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GENOMIC AND PHYLOGENETIC ANALYSIS OF HEPATITIS C VIRUS STRAINS FROM ARGENTINA

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Abstract HCV genomic characterization was performed by nucleotide sequence analysis (n=50) combined with restriction fragment length polymorphism (RFLP) of the 5' UTR region in 82 isolates corresponding to different Argentine groups. Genotype 1 was detected in 70.7 % of the samples (58 out of 82), genotype 2 in 21.9% (18 of 82) and genotype 3 in the remaining 6 sera (7.3%). HCV 1b subtype contributed with 35.3 % to the whole population studied (29 of 82) and was detected in 6 out of 21 sporadic cases. Besides their epidemiological significance, these results should be taken into account when future vaccines are considered on the basis of geographical HCV genotypic prevalence.

Resumen ***Análisis genómico y filogenético de cepas del virus de Hepatitis C de Argentina.*** El análisis del RNA del virus de la hepatitis C (HCV) permite clasificar diferentes aislamientos por lo menos en seis genotipos que a su vez abarcan diverso número de subtipos. Algunos de ellos se asocian a diferencias en el curso evolutivo de la infección y a una distinta sensibilidad al tratamiento antiviral. Este estudio muestra el análisis mediante secuenciación nucleotídica combinado con el del polimorfismo del tamaño de fragmentos de restricción de la región 5' UTR de 82 cepas de HCV de Argentina provenientes de múltiples grupos poblacionales. El genotipo 1 fue detectado en el 70,7% de las muestras (58 / 82), el tipo 2 en el 21,9% (18 / 82) y el genotipo 3 en los restantes 6 sueros (7,3%). El subtipo 1b contribuyó con un 35,3 % al total de la población estudiada (29 / 82) y fue detectado en 6 de 21 casos esporádicos (28,5%). Además de aportar nuevos datos a la epidemiología molecular regional del HCV, la prevalencia de los genotipos aquí descriptos deberá ser considerada al momento de evaluarse futuros ensayos de vacuna.

Key words: hepatitis C virus, HCV genotyping, HCV nucleotide sequence.

Hepatitis C virus (HCV) is the etiologic agent of most parentally transmitted non A - non B hepatitis. A chronic course is observed in more than 50% of infected patients, who may develop cirrhosis and even hepatocellular carcinoma[1].

HCV is a single strand RNA virus with a positive polarity having a nucleotide extension of approximately 10,000 bases. Its viral genome shows two non-coding regions (5'UTR and 3'UTR) which flank a central region coding for a polyprotein, from which derive after post-translational cleavage structural (envelope and core) and non-structural proteins (NS2 - NS5). Most conserved regions are those located at the 5' end and within a subregion of the 3' end[2]. In contrast, regions showing greater variability are present within coding regions for gp1 and gp2 glycoproteins[3].

HCV presents a hierarchical distribution mainly based on a dissimilar degree of nucleotide sequence homology, which enables its classification in various groups (types) and subtypes. Each group consists of one or multiple HCV nucleotide sequences obtained from infected individuals (isolates). Within each infected individual, multiple related viable viral genomes (quasispecies), cocirculate, which may be differentiated by only a few nucleotides. This is a feature of RNA viruses and is mainly associated to their genomic replication. Such genomic variability is also related to both the possibility to induce viral persistence and the appearance of drug resistance, as well as posing a major difficulty to obtain a vaccine.

The high degree of HCV nucleotide heterogeneity has led to a considerable controversy in nomenclature and classification of this virus.

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A convenient system has been proposed[4] which includes 6 different types (namely 1,2,3, etc.) with a sequence homology below 69% *inter se*. Each type includes one or several subtypes (named a, b, c, etc. according to their date of discovery). Among subtypes belonging to the same type roughly 79% sequence homology is observed. Finally, such subtypes include a variable number of isolates, which exhibit more than 88% nucleotide sequence homology[5].

Among non-commercial techniques for HCV genotyping, analysis by restriction fragment length polymorphism (RFLP) of amplicons obtained by reverse transcription coupled to Nested PCR of the 5' UTR region[6,7] and subtype specific core-based PCR amplification[8,9,10] have been widely used. However, none of these HCV typing methods -as well as others recently manufactured- are as conclusive as the complete genomic sequencing of each isolate. However, despite being the gold standard, such method cannot be usually applied due to its high complexity, raised costs, and time requirements which are incompatible with diagnostic needs.

