow source of infection and not a multifocal event, and are consistent with the low degree of divergence found in the Argentine general population, despite the fact that the subjects studied resided in three different provinces (Buenos Aires, Córdoba, and Río Negro), and from whom serum sampling was performed, several hundred of kilometers apart from each other.

HCV-1b sequences grouped with those from all over the world, including Europe (Spain, Russia), Asia (China), Africa (Madagascar, Tunisia) and North America (United States), and represented a heterogeneous population (Table 3).

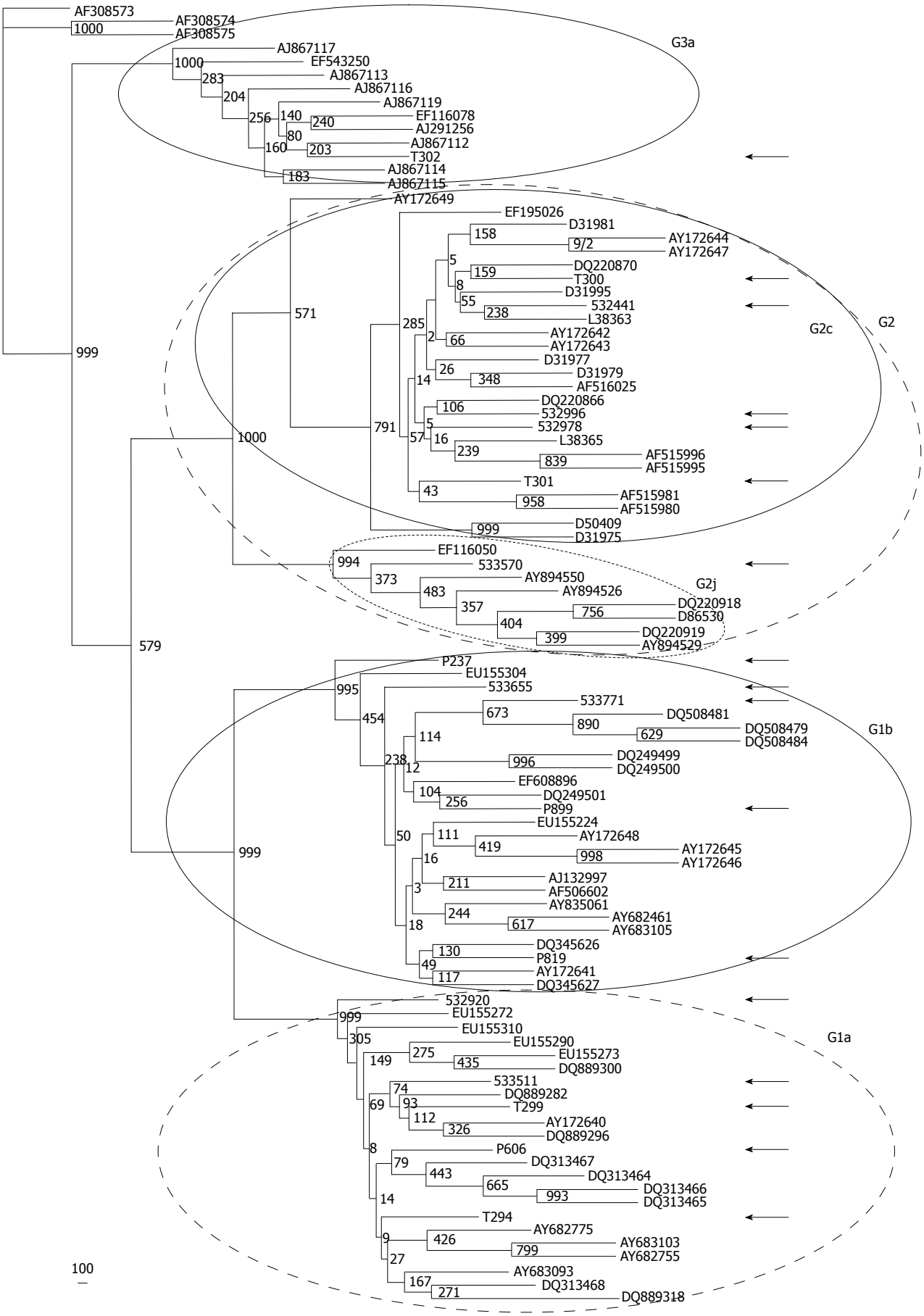
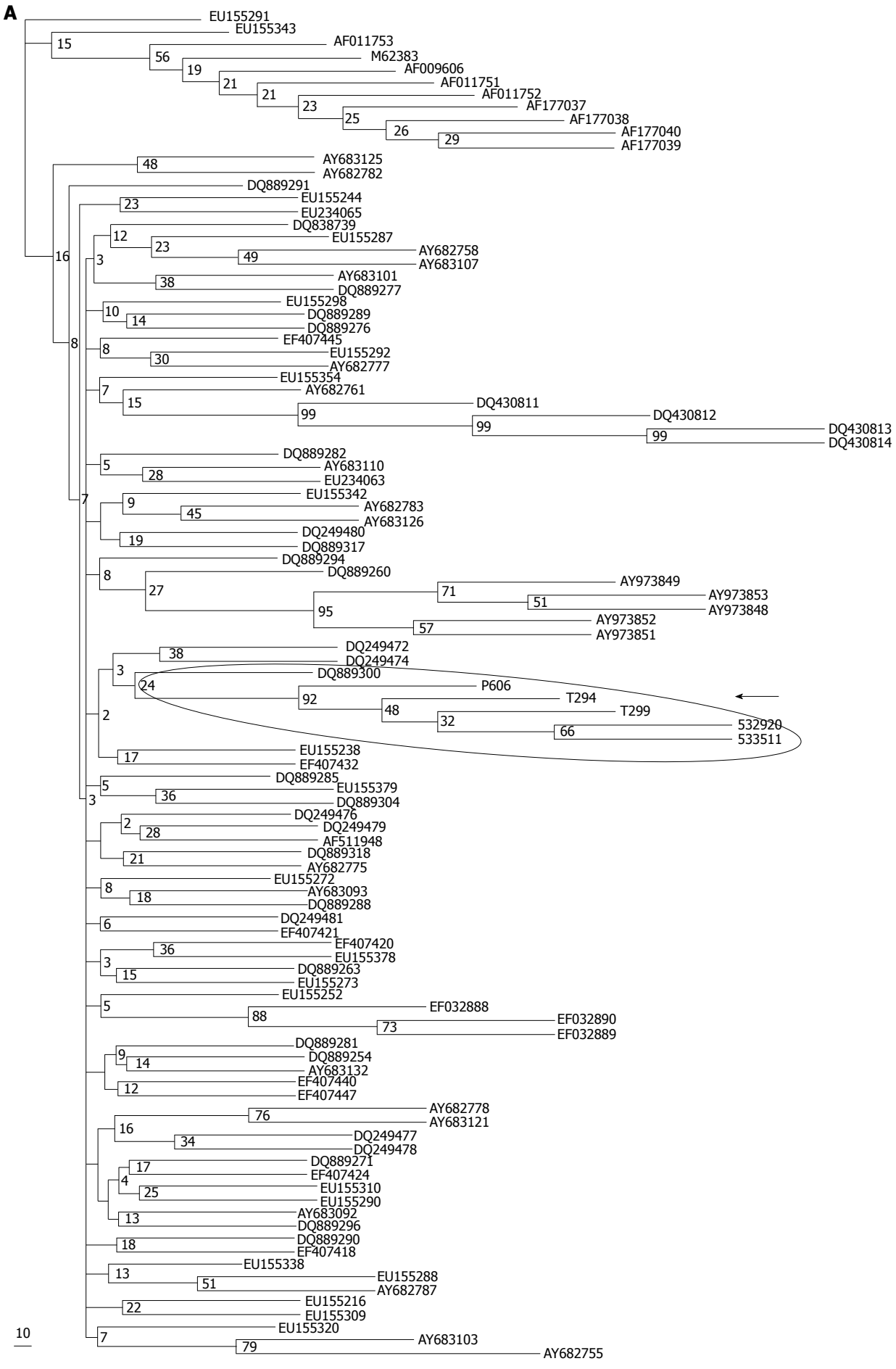


