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Diversity of Hepatitis B and C Viruses in Chile

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Although there is a low prevalence rate (around 1% of the population) of infection with hepatitis B virus (HBV) and hepatitis C virus (HCV) in Chile, little is known about the diversity and molecular characteristics of the circulating viruses. In the present study, 40 HBV and 57 HCV samples from Santiago City, Chile, were examined. The phylogenetic analysis of HBV samples showed the autochthonous genotype F as the most represented genotype in the study (67.5%), while genotypes A, B, C, and D were less frequent (7.5%, 5%, 7.5%, and 12.5%, respectively). The frequency of circulation of HBV genotypes observed is in accordance with the genetic background of the Chilean population. Most of the HCV samples tested belonged to subtype 1b (82%). The coalescent analysis conducted for both the NS5A and NS5B regions of the HCV strains showed similar population growth rates, with a most recent common ancestor estimated to date between 1893 and 1901. This result may indicate that genotype 1b strains circulating in Chile have epidemiological features similar to those described for HCV genotype 1b in Brazil and the United States. However, the most recent common ancestor for Chile is older than that reported recently for genotype 1b in Argentina. ***J. Med. Virol. 81:1887–1894, 2009.***

KEY WORDS: HCV; HBV; genotypes; coalescence; phylogeny

INTRODUCTION

It has been estimated that more than 170 million people are infected with hepatitis C virus (HCV) worldwide and that the burden of hepatitis B virus (HBV) infection is significantly greater, with more than 400 million people infected. Chronic infection by HBV or HCV may progress to cirrhosis and hepatocellular carcinoma which are associated with high morbidity and mortality [Lai et al., 2003; Pawlotsky, 2004].

Eight HBV genotypes (A–H) have been described so far [Norder et al., 1994; Stuyver et al., 2000; Arauz-Ruiz et al., 2002]. The worldwide prevalence of HBV genotypes is the result of the distribution of humans across continents [Robertson and Margolis, 2002]. The most cosmopolitan genotypes are A and D. Genotype A is predominant in Europe, Africa, and North America, whereas genotype D is predominant in countries of the Mediterranean and the Near and Middle East. Genotypes B and C are found in East and South-east Asia, and genotype E in West Africa. Genotype G has been isolated in different countries, from Germany and France to Japan and the USA. Genotypes F and H are considered indigenous to the native population of the Americas, with genotype F restricted to Central and South America [Arauz-Ruiz et al., 1997; Blitz et al., 1998; Piñeiro y Leone et al., 2003].

Chile has been described within the group of countries with a low prevalence of HBV infection [Paraná and Almeida, 2005]. A recent report has shown that genotype F is the most prevalent, with a prevalence of 84%, whereas genotypes A, B, C, and D have been found at a prevalence of 3.8%, 3.8%, 6.1%, and 2.3%, respectively [Venegas et al., 2008].

HCV is divided into at least six major genotypes (1–6), with more than 90 subtypes. HCV subtypes 1a, 1b, 2a, 2b, 2c, and 3a are responsible for over 90% of the infections in North and South America, Europe, and Japan [Simmonds and Smith, 1999; Simmonds et al.,

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2005]. From these HCV subtypes, 1b is the most prevalent genotype found in several parts of the world, including the United States, Europe, Japan, Chile, and Latin America [Muñoz et al., 1998; Soza et al., 2004; da Fonseca and Brasil, 2004; Fay et al., 2006; Dehesa-Violante and Nuñez-Nateras, 2007]. Subtypes 1a, 1b, and 3a have been found in high prevalence worldwide during the last 100 years and have been probably transmitted by transfusions and intravenous drug use [Simmonds and Smith, 1997; Kuiken et al., 2005].

The evidence that the genotype of HBV and HCV may be one of the factors influencing the severity of infection, the response to the treatment, and the outcome of liver disease justifies the analysis of the prevalence of genotypes and its genetic characteristics [Pawlotsky, 2003; Fung and Lok, 2004; Pujol and Devesa, 2005].

The aim of this study was to gain insight into the current molecular epidemiology and phylogenetic analysis of HBV and HCV genotypes and subgenotypes in Chile.