HCV genomic characterization is relevant not only for molecular epidemiological studies[11] but also for a proper interpretation of diagnostic tests currently marketed to detect specific antibodies or viral genomes[6,12,13].

Viremia levels[14] and HCV genotypes[15] have been implicated among predictive factors of the severity of chronic infection. Moreover, dissimilar incidence of hepatocellular carcinoma among American and Japanese patients despite a similar seroprevalence for HCV, has been associated to differences in genotype prevalence in both countries. In Japan a greater prevalence of 1b subtype is observed in such tumors, where cirrhosis does not appear to be a mandatory step[16] as usually observed in Western countries. Thus, 1b overexpression in Italian patients has not been attributed to a mere genotypic difference related to older infected individuals. Moreover, such subtype is also associated to tumor development in cirrhotic patients, independently of age or sex[17]. On the other hand, 1b subtype[18], quasispecies complexity distribution within a given patient[19] and the existence of point mutations[20] have been reported as predictive factors of viral resistance to interferon therapy.

Traditionally, viruses have been classified according to their antigenic properties, but within the last few years - and due to advances in molecular biology- genotypic classification has also been feasible. The potential significance of such findings lies in the possibility to investigate virus-host interactions, as well as viral factors associated to both infection severity and treatment response.

The aim of the present study was to perform HCV genotyping in chronic carriers by means of nucleotide sequencing combined with RFLP in Argentine strains.

Material and methods

Population: 82 HCV chronically infected patients, 71 adults and 11 children (47 male; mean age 27.5 yr-old, range 2 - 71 yr) were studied.

These subjects were classified within 3 groups: those who had parenteral risk of infection (n=56: 28 who received blood transfusions, 17 intravenous drug users [IVDU], 8 dialyzed - referred to the Faculty of Medicine from a Center located at Buenos Aires Province- 2 health workers, and 1 patient who reported a past surgery); those with non-parenteral risk (n=5) and sporadic cases (n=21).

Sample collection. All blood samples were obtained under appropriate conditions for RNA handling. Three to 5 ml of whole blood were collected by vein puncture. Sera were separated by centrifugation within 3 hs, aliquoted and kept at -70ºC until further processing.

RNA extraction, reverse transcription and amplification by Nested PCR of the 5' UTR: 200 µl of serum were treated with guanidinium isothiocyanate and acidic phenol following a protocol previously described[21].

The equivalent to 100 µl of serum was processed for reverse transcription at 70ºC using the thermostable enzyme *Tth* (Promega, WI, USA) or the recombinant r*Tth* (Perkin Elmer, Roche Molecular Systems, Branchburg, NJ, USA). Amplification of the initial cDNA was carried out by using the same enzyme. Nested PCR was performed using *Taq* polymerase (Promega, Perkin Elmer or Boheringer Mannheim, Germany).

Primers used were HCV1 (outer antisense) HCV2, (outer sense) HCV3 (inner antisense) and HCV4 (inner sense) as described[22], thus allowing the synthesis of 210 bp amplicons.

Throughout the whole procedure, Kwok and Higuchi rules[23] were strictly followed, except that from serum collection to agarose gel loading, different sets of micropipettes and special aerosol resistant tips (ART, Molecular Bio-Products, Inc.) were used. To validate results a negative control was included from the extraction step every four samples and another negative control was also added from reverse transcription. A positive control was included from RNA extraction.

RFLP genotyping: This procedure was developed following the methodology proposed by Davidson et al.[7] slightly modified by the authors. Briefly, 10 µl of products obtained by RT - Nested PCR were digested with the following sets of endonucleases: *Hae* III / *Rsa* I, *Hinf* I / *Bst*N I. According to the result obtained, further digestions were carried out with *Bst*U I (for type 1) or with *Scr*F I (for types 2 or 3). Enzymatic treatment was carried out at 37ºC for 2 hs, except for *Hinf* I/*Bst*N I which was later treated for 2 hs at 60ºC, as performed with *Bst*U I. This methodology has been reported to allow discrimination between subtypes "a" and "b" for types 1, 2 or 3[7]. RFLP was carried out in 32 out of the 82 samples, which have not been sequenced.