Figure 1 Assignment of Hepatitis C virus subtype isolates according to the phylogenetic tree performed by the Neighbor-Joining method, after an NS5B alignment of Hepatitis C virus sequences obtained from the 17 Argentinian volunteers studied herein, as well as from 89 reference sequences (*e.g.*, those composing subgenotype groups G1a, G1b, G2c, G2j, G3a) downloaded from GenBank. A consensus tree is shown after analyzing 1000 replicate trees. A distance scale (in nucleotide substitutions per position) is shown. Arrows indicate Hepatitis C virus sequences derived from Argentinian volunteers.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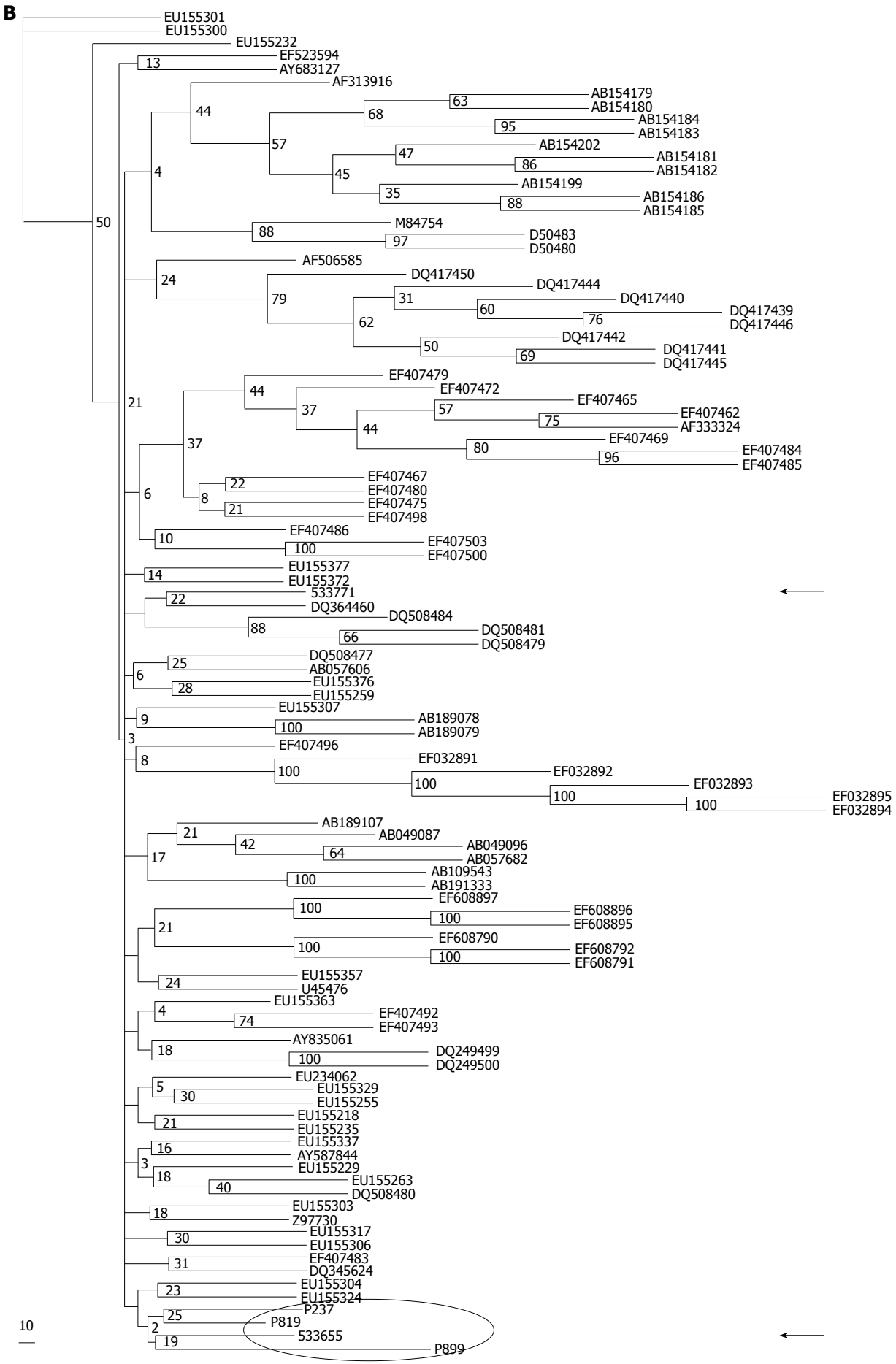
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AB154186
AB154185
M84754
D50483
D50480
AF506585
DQ417450
DQ417444
DQ417440
DQ417439
DQ417446
DQ417442
DQ417441
DQ417445
EF407479
EF407472
EF407465
EF407462
AF333324
EF407469
EF407484
EF407485
EF407467
EF407480
EF407475
EF407498
EF407486
EF407503
EF407500
EU155377
EU155372
533771
DQ364460
DQ508484
DQ508481
DQ508479
DQ508477
AB057606
EU155376
EU155259
EU155307
AB189078
AB189079
EF407496
EF032891
EF032892
EF032893
EF032895
EF032894
AB189107
AB049087
AB049096
AB057682
AB109543
AB191333
EF608897
EF608896
EF608895
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EU155329
EU155255
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EU155337
AY587844
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10