METHODS

Origin of the Samples

All the serum samples, 40 HBV positive samples (collected between 2002 and 2006), and 57 HCV positive samples (collected between 1997 and 2006), were obtained from patients infected chronically from the Laboratory of Molecular Biology of the Gastroenterology Section of the Clinical Hospital of Chile University, Santiago City, Chile. All but three HBV positive and two HCV positive patients were from Santiago de Chile.

Seventeen out of the 40 HBV samples were HBeAg positive, 10 were HBeAg negative and 13 were not determined. There was no evidence of co-infection with either HCV or HIV.

Seventeen of the 57 patients with HCV which had been treated with standard IFN (3 mU thrice weekly) plus ribavirin (1,000–1,200 mg daily based on body weight) for 48 weeks and their pre-treatment sera were tested in this study. All treated patients were followed for at least 6 months after therapy to determine its response to treatment. Seven patients were considered to have a sustained virological response to the therapy because serum alanine aminotransferase levels were normal 6 months after therapy, with no evidence of serum HCV RNA as assessed by nested RT-PCR. The remaining 10 patients were considered non-responders.

Written informed consent for participating in this study was obtained from all patients and the study protocol was approved by the local ethics committee in accordance with the 1975 Helsinki Declaration.

Laboratory Tests for HBV and HCV

HBsAg and anti-HBc positive sera were detected by AxSYM® (Abbott Laboratories, North Chicago, IL).

Anti-HCV antibodies were detected by third generation ELISA using the HCV EIA 3.0® detection kit (Abbott Laboratories).

DNA–RNA Extraction, Amplification, and Sequencing

DNA from HBsAg positive samples was extracted from 200 µl of sera by the QIAamp DNA mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. A 753-bp fragment encompassing the whole HBsAg gene was obtained following a nested-PCR protocol. The first round was carried out with primers P1 and P2 under the conditions described by Günther et al. [1998]. The second round was carried out with primers HBV5 5′-CCT GCT GGT GGC TCC AGT TC-3′ (nt 55-74) and HBV6 5′-CCA ATT ACA TAT CCC ATG AA-3′ (nt 893-874) [Mbayed et al., 2001]. The amplified PCR products were purified with the QIAquick Gel Extraction Kit (Qiagen) following the manufacturer's instructions.

RNA from HCV positive sera was extracted with Trizol (Gibco-BRL, Carlsbad, CA) and then dissolved in 9 µl of mili Q water, denatured at 80°C for 5 min and primed with random hexamers (Biodynamics, Buenos Aires, Argentina). The reverse transcription reaction was performed at 37°C for 90 min using M-MLV reverse transcriptase (Promega, Madison, WI). The partial NS5A and NS5B regions were amplified by nested PCR with the following primers: for NS5A: external primers ES: (5′-TGG ATG GAG TGC GGT TGC ACA GGT A-3′, sense) and EA: (5′-TCT TTC TCC GTG GAG GTG GTA TTG G-3′, antisense), and internal primers: IS: (5′-CAG GTA CGC TCC GGC GTG CA-3′, sense) and IA: (5′-GGG GCC TTG GTA GGT GGC AA-3′, antisense), which generate a 573-nucleotide fragment; for NS5B: external primers ES: (5′-G CCG TGA TGG GCT CCT CAT ACG-3′) EA: (5′-CCR GAT GCR TCG TGC GCG AC-3′) and internal primers IS: (5′-G ACA CCC GCT GTT TTG ACT CAA C-3′) and IA: (5′-GTA CCT AGT CAT AGC CTC CGT G-3′).

The HBV and HCV amplicons obtained were sequenced in both senses by Macrogen, Inc. (Seoul, Korea).

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this article are FJ709457-96 (HBV) and FJ709513-96 (HCV).

Identification of HBV Genotypes, Subgenotypes, and Serotypes

Genotype and subgenotype assignment was based both on phylogenetic analysis and amino acid comparisons along both the S and P genes. For phylogenetic analysis, sequences from complete genomes were used as reference samples. The amino acid analysis was carried out with the VESPA software [Korber and Myers, 1992], considering that a candidate signature pattern should be present in more than 90% of the sequences of the query set and in less than 10% of the samples of the background set. Numbering of amino

acid positions was established according to subgenotype A2 small S and P ORFs.

