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Geographic distribution of hepatitis C virus genotypes in Brazil

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Abstract

Brazil is a country of continental dimension with a population of different ethnic backgrounds. Thus, a wide variation in the frequencies of hepatitis C virus (HCV) genotypes is expected to occur. To address this point, 1,688 sequential samples from chronic HCV patients were analyzed. HCV-RNA was amplified by the RT-PCR from blood samples collected from 1995 to 2000 at different laboratories located in different cities from all Brazilian States. Samples were collected in tubes containing a gel separator, centrifuged in the site of collection and sent by express mail in a refrigerated container to Laboratório Bioquímico Jardim Paulista, São Paulo, SP, Brazil. HCV-RNA was extracted from serum and submitted to RT and nested PCR using standard procedures. Nested PCR products were submitted to cycle sequencing reactions without prior purification. Sequences were analyzed for genotype determination and the following frequencies were found: 64.9% (1,095) for genotype 1, 4.6% (78) for genotype 2, 30.2% (510) for genotype 3, 0.2% (3) for genotype 4, and 0.1% (2) for genotype 5. The frequencies of HCV genotypes were statistically different among Brazilian regions (P = 0.00017). In all regions, genotype 1 was the most frequent (51.7 to 74.1%), reaching the highest value in the North; genotype 2 was more prevalent in the Center-West region (11.4%), especially in Mato Grosso State (25.8%), while genotype 3 was more common in the South (43.2%). Genotypes 4 and 5 were rarely found and only in the Southeast, in São Paulo State. The present data indicate the need for careful epidemiological surveys throughout Brazil since knowing the frequency and distribution of the genotypes would provide key information for understanding the spread of HCV.

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Introduction

Hepatitis C is an infection of worldwide occurrence with more than 170 million infected people around the world. Most of these hepatitis C virus (HCV) cases are asymptomatic carriers, but chronic infection becomes established in most (85%) of the infected individuals. Consequently, hepatitis C is frequently diagnosed in advanced clinical stages or when asymptomatic carriers present themselves as blood-donor candidates. The chronic stage evolves to severe liver disease in almost 20% of the infected individuals (1).

Six major viral genotypes and over 50 proposed subtypes of HCV have been identified worldwide (2). Viral genotype determination before treatment is currently advised as a routine assay. Patients infected with genotypes 1 and 4 with viral loads higher than 800,000 IU/ml must be treated for one year, while those infected with other genotypes may be treated for only 6 months (3,4). Therefore, HCV genotyping is an important tool for the prognosis and follow-up of infected patients.

Table 1. Geographical origin of the samples according to Brazilian regions and states.

Region/State	N
North	85
Acre	59
Amazonas	14
Other Northern states	12
Northeast	237
Alagoas	28
Maranhão	38
Paraíba	35
Pernambuco	122
Other Northeast states	14
Center-West	79
Goiás	35
Mato Grosso	32
Other states of the Center-West	13
Southeast	1,111
Espírito Santo	9
Minas Gerais	28
Rio de Janeiro	234
São Paulo	840
South	176
Paraná	156
Rio Grande do Sul	8
Santa Catarina	12
Total	1,688

To our knowledge, only one population-based study has been carried out to analyze HCV prevalence in Brazil. Focaccia and collaborators (5) have reported an estimated prevalence of 1.42% (0.70-2.12%) for hepatitis C in the general population of São Paulo municipality. An extensive review on HCV infection data in Brazil showed the following prevalence in healthy adults and/or blood donors for the different regions of Brazil: 0.9 to 2.4% for the North, 1.7 to 3.4% for the Northeast, 1.0 to 1.4% for the Center-West, 0.8 to 2.8% for the Southeast, and 1.1 to 2.1% for the South (6). None of these studies have analyzed the frequency of the different genotypes.

Since Brazil is a large country with many different population backgrounds, a wide variation in the frequencies of HCV genotypes would be expected to be found throughout its territory. The aim of the present study was to determine the frequency of HCV genotypes in a large number of samples from different regions in Brazil using a standardized methodology.

Material and Methods

Samples

Sequential samples of 1,688 viremic chronic hepatitis C patients from different Brazilian regions were studied. Samples were collected from 1995 to 2000 at, or sent to, Laboratório Bioquímico Jardim Paulista, São Paulo, SP, Brazil, for HCV genotyping. Blood samples were collected in tubes containing a gel separator, centrifuged in the site of collection and then send to Bioquímico Jardim Paulista by express mail in a refrigerated container. The geographical origin of the samples is shown in Table 1. Serum samples

were stored at -20ºC and thawed immediately before use.