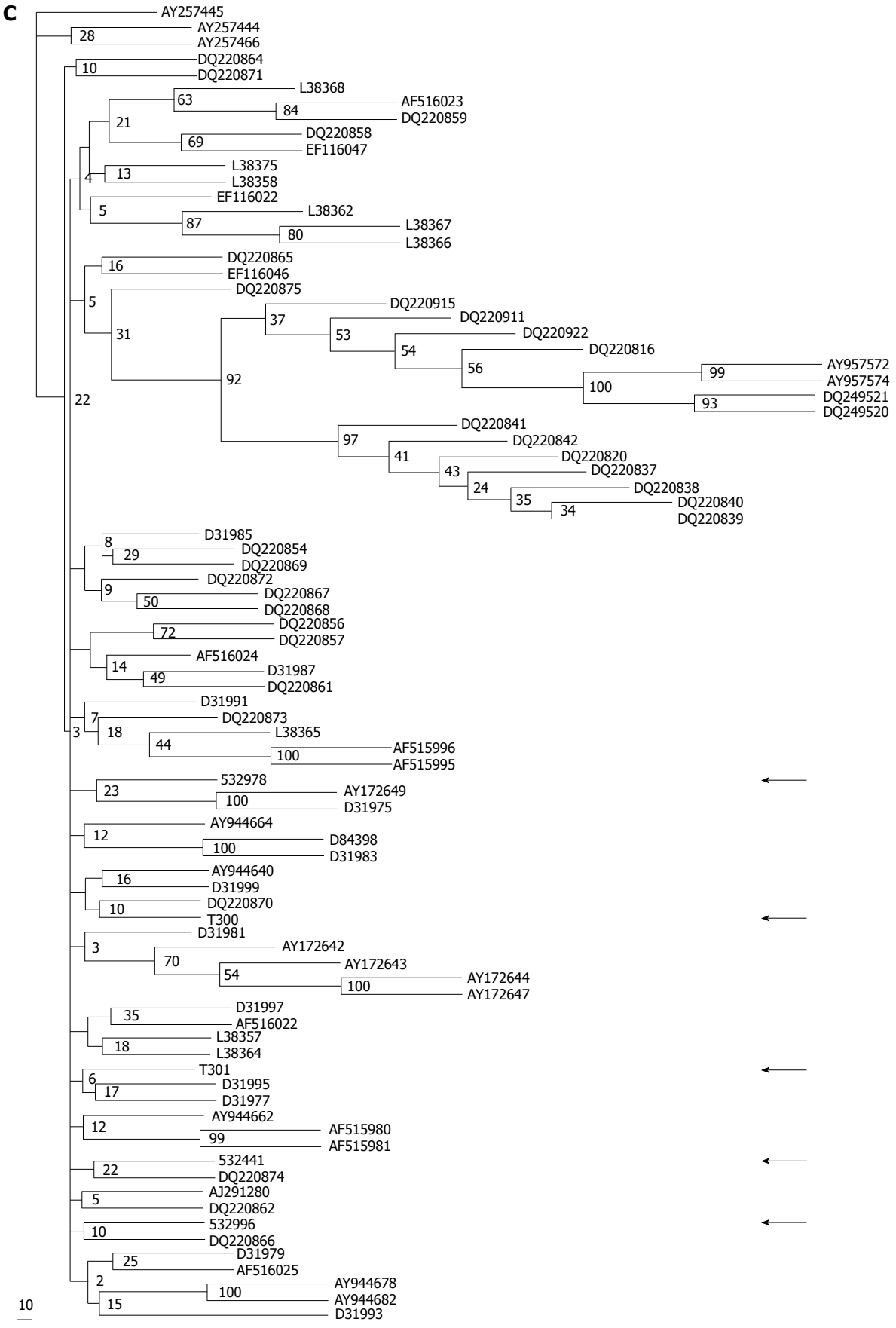

Figure 2 Phylogenetic trees performed by the Neighbor-Joining method. NS5B sequences obtained from the volunteers whose respective hepatitis C virus (HCV) isolates had been classified as HCV-1a (n = 5), HCV-1b (n = 5) or HCV-2c (n = 5) were respectively analyzed in panels A, B and C together with 100 HCV-1a, 100 HCV-1b or 81 HCV-2c sequences downloaded from the GenBank. The respective consensus trees are shown after analyzing 100 replicate trees for each HCV subtype. A distance scale (in nucleotide substitutions per position) is shown in each panel. Arrows indicate sequences obtained from Argentinian volunteers.