Serotypes were deduced from the amino acid sequences according to Purdy et al. [2007].

HCV Genotype and Subtype Assignment

HCV genotypes were determined by RFLP analysis after amplification of the 5′-UTR region as described previously [Muñoz et al., 1998]. Subtypes were then assigned by phylogenetic analysis of the NS5B region.

Phylogenetic Analysis

The HBV and HCV sequences were subjected to alignment with CLUSTALX v1.83 software [Thompson et al., 1997].

The HBV phylogenetic tree was obtained using the Neighbor-Joining algorithm implemented in the MEGA v 4.0 software [Tamura et al., 2007] with the Kimura-two-parameter model of molecular evolution. The reliability of the inferred tree was assessed by a bootstrap resampling test including 1,000 replications. The intragenotype HBV distances were obtained using the same parameters.

HCV phylogenetic trees were obtained using the Maximum Likelihood algorithm with the PAUP v 4.0b10 software [Swofford, 2002] using $GTR + I + \Gamma$ model of molecular evolution. The reliability of the inferred tree was assessed by a bootstrap resampling test including 1,000 replications based on the Neighbor-Joining algorithm.

Amino Acid Analysis of HCV

The VESPA program was also used to search a signature pattern between Chilean and Argentinean HCV sequences. The comparative analysis was extended to a Genbank world database that comprises non-related HCV genotype 1b sequences.

Analysis of Substitution Rates and Divergence Times for HCV-1b

Rates of nucleotide substitutions per site and divergence times were estimated using the Bayesian MCMC (Markov Chain Monte Carlo) analysis implemented in the BEAST program [Drummond et al., 2002, 2005]. For this analysis, five population dynamics models were used: bayesian, constant, expansional, exponential, and logistic, using the Akaike's Information Criterion to determine the model that best fit the data. Both strict and relaxed molecular clocks were used to explore the extent of rate variation in the data as well as to estimate the rate of nucleotide substitution and the age of the most recent common ancestor. In all cases, the GTR model of nucleotide substitution was used with chain lengths of 50 million for both the strict and the relaxed molecular clock, with the extent of convergence assessed using the Tracer program (http://evolve.zoo.ox.ac.uk/beast/). This analysis was run for the amplified genotype 1b samples, on two different data sets: the NS5A region ($n = 40$) and the NS5B region ($n = 40$) sequences. An extensive analysis was undertaken in order to discard the possibility of recombination with the SimPlot program [Lole et al., 1999]. The window width and the step size were set to 100 and 20 bp, respectively. The results obtained in the recombination analysis were confirmed using a bootscanning analysis [Salminen et al., 1995]. In this case, a sliding window of 100 nucleotides, moving 20 nucleotides at a time, was used.

RESULTS

HBV Genotype, Subgenotype, and Serotype Assignation

The 40 Chilean samples grouped by phylogenetic analysis in genotype A (3 samples), genotype B (2 samples), genotype C (3 samples), genotype D (5 samples), and genotype F (27 samples) (Fig. 1). Distances within and between HBV genotypes coexisting in Chile corroborated the phylogenetic analysis (data not shown).

Further analysis showed that all genotype F isolates conformed subgenotype F1, clade 1b. Genotype A samples belonged to subgenotype A2. Genotype D samples conformed three phylogenetic subgenotypes: D2 (three samples), D3 (one sample), and a third one denominated Du (which did not correspond to any described subgenotype) composed of one sample from this study (CHI11) and two samples retrieved from GenBank (EU414141 and DQ111987). Genotype B samples grouped within subgenotype B2. Genotype C samples seemed to belong to subgenotype C1 and C2, although an unequivocal discrimination was not possible to obtain for genotype C with the fragment in analysis (Fig. 1). The amino acid signature patterns along the S and P ORFs corroborated the abovementioned subgenotype discrimination (data not shown).

According to their deduced amino acid sequences HBV Chilean samples belonging to genotype A displayed serotype adw2, as well as genotype B samples. Genotype C and F samples were serotypes adr and adw4, respectively; while four genotype D samples were serotype ayw2 and only one serotype ayw3.