Sequencing of 5' UTR amplicons: 100 µl of Nested PCR products were purified from 6% polyacrylamide gel. Bands were eluted using a solution with 0.5% ammonium acetate, 0.01 M magnesium acetate, 1mM EDTA and 0.1% SDS with slow agitation for 12 hs. After centrifugation, supernatants were collected and a phenol - chloroform extraction was carried out followed by ethanol precipitation in presence of ammonium acetate 3M.

DNA was resuspended in 5 - 20 µl of sterilized bidistilled water, according to product yield, as measured by spectrophotometric reading at 260 nm.

Sequencing was carried out according to the method of Sanger[24], partially modified by the cycle sequencing procedure, alternately using for each sample both internal primers (HCV2 and HCV4) with 5' end fluorescein-labelled dideoxynucleotides in an automatic sequencer (ABI 373A, Applied Bioosystems, Foster City, CA, USA). To avoid misinterpretations, each tem-

plate was obtained at least from 2 different aliquots of RNA and sequenced bidirectionally, using HCV3 and HCV4 alternately.

In turn, when nucleotide sequences at positions corresponding to endonuclease recognition sites were established, computarized predictive mode RFLP analysis (Lassergene Program for Windows, MAPDRAW) was carried out. In addition multiple and pairwise sequence alignment (MEGALIGN Program) allowed strain classification within HCV genomic types using Clustal method[25]. Fifty HCV isolates were analyzed by this methodology.

Results

Type 1 was detected in 58 out of 82 samples (70.7%), type 2 in a further 18 (21.9%) and type 3 in the remaining 6 sera (7.3%) (Fig.1). Within the RFLP discrimination ranges, various HCV subtypes - except for 3b- were observed, as shown in Fig. 2 and Table 1. However, no samples belonging to types 4, 5 or 6 were detected.

In sporadic cases genotype 1 accounted for 71.4%, with 42.8% for subtype 1a (9/21) and 28.6% for 1b (6/21), and subtype 2a/c for the remaining 28.6% (n=6).

Among the 56 cases with parenteral transmission risk (hemodialyzed, polytransfused and IVDU), 38 (67.8%) belonged to genotype 1 (18 to subtype 1a - 32.1%- and 20 to 1b -35.7%-), 12 (21.4%) to type 2 (10 to 2a/c - 17.8%- and 2 to 2b -3.6%-) and the remaining 6 to type 3 (10.7%, all subtype 3a).

Among the 5 patients with non-parenteral transmission risk, only genotype 1 was demonstrated (1a in 2 and 1b in 3).

Partial nucleotide sequence alignment is observed in Figure 3, while a phylogenetic tree is depicted in Figure 4.

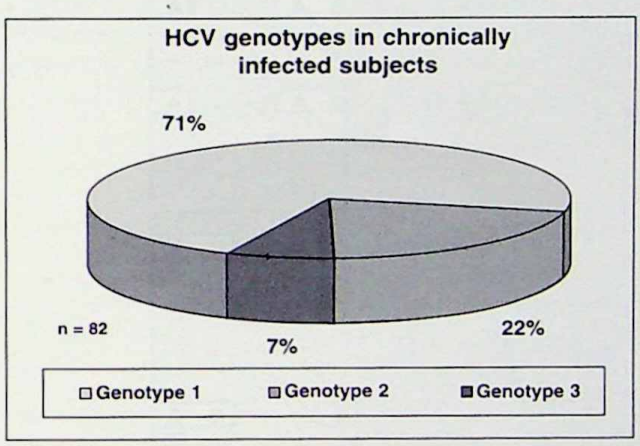

Fig. 1.- HCV genotypes in chronically infected subjects.

Table 1. HCV types and subtypes distribution among Argentine patients

Genotype	Parenteral risk	Non-parenteral risk	Sporadic cases	Total
1 a / c	18	2	9	29
1b	20	3	6	29
2 a / c	10	0	6	16
2 b / c	2	0	0	2
3 a / c / d / e	6	0	0	6
Total	56	5	21	82