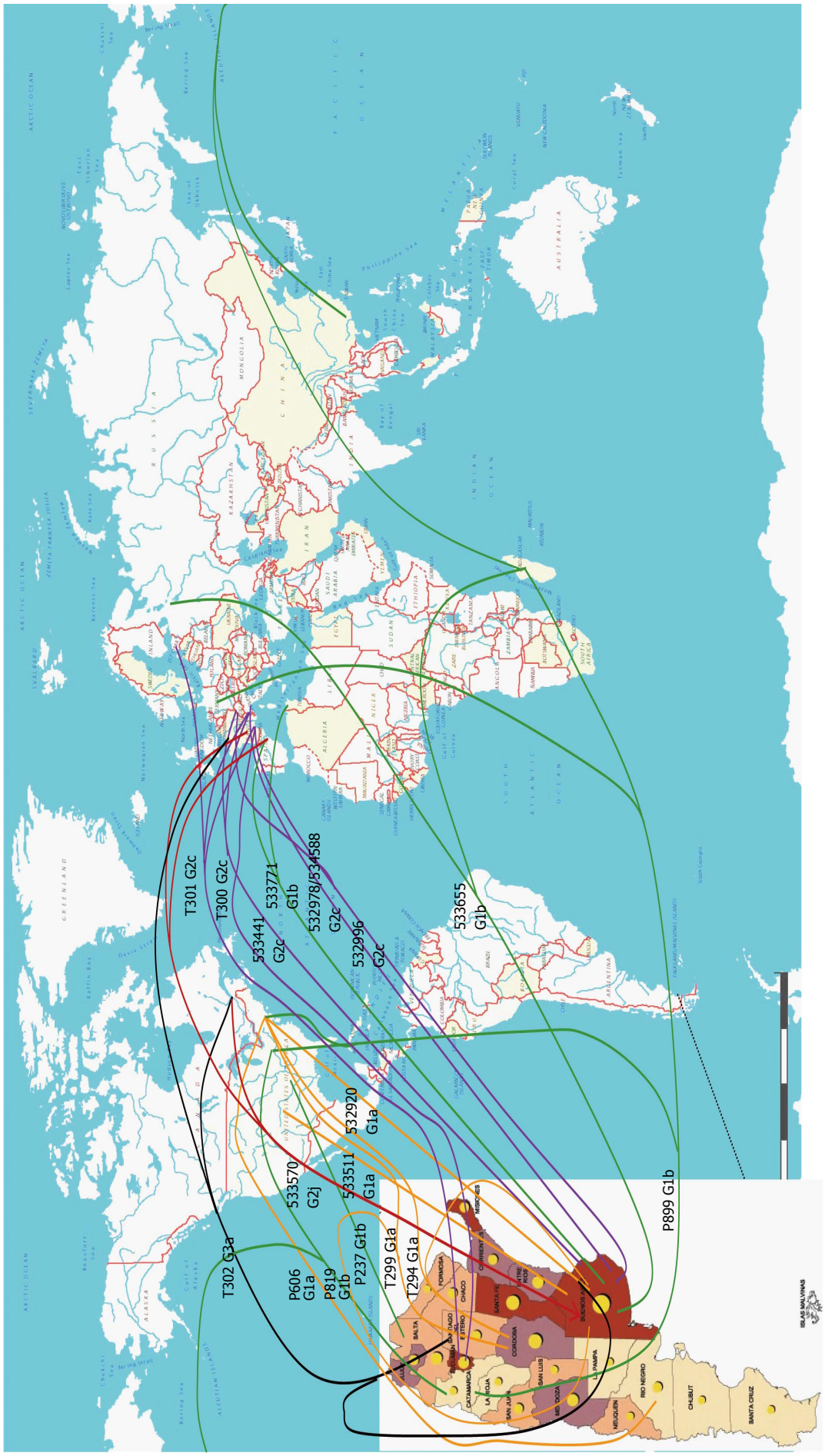

Figure 3 Each line links the Argentinian location of the sequence named on the top of the line with the world location/s of the most closely related sequence/s published in GenBank.