HCV Genotype and Subtype Assignation

HCV genotype 1b was detected in 47 patients (82.4%), genotype 1a in 6 patients (10.5%), genotype 2 in 3 patients (5.25%), and genotype 3a in 1 patient (1.7%). Only the sample Ch10NT, identified previously as 1b by RFLP of 5′-UTR, has genotype 1a by the phylogenetic analysis of NS5B.

HCV-1b Phylogenetic Analysis

Since genotype 1b is by far the most prevalent genotype in the region examined, and due to its severe pathological characteristics and its poor response to treatment, the remaining tests conducted were

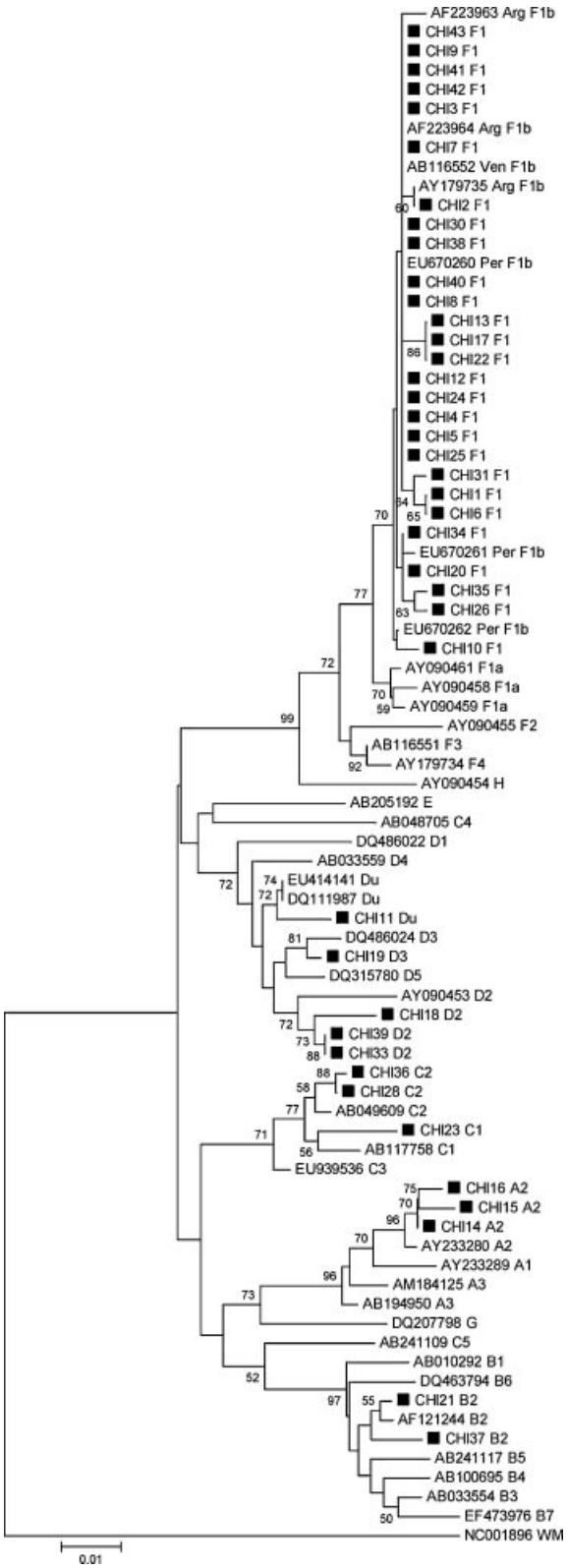

Fig. 1. Neighbor-Joining phylogenetic tree based on Kimura-two-parameter model including the 40 new HBV samples of this study. Both the genotypes and the subgenotypes are indicated in capital letters. The numbers at each node correspond to bootstrap values obtained with 1,000 replicates (values lower than 50 are not shown). HBV Woolly Monkey sequence NC001896 was used as outgroup.

restricted to the 47 Chilean sequences of this particular genotype.

A total of 40 and 43 out of the 47 samples were amplified successfully for the NS5B and NS5A regions, respectively.