This project was approved by the Ethics Committee of the University of São Paulo School of Medicine.

Viral RNA extraction

Viral RNA was extracted from 100 µl of serum using the guanidinium isothiocyanate-phenol-chloroform method (7). Reagents for RNA extraction, cDNA synthesis and PCR were obtained from Life Technologies (São Paulo, SP, Brazil). Each batch of samples was processed with positive and negative controls during all steps. To avoid cross-contamination between samples, the procedures proposed by Kwok and Higuchi (8) were strictly followed.

Primers

Primers NCR2 (5' ATACTCGAGGTGC ACGGTCTACGAGACCT 3'), PTC1 (5' CG TTAGTATGAGTGTCGTGC 3'), PTC3 (5' AGTGTCGTGCAGCCTCCAGG 3') and NCR4 (5' CACTCTCGAGCACCCTATCA GGCAGT 3') were used (9,10). As shown in Figure 1, these primers cover the following nucleotide positions in the 5' untranslated region (5'UTR): PTC1 (81-98), PTC3 (99-108), NCR4 (288-313), and NCR2 (323-341) according to the HCV sequence described by Choo et al. (11), locus HPCPLYPRE, GenBank accession No. M62321 (Figure 1).

Complementary DNA synthesis

After extraction, viral RNA was resuspended in a total volume of 20 µl and reverse transcription was carried out with 200 U of Moloney murine reverse transcriptase and the reverse specific primer NCR2 (0.5 µM) in 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, and 0.5 mM each dideoxynucleotides (dATP, TTP, dCTP, and dGTP) for 1 h at 37ºC, followed by an enzyme inactiva-

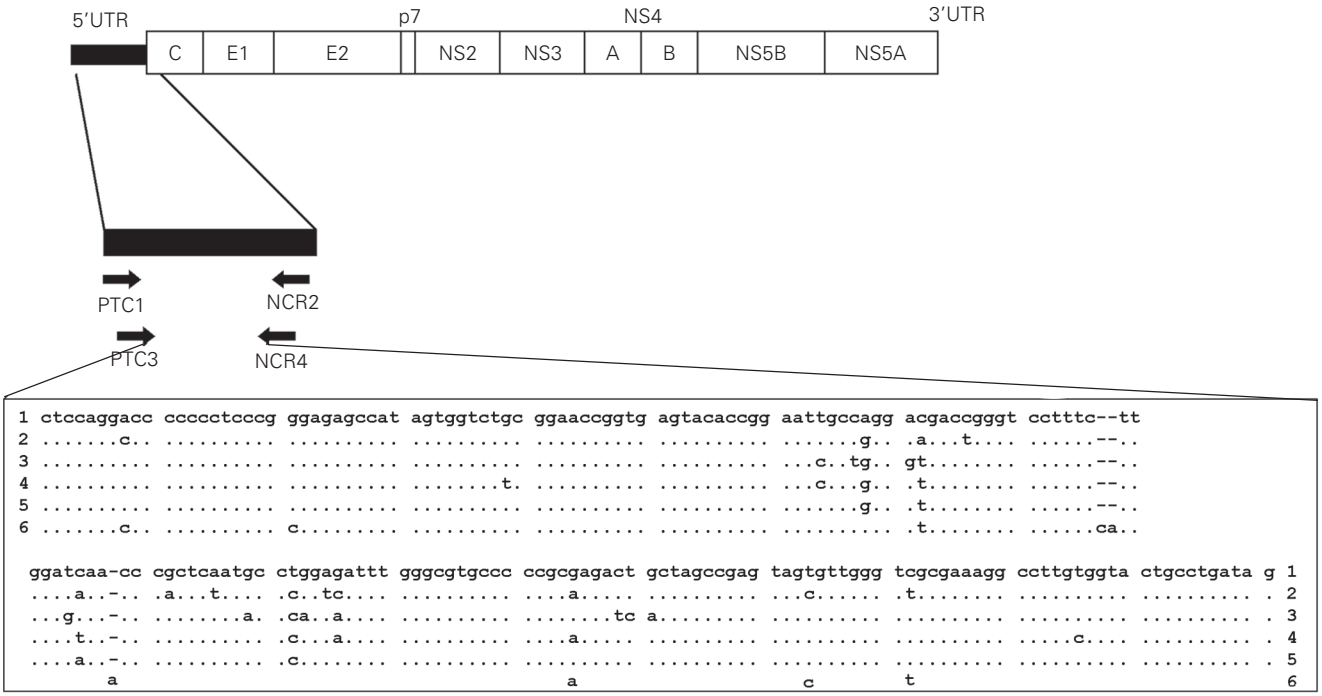

Figure 1. Coordinates of the primers used in the hepatitis C virus amplification and sequences from the amplified region representing different genotypes: 1 (AF333324), 2 (D00944), 3 (D17763), 4 (Y11604), 5 (Y13184), 6 (Y12083). 5'UTR = 5' untranslated region.

tion step at 95ºC for 15 min in a PTC-100 thermocycler (MJ Research, Watertown, MA, USA).