Table 4 Subtype assignment of each hepatitis C virus Argentinian sequence obtained in this study

Isolate	NS5B genotype	Closest GB deposited sequences	Origin
532920	1a	DQ8893001bos	United States (Massachusetts, Boston area)
533511	1a	EU155310usa	United States
		EU155290usa	
		AY683093usa	
		DQ8893001usa	
P606	1a	AY682755alb	United States (Albany, NY)
		AY683103alb	
		AY682775alb	
		DQ889318bos	United States (Massachusetts, Boston area)
		DQ889300bos	
T294	1a	DQ313467arg	Argentina
		DQ313464arg	
		DQ313465arg	
		DQ313466arg	
		DQ889300bos	United States (Massachusetts, Boston area)
T299	1a	DQ889300bos	United States (Massachusetts, Boston area)
		DQ889296bos	
		AY172640arg	Argentina
		DQ889296bos	United States (Massachusetts, Boston area)
533655	1b	AF506602rus	Russia (Western Siberia)
		DQ345627mad	Madagascar (Antananarivo)
533771	1b	DQ508484tun	Tunisia (Tunis)
		DQ508481tun	
		DQ508479tun	
		EF608896bcn	Spain (Barcelona)
P237	1b	EU155224ten	United States (Tennessee)
		EU155304ten	
P819	1b	AY835061chn	China (Foshan)
		DQ249500usa	United States (factor Ⅷ concentrate)
		DQ249499usa	
		DQ345626mad	Madagascar (Antananarivo)
P899	1b	DQ345626mad	Madagascar (Antananarivo)
		AJ132997ger	Germany
		AY682461alb	United States (Albany, NY)
		AY683105alb	
		DQ249501usa	United States (factor Ⅷ concentrate)
532441	2c	L38363swi	Switzerland
		D31981ita	Italy (France from Italians)
532996	2c	L38365swi	Switzerland
		AF15995mar	France (Marseille)
		AF515996mar	
		DQ220866tou	France (Toulouse)
532978	2c	D31975ita	Italy (France from Italians)
534588		AY172649ita	
		D50409(BEBE1)ita	
T300	2c	DQ220870fr	France (Toulouse)
T301	2c	AF516025mar	France (Marseille)
		EF195026tall	Estonia (Tallinn)
		AY944641gen	Italy (Genoa)
		D31977ita	Italy (France from Italians)
533570	2j	AY89526que	Canada (Quebec)
		AY894529que	
		AY894550que	
		EF116050que	
		DQ220919tou	France (Toulouse)
		DQ220918tou	
		D86530bcn	Spain (Barcelona)
T302	3a	EF116078que	Canada (Quebec)
		AJ291256ssd	France (Seine Saint Denis district)
		AJ867113arg	Argentina
		AJ867159arg	
		AJ867116arg	
		AJ867115arg	
		AJ867114arg	

The 3rd column from the left shows the GenBank (GB) sequences that are most similar to each of our samples, and the 4th column from the left exhibits the origin of the GB already deposited sequences.

HCV-2c sequences from the Argentine general population formed a heterogeneous group with a completely different pattern as compared with HCV-1b. The sequences clustered with GB sequences originated from HCV patients in Italy. Even the sequences reported from Southeastern France were obtained from Italians living in this region. Only one of the clustered GB sequences was documented in Estonia. Three of our 2c sequences were detected in Buenos Aires/C.A.B.A. and two in Tucumán (approximately 1200 km from C.A.B.A.).

When blasted with GB sequences, the single sequence assigned to HCV-2j clustered with those from Canada, France, and Spain, showing the high heterogeneity among the HCV-2j sequences analyzed.

Regarding the single HCV-3a local sequence, similarities were found with one sequence from Canada and another one from France, and with an additional group of five sequences reported in Argentina.