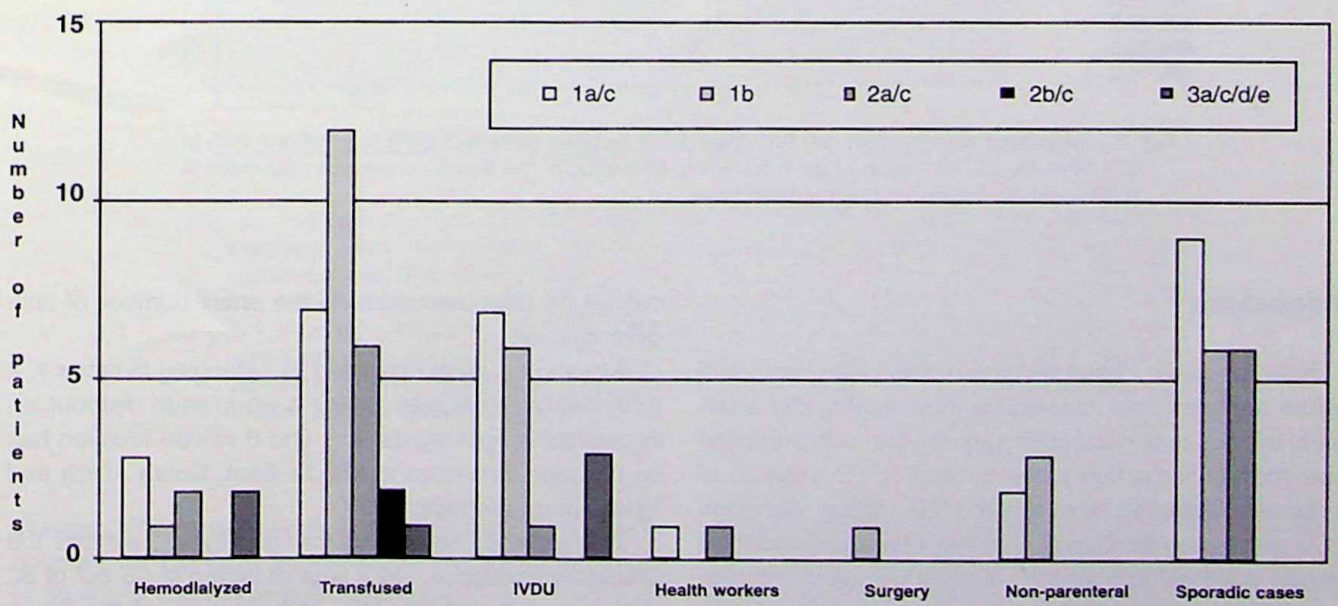

Fig. 2.- HCV subtype prevalence within different Argentine groups.