The phylogenetic study of this region revealed an extensive diversity, where most of the samples did not cluster together, indicating the presence of a polyphyletic group. However, we identified three small clusters: Chile 1, made of Ch5NT and Ch25NT, with a bootstrap value of 70%; Chile 2, made up of samples Ch19NT and Ch22NT, with a bootstrap value of 81%; and Chile 3, formed by the samples Ch2NT and Ch16NT, with a bootstrap value of 99% (Fig. 2).

A similar tree topology was observed after the phylogenetic analysis of the NS5A region, which also showed an intermingling behavior and the formation of three clusters: Chile 2+, with a bootstrap value of 76%, which consisted of Ch19NT and Ch22NT (cluster Chile 2 in NS5B), plus Ch4NR and Ch13NT; Chile 3, which matched that for NS5B, with a bootstrap value of 99%; and a new cluster (Chile 4), which showed the association of two samples (Ch9NT and Ch29NT) with a bootstrap of 74% (Fig. 3).

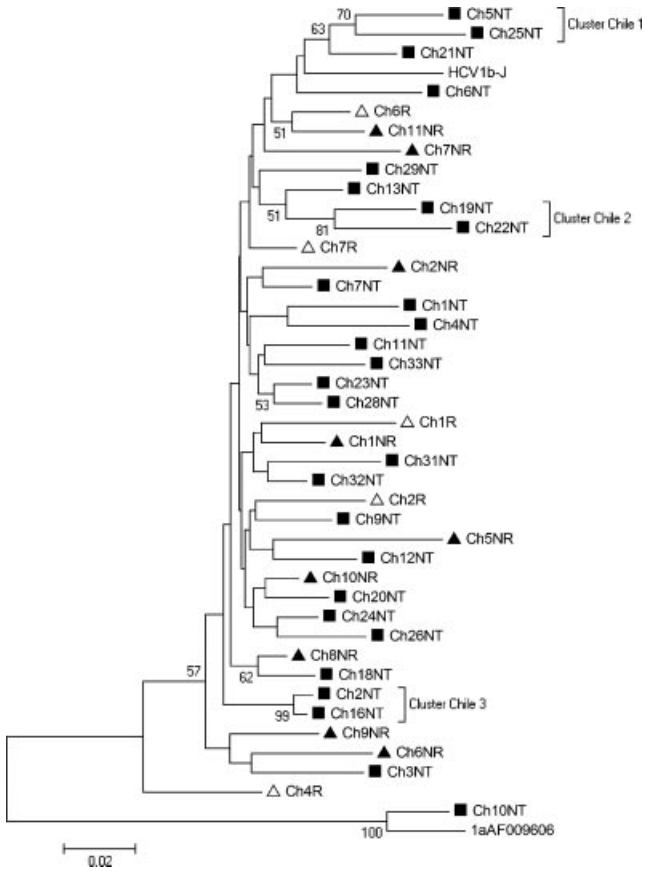

Fig. 2. Maximum Likelihood phylogenetic tree of 40 HCV sequences obtained with the GTR + I + Γ model. The numbers at each node correspond to bootstrap values from a congruent Neighbor-Joining tree obtained with 1,000 replicates (values lower than 50 are not shown). HCV-J 1b was added as a prototype and 1aAF009606 as outgroup. NS5B, 342 nt.

Fig. 3. Maximum Likelihood phylogenetic tree of 43 HCV sequences obtained with the GTR + I + Γ model. The numbers at each node correspond to bootstrap values from a congruent Neighbor-Joining tree obtained with 1,000 replicates (values lower than 50 are not shown). HCV-J 1b was added as a prototype and 1aAF009606 as outgroup. NS5A, 450 nt.