DISCUSSION

Here we report a very low prevalence of HCV infection (0.32%) in a large cohort of random volunteers from Argentina, contrasting with the 2% prevalence previously reported in studies based on selected populations in small communities, or even higher rates among highly vulnerable groups[12,29] (Hepatitis C Argentinian Consensus, 2007). The observed prevalence is lower than that reported in neighboring countries, such as Brazil (1.5%), Uruguay (1%), and Chile (0.85%)[12]. The highest prevalence was detected in Buenos Aires province and C.A.B.A., both making up the region that received the greatest number of European immigrants, especially during the first half of the 20th century (70.38% of all immigrants, 20.8% residing in C.A.B.A.; http://www.mininterior.gov.ar/poblacion/estadisticas.asp Censo2001). In this regard, recent data shows that at present most of the European immigrants from Italy and Spain are over 60-year-old (http://www.mininterior.gov.ar/provincias/archivos_prv25/6-%0Perfil_Migratorio_de_la_Argentina.pdf).

The HCV isolates studied here did not form a close national cluster separate from the GB sequences. Interestingly, genetic divergence and phylogenetic analyses showed a different profile depending on the subtype analyzed. In this sense, the HCV-1a samples, detected from subjects residing in distantly placed cities/towns (hundreds of kilometers apart from each other) from three provinces, made up a highly homogeneous population, whereas the HCV-1b and HCV-2c samples were more heterogeneous, suggesting a different profile of epidemiological origin/transmission of infection for each subtype. The high homogeneity of subtype 1a and its similarity with sequences reported from United States suggest that HCV-1a was introduced in Argentina by a common infectious source from this geographic area. This finding agrees with the model of recent HCV genotype diversification in Central and South America[30-32] compared with other continents. HCV-1b isolates formed separate clusters that were most similar to European sequences, suggesting multiple focal transmission events, likely with independent geographical origins. Interestingly, the subject whose HCV isolate showed an HCV-1b phylogenetic relationship with a Russian HCV-1b sequence stated such ethnicity. HCV-2c represents an important contribution to Argentinian HCV epidemiology (at least, 25% in this study), supporting previous observations (23%)[15]. Most of the 2c isolates clustered close to sequences reported from Italy and Southern France. In general, the 2c sequences deposited in GB represent a highly heterogeneous population with huge genetic diversity in Ghana, Guinea, Benin, and Burkina Faso in Africa[33], suggesting that HCV-2c has long been present in human populations, especially in these parts of Africa, and that it spread to Egypt, Europe and elsewhere in the 20th century[34]. It has been proposed that the introduction of HCV-2c in Italy was a consequence of close contacts between native Africans and soldiers and colonials during the colonial wars in 1882-1896 and 1911-1912[35,36]. A high prevalence of HCV-2c was observed among individuals in Italy[37-40] and Southern France, all related with Italian immigrants[41]. Coincidentally, a substantial percentage of the Buenos Aires population descends from Italian immigrants that arrived in the 20th century. Taken together, our results suggest that the introduction of HCV-2c in Argentina may have been the result of a multiple event, likely related to waves of Italian immigration. In this regard, it is worth mentioning that a high prevalence (approximately 50%) of this genotype has been reported among chronic HCV patients from Córdoba province[19,20,29], as compared with data from Buenos Aires and C.A.B.A. patients[15] and even higher rates (90%) from patients residing in Cruz del Eje, a small rural town located in the Northern region of Córdoba province, where HCV prevalence was reported to be 5%[29]. In contrast, the present study could not detect the circulation of such genotype from the general population studied in the city of Córdoba (encompassing the whole group from the homonymous province). Several hypothetical factors might have contributed to the observed discrepancy, among them, it seems worth mentioning: (1) the previously reported overall low prevalence of infection in the city of Córdoba[29] and in this study; (2) the lower contribution of HCV-2c to the total HCV genotype prevalence in such capital city located in the central region of the province, as compared with Cruz del Eje[20,29]; (3) the dissimilar nature of studied groups (patients versus general population), hence showing a dissimilar HCV infection prevalence, and consequently having lower probability to pick up HCV positive samples; and (4) the mean age ± SE of all the analyzed populations (49.77 ± 2.15 for patients from Córdoba and other locations of Córdoba Province (n = 26)[29], 66.15 ± 1.52 years for patients from Cruz del Eje (n = 49)[29], as compared with 37.1 ± 0.4 in this study (SD = 12.5; median age = 35 years; n = 668). However, the last mentioned factor failed to reach statis-

tical significance when the one way analysis of variance was carried out ($P = 0.1177$).

The recorded HCV-3a sequence exhibited similarities with isolates from France and Canada and other Argentinian isolates, in concordance with a more recent, worldwide expansion of this subtype[22]. The HCV-2j sequence showed similarities with French, Canadian, and Spanish HCV sequences. No other genotypes (4, 5 or 6) were detected in the Argentine general population studied.