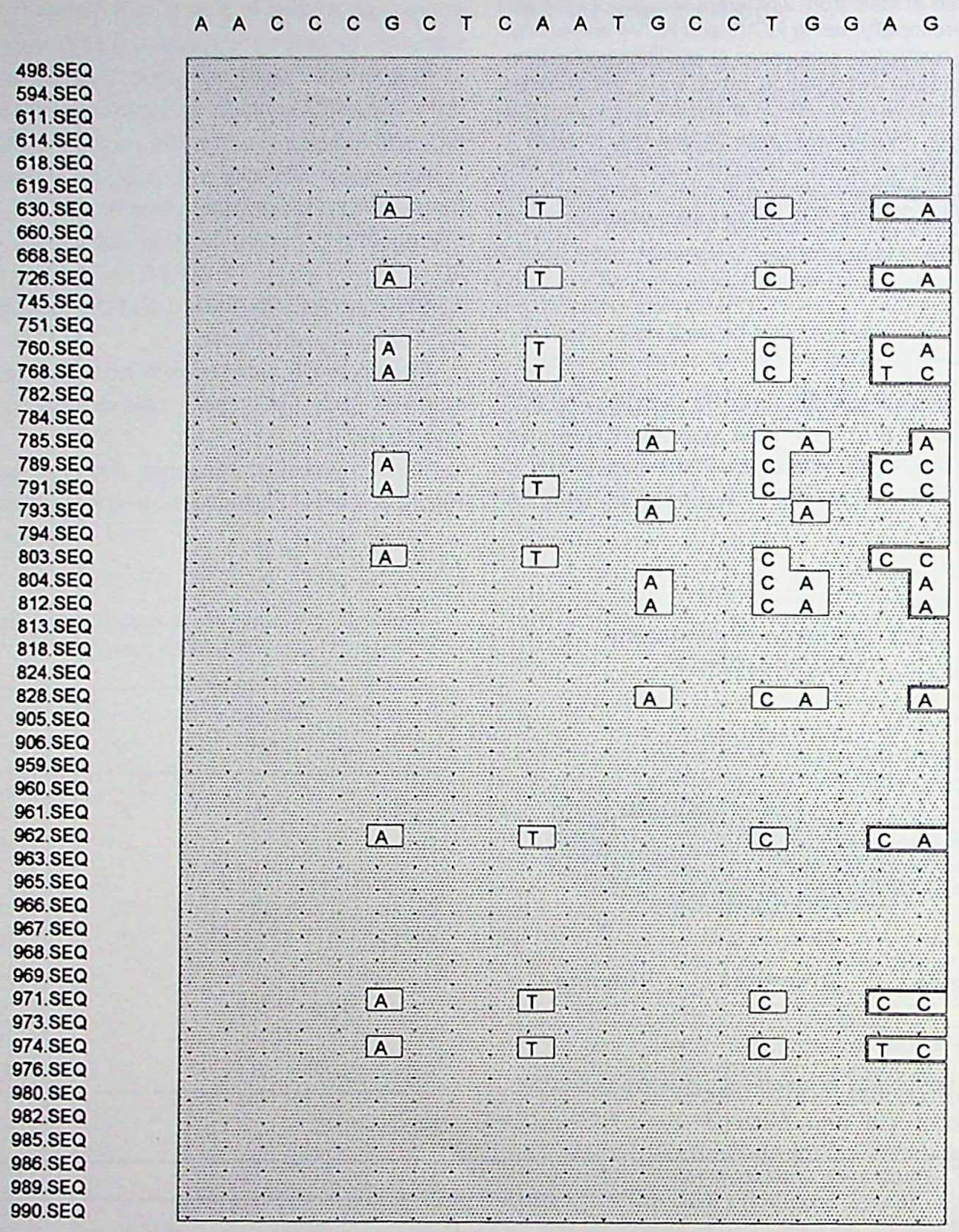

Fig. 3.- Sequence alignment of 50 Argentine HCV isolates corresponding to positions 205 to 224 from the 5'UTR region of genomic RNA. At the top of the figure consensus sequence is shown. Boxes represent polymorphic sites.

Discussion

HCV genomic characterization was performed with serum samples from chronically infected Argentine patients by means of nucleotide sequencing -with predicted type-specific restriction patterns- and RFLP analysis of amplicons obtained from the 5' UTR region. Although HCV genotype distribution was not apparently different among children and adults, it must be taken into account that an appropriate comparison according to age cannot be performed, due to the small number of children studied.

All samples were classified as belonging to types 1, 2 or 3. These genotypes exhibit a world-wide distribution, in contrast to genotypes 4, 5 and 6 whose location has so far been restricted to Middle East, South Africa and Hong Kong, respectively.

This study demonstrates that genotype 1 exhibits the greatest prevalence, as it was detected in 58 out of 82 samples analyzed (70.7%), irrespectively of the group