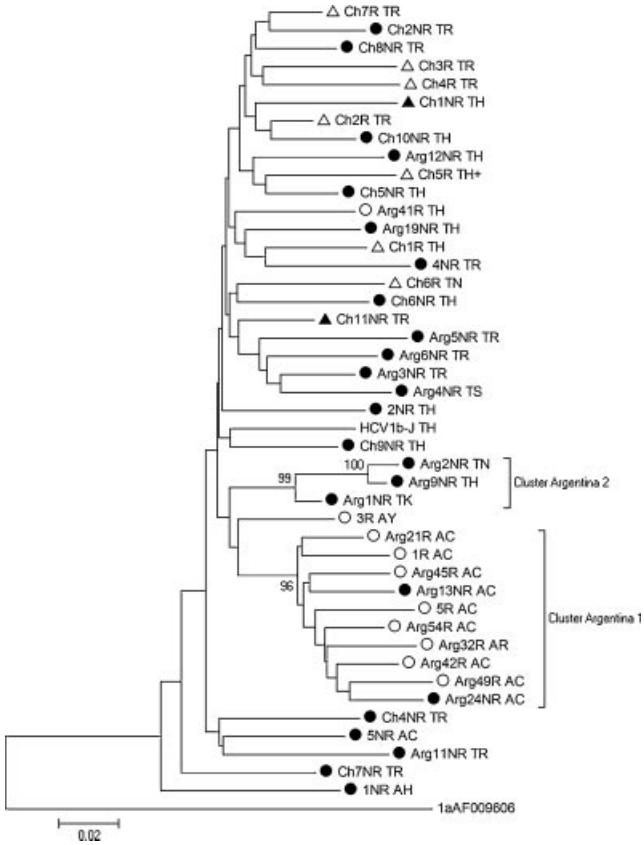

Fig. 4. Maximum Likelihood phylogenetic tree of 17 Chilean HCV sequences and 26 Argentinean sequences from pre-treated patients obtained with the GTR + I + Γ model. The numbers at each node correspond to bootstrap values from a congruent Neighbor-Joining tree obtained with 1,000 replicates (values lower than 50 are not shown). HCV-J 1b was added as a prototype and 1a AF009606 as outgroup. Empty triangles and circles for R and full triangles and circles for NR, Chile, and Argentina, respectively. NS5A, 450 nt.

Analysis of Treated Patients With HCV-1b

Seven out of the 17 genotype 1b treated patients achieved a sustained virological response, while 10 did not respond to the antiviral therapy. The association between the patient's age and the response to the treatment showed no statistical significance ($P = 0.1134$).

A phylogenetic analysis of Argentinean HCV samples has revealed a different genetic lineage of genotype 1b samples, related to the response to treatment [Di Lello et al., 2008]. In order to compare the sequences from these Argentinean pre-treatment serum samples with those from Chile, a phylogenetic analysis of NS5A and an amino acid analysis were carried out. As shown in Figure 4, the Chilean samples presented a situation different from that of the Argentinean samples, almost half of which constituted a cluster associated with response to treatment (cluster Argentina 1). The 17 NS5A Chilean sequences appeared intermingled with the Argentinean samples, but none of them was part of cluster Argentina 1.

In addition, the analysis showed that the Chilean samples had an amino acid pattern similar to that from both the world database and the Argentinean no-cluster 1 samples (data not shown).

Rates and Dates of the Evolution of HCV-1b

In order to investigate the origin and spread of the 40 Chilean HCV samples of genotype 1b, a demographic reconstruction analysis was carried out for both the NS5B and NS5A regions. In all cases, the best population dynamics model was the logistic one. The date for the most recent common ancestor of these Chilean samples was estimated to be around 1893 (ESS 270.7) for NS5B and 1901 (ESS 176) for NS5A (Table I). As regards the extent of rate variation, the relaxed clock fit best these samples.

In view of the fact that viral recombination could affect potentially coalescent analyses strongly [Schierup and Hein, 2000; Worobey, 2001], the SimPlot program was used to discard this possibility. No recombination was observed.

TABLE I. Estimated Evolutionary Parameters for Each Dataset Analyzed

Dataset	Description	Substitution rate	Date of MRCA (year)
1	NS5A Chile	7.5 E-4 (4.39 E-4/1.1 E-3)[a]	1901 (1859–1937)[a]
2	NS5B Chile	5 E-4 (pre-set)[a]	1893 (1864–1922)[a]

MRCA, most recent common ancestor.
[a]95% HPD are in parentheses.

DISCUSSION

In this study, further molecular evidence of HBV and HCV genotypes and subgenotypes circulating in Santiago de Chile is described.