In conclusion, NS5B analysis allowed an accurate classification of subtypes and enabled to perform the study of the evolution and origin of HCV infection. Here, we report a very low prevalence of HCV in the Argentine general population (0.32%). Phylogenetic analysis suggests diverse profiles of epidemiological expansion of HCV in Argentina: HCV-1a might have occurred from a putative common source, whereas HCV-1b and HCV-2c might have been introduced into the country following fluxes of immigration from other endemic areas, especially from Europe. The significantly high number of HCV-2c sequences compared to the reported data from neighboring countries may be the consequence of the high percentage of Italians migrating to Argentina from an area where such subtype was (and still is) highly prevalent. Argentina is a good example of how human practices, together with global expansion and human migration flows, have increased the HCV spread over the world. Adherence to standard universal precautions to avoid transmission should be strictly followed even in countries with a low prevalence of HCV.

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COMMENTS

Background

Hepatitis C virus (HCV) is a leading cause of chronic liver disease. HCV is distributed globally, affecting all countries with an estimated worldwide prevalence of 2.3% (approximately 160 million people) of the whole general population. Comparisons of HCV nucleotide sequences derived from individuals from different geographical regions revealed the circulation of at least six major HCV genotypes and more than 50 subtypes. Accurate HCV genotyping in chronically infected patients is crucial not only for epidemiological studies but also from a clinical standpoint, since the HCV genotype orientates the treatment strategy.

Research frontiers

Direct sequencing, also referred as "population" sequencing, is the gold standard for HCV genomic sequence analysis. The viral genome region(s) sequenced must be carefully chosen, because not all of them provide accurate typing and subtyping. Since genotyping methods based on the exclusive analysis of the 5'NCR may lead to up to 10% mistyped results, there is a need to extend the analysis to coding regions such as NS5B or core. In this regard, the knowledge of the implicated HCV genotype in each patient contributes to select the appropriate treatment. Those infected with HCV genotype 1 must be treated with a triple combination of pegylated interferon-α (IFN-α), ribavirin and either telaprevir or boceprevir, whereas patients infected with other genotypes must still be treated with pegylated IFN-α and ribavirin alone. Moreover, HCV genotyping based on phylogenetic analysis, and - in case a representative sampling of a given (sub)genotype is obtained from an area - Monte Carlo Markov Chains Bayesian coalescent analysis may respectively lead to trace the origin and if such condition is met - the putative date of the Most Recent Common Ancestor of sequences.

Innovations and breakthroughs

This is a molecular epidemiological study performed in a large cohort of the local general population from 12 out of 23 Argentine provinces, as well as from the Autonomous city of Buenos Aires (the national capital). Unexpectedly, it shows a low prevalence of HCV (about 0.32%) in a general population cohort which included 6251 individuals. This low prevalence suggests that HCV could have been "recently" introduced in Argentina, as proposed by coalescent studies performed in restricted local areas of this country by other authors, where a predominant (sub)genotype was found, allowing such analysis. HCV subtypes were distributed as follows: 1a (25%), 1b (25%), 2c (25%), 3a (5%), and 2j (5%). HCV-1a sequences comprised a highly homogeneous population and clustered with United States sequences. HCV-1b sequences represented a heterogeneous population, suggesting that this genotype might have been introduced from different sources. Most HCV-2c sequences clustered close to the 2c reported from Italy and Southern France. Phylogenetic analysis is used by the authors to trace the putative source of HCV transmission and suggests that introduction of local HCV in this country is a consequence of multiple events that differed for each subtype studied. Diverse epidemiological patterns of HCV spread in Argentina might have occurred.

Applications

These new data could be useful to implement suitable measures regarding HCV surveillance by Argentine Public Health authorities.

Terminology

HCV genotype: group of HCV variants assigned to a given genetic groups (1-6) which differs from others by 31%-33% at the nucleotide level. HCV subtype (sub-genotype): group of more closely related HCV variants assigned to a given genetic group which differs from others by 20%-25% at the nucleotide level (named with lower case letters: *i.e.*, a, b, c, *etc.*).

Peer review

This is a very well done and written molecular epidemiological study which considers the investigation of the prevalence of HCV infection and subtype frequencies among adults in Argentina. It should be underlined that authors have investigated a large amount of general population from 12 provinces representing all the geographical regions of the country.

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