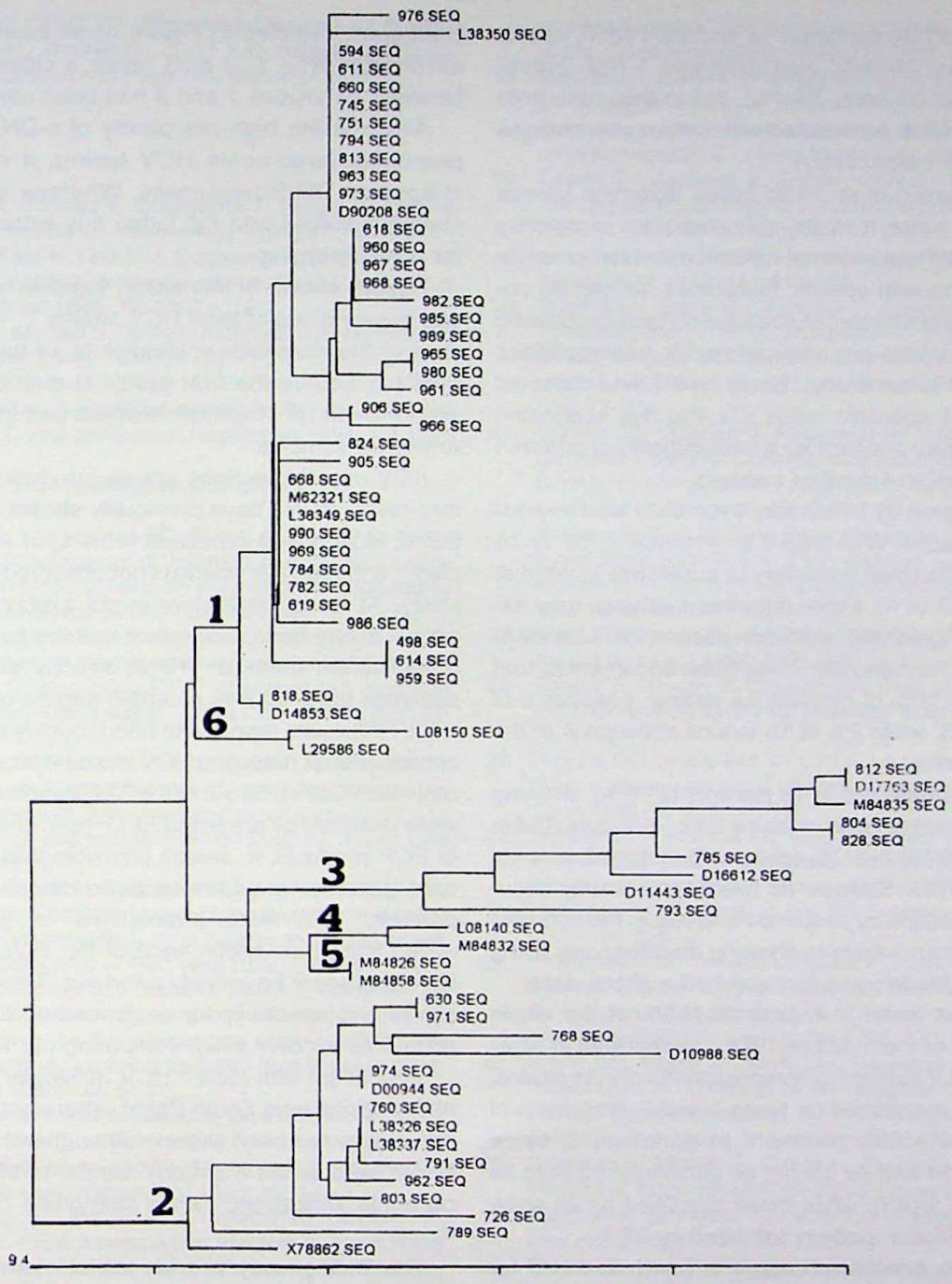

Fig. 4.- Phylogenetic tree of 5' UTR region from HCV genomes, using Clustal method with weighted residue weight table. Fifty Argentine sequences are named solely with a number: samples # 498, 594, 611,614,618, 619, 630, 660, 668, 726, 745, 751, 760, 768, 782, 784, 785, 789, 791, 793, 794, 803, 804, 812, 813, 818, 824, 828, 905, 906, 959 - 963, 965 - 969, 971, 973, 974, 976, 980, 982, 985, 986, 989 and 990, correspond to Accession numbers from GenBank AF041264 to AF041313, respectively; those sequences obtained from GenBank used as references for phylogenetic tree construction are identified by their accession number (preceded with a letter).

studied. Within genotype 1, subtypes 1a and 1b accounted for identical percentages, corresponding each to 35.4% of the whole population (Fig.1).

Comparative genotypic analysis of diverse groups showed interesting features (Fig.2). For example, despite of their common origin from a dialysis unit, these patients exhibited dissimilar and evenly distributed HCV genotypes, suggesting a different source of infection. In contrast, among transfused patients (n=28) subtype 1b was predominant, since it was detected in 12 sera

(42.8%) followed by subtypes 1a and 2a/c (n=6, each). The IVDU group showed also genotype 1 high prevalence (13 out of 17 sera, 76.4%), but in this case both subtypes 1a and 1b contributed with similar percentages (41.1 and 35.3, respectively).

HCV genotyping of sporadic cases deserves special consideration, since it might truly represent circulating local strains, without external influences which promote their association with specific (sub)types, as can be observed when concentrates of coagulation factors obtained in Western countries are administered to haemophiliacs (only 1 patient in our study). Since type 1 was detected in 15 out of 21 sporadic cases (71.4%) it is suggested that this type may account for a vast majority of community acquired HCV Argentine patients.