Phylogenetic analysis showed that genotype F is the most common among Chilean isolates (67.5%). This genotype has been described as the indigenous representative of the virus in the Americas, since it is almost exclusive among diverse Amerindian groups [Blitz et al., 1998; Nakano et al., 2001; Livingston et al., 2007; Devesa et al., 2008].

A further discrimination showed that subgenotype F1 is the only member of HBV genotype F circulating in Santiago City, as is the case in Peru and Alaska [Livingston et al., 2007; von Meltzer et al., 2008]. In addition, subgenotype F1 is co-circulating with subgenotype F4 in the Provinces of Formosa and Chaco and in the city of Buenos Aires, in Argentina [Piñeiro y Leone et al., 2008]. Interestingly, subgenotype F4, the only subgenotype detected in Bolivia and in the northern Argentinean provinces of Jujuy and Salta, was not detected among the Chilean samples.

Further phylogenetic analysis of subgenotype F1 using complete genome sequences suggests a further grouping of the sequences in two clades, 1a and 1b [Piñeiro y Leone et al., 2003]. The Chilean samples appeared included into the denominated clade 1b with other samples from Argentina, Peru, and Venezuela.

The subgenotype distribution of HBV genotype F observed in South America suggests a viral diversification process with a co-circulation of multiple lineages associated with specific geographical regions. Additional studies using full genome sequences may provide valuable information with respect to the origin and evolution of genotype F in this region.

The circulation of foreign HBV genotypes A, B, C, and D in Santiago City (7.5%, 5.0%, 7.5%, and 12.5%, respectively) seems to reflect the impact of diverse migratory waves that have played important roles at different stages of the demographic history of Chile. The presence of subgenotype A2, as well as that of subgenotypes D2 and D3, may be the result of the European colonization, whereas the presence of genotypes B and C may result from the more recent migration from East and Southeast Asia.

With respect to the distribution of the HCV samples examined, genotype 1b was predominant (82.4%). This is in agreement with previous studies carried out in Chile, which reported values around 80% of genotype 1b [Muñoz et al., 1998; Soza et al., 2004]. Nevertheless, the prevalence of genotype 1b in the rest of Latin American countries is around 30% [Simmonds et al., 2005; Pujol and Loureiro, 2007]. The higher prevalence of HCV genotype 1b in Chilean samples is of interest due the lower response to treatment and its pathological characteristics. However, further studies are needed for a better description of the HCV infection in this region.

In the phylogenetic analysis of the samples obtained from HCV genotype 1b infected patients, both regions showed a similar tree topology, where most samples had an intermingling behavior, thus indicating the presence of a polyphyletic origin. This situation is different from that observed previously in Argentina, where almost half of the samples form a single cluster related to treatment response [Di Lello et al., 2008]. In the same way, the Chilean samples did not have characteristic amino acids and showed a pattern very similar to that of the Genbank world database.

The most recent common ancestor for these Chilean samples was shown to date from 1893 to 1901. The analysis of the epidemiological histories presented a similar population growth rate for both the NS5A and NS5B regions. The best population dynamics model was the logistic model, thus suggesting a rapid exponential growth followed by a stationary phase. This result is comparable to those determined for genotype 1b in Brazil and the United States (most recent common ancestors dating from 1880 to 1920), but older than that calculated for Argentina, which presents an exponential population dynamics model (1964–1979) [Nakano et al., 2004; Di Lello et al., 2008]. The disparity in the ancestral population dynamics between Chile–Brazil and Argentina reveals the divergence at genetic level of the virus and might indicate an increasing diversification of HCV genotype 1b in South America. These differences are supported by previous studies carried out in Uruguay, Chile, Brazil, and Argentina, which have described several lineages in these neighboring countries [Colina et al., 1999; Vega et al., 2001; Gismondi et al., 2004; Cristina, 2005].

In conclusion, the distribution of HBV and HCV genotypes in Santiago de Chile is in agreement with that expected for such a large and cosmopolitan South American city, where the current infection with HBV and HCV may be the result of several independent viral introductions. The diversity of HBV observed seems to correspond with the demographic history of Chile. The diversity of HCV genotype 1b in Chile would represent a local example of the epidemic behavior of this subtype, also observed in other South American countries.

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