HCV subtyping by RFLP may eventually lead to erroneous conclusions. With regard to genotype 1, 1a or 1b subtypes are ascribed according to nucleotide located at position 243 (G or A). However, other subtypes may exhibit an indistinguishible restriction pattern: i.e. 1c is identical to 1a[7, 27]. Furthermore, it has been documented that approximately 10% of genuine 1a strains possess a G at position 243, while 2% of 1b strains show an A at the same location[26].

Type 2 was detected in 18 patients (21.9%), showing a strong predominance of subtype 2a/c -indistinguishible at 5' UTR- (16/18) over 2b subtype. Genotype 2 was not detected in IVDU. Subtype 2c has been recently documented in Argentina by sequence analysis in the core and NS5 regions from a sample showing discrepancies using 2 different methodologies (unpublished authors' data)

Type 3 was found in 6 patients (8.3% of the whole population), 3 of them among IVDU, as reported in other studies[27]. RFLP subtyping demonstrated only 3a strains. As previously mentioned for types 1 and 2, limitations of this method are also pertinent to genotype 3, since subtypes 3c, 3d and 3e cannot be distinguished from 3a at the 5' UTR region, while those classified as 3b show the same restriction pattern exhibited by 3f[7, 28].

From these considerations, it is concluded that for subtype assignment it is mandatory to perform simultaneous sequencing of coding genomic regions which exhibit a greater degree of nucleotide heterogeneity within a given type (i.e. E1, core or NS5). However, 5'UTR amplicons analyzed by RFLP are still one of the most widely used methods. Significant nucleotide conservation at such location among different strains, which allows the use of universal primers, and therefore maximal sensitivity for detection and subsequent typing, explains RFLP current acceptance. Paradoxically, such conservation is at the same time a hindrance for conclusive subtyping.

Computer analysis allowed nucleotide sequence alignment (Fig. 3) as well as a comparison of local strains with subtype-specific prototypes. As observed in the cladogram depicted in Figure 4, all local strains were ascribed to type 1, 2 or 3, while a closer relationship between genotypes 1 and 3 has been confirmed.

Although the high complexity of c-DNA sequencing precludes large-scale HCV typing, it allowed us to strengthen RFLP usefulness. Whereas genomic information is limited with the latter, it is extremely valuable for massive typing.

Results shown in this study, together with our previous observations of local HCV strains [21], show a partial view of the molecular epidemiology of this agent in Argentina. This is the first study of multiple nucleotide sequence and phylogenetic analysis carried out with Latin American samples.

HCV mixed infections are an interesting field of current research.We have previously shown a high proportion of HCV mixed infections (45.4%) in Argentine samples[21], although this rate was not observed in the present study. At least two factors might explain this discrepancy. On one hand, it is known that the core-based PCR amplification method[8-10] may readily detect different subtypes but produces a certain degree of mispriming[34], while RFLP was reported to need roughly equimolar concentrations for detecting HCV mixed infections[6, 7]. On the other hand, since 50 out of the 82 characterized genomes were analyzed by predicted RFLP from direct sequencing of PCR products, it seems plausible that only predominant genomes would have been detected. Therefore, possible HCV minor populations -i.e. contributing to mixed infections- within each of the directly sequenced isolates cannot be entirely ruled out. Thus, diverse sensitivity and specificity for each methodology should be taken into account when comparing our two studies.

In contrast with recent HCV molecular epidemiologic observations from South Brazil, where genotype 1> 3> 2 prevalence has been shown -although without subtyping- [32], our data depict a pattern similar to that recently reported in Venezuela[33], while strengthen our initial study [21].

Our findings may provide useful information for diagnostic detection of genomic HCV[13] and for a better interpretation of genotype-dependent serology[6, 12]. Bearing in mind that HCV superinfection has been documented in non-human primates infected with different genotypes[29], experimental vaccines currently in preparation[30, 31] should consider not only the efficacy of protection against challenge with a genotype identical to the immunogen, but also to heterologous genotypes, on occasion genetically distant as 1b vs 2a/c.

The proper knowledge of prevalent HCV genotypes in different world areas will contribute to develop an adequate prophylaxis to avoid infection by this agent.

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