Polymerase chain reaction

Primers PTC1 and NCR2 were used in the first amplification round and primers PTC3 and NCR4, in the second round. The reactions were carried out with *Taq* DNA polymerase (5 U), 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 0.2 mM each of the dideoxynucleotides (dATP, TTP, dCTP, and dGTP), 0.5 µM of each primer, and 5 µl of the previous reaction mix.

First round cycles were: denaturation at 94ºC for 55 s, annealing at 55ºC for 40 s, extension at 72ºC for 40 s, and a final extension at 72ºC for 10 min in a PTC-200 thermocycler (MJ Research). The second round (nested PCR) of amplification was carried out with 10 µl of the first round product and the internal primers PTC3 and NCR4, using the same cycles as described above.

PCR fragments of 188 to 190 bp were observed under ultraviolet light after 2% agarose gel electrophoresis followed by ethidium bromide staining.

Sequencing reaction

Nested PCR products were submitted to cycle sequencing reactions without prior purification, using the ABI Prism® BigDye™ Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA, USA). The reaction mixture contained 8 µl of Terminator Ready Reaction Mix, 8 µl of MilliQ water, 1 pmol of each primer (second-round primers; see above), and 2 µl of the PCR products. Cycle sequencing was carried out with the PTC-200 thermocycler (MJ Research) under the following conditions: 25 cycles of 96ºC for 30 s, 50ºC for 15 s, and 60ºC for 4 min. Sequencing reaction products were separated and analyzed using the Automated DNA Sequencer ABI377 (Applied Biosystems).

Sequence analysis

Reverse and forward strand sequences were aligned and a consensus sequence was obtained for each case. The consensus sequence was then compared with the sequences of all HCV genotypes from the database. The alignment included 311 5'UTR sequences, of which 98 were from genotype 1, 38 from genotype 2, 76 from genotype 3, 66 from genotype 4, 24 from genotype 5, and 9 from genotype 6. This alignment was obtained from Smith et al. (12). For this purpose, the EditSeq and MegAlign programs from the DNAstar package (LaserGene Inc., Madison, WI, USA) were used. One sequence from each genotype is shown in Figure 1 and the features used to identify the genotypes are highlighted. Sequences from Brazilian patients were deposited in GenBank under the accession numbers AY306229-AY306686, AY309974-AY310119, and AY310921-AY311334.

Statistical analysis

Statistical analysis was carried out using the Epi-Info software version 6.04d (Centers for Disease Control & Prevention, Atlanta, GA, USA, and World Health Organization, Geneva, Switzerland). Unless otherwise stated, the statistical analysis for each group (Region or State) was carried out by comparing each group with the universe, i.e., the total number of cases in the other regions or the total number of cases in the other states from the same region.

Results

Genotypes 1, 2, 3, 4, and 5 of HCV were found among the 1,688 Brazilian samples studied. The general frequencies were 64.9% (1,095) for genotype 1, 4.6% (78) for genotype 2, 30.2% (510) for genotype 3, 0.2% (3)

for genotype 4, and 0.1% (2) for genotype 5. As shown in Figure 2, the distribution of HCV genotypes was statistically different among the Brazilian regions (P = 0.00017). Genotype 1 was the one most frequently found in all regions. In the North, it was found in 74.1% of the samples, followed by 66.7% in the Northeast, 66.4% in the Southeast, 57.0% in the Center-West, and 51.7% in the South. The difference in the lowest frequency of genotype 1 between the South and the other regions was statistically significant (P = 0.001), whereas a trend for significance (P = 0.08) was observed for the highest frequency in the North region. Genotype 2 was more prevalent in the Center-West region (11.4%, P = 0.0088), decreasing in the South (5.1%), Southeast (4.7%), Northeast (3.0%), and North (1.2%). The frequency of genotype 3 was higher in the South region (43.2%, P = 0.001) than in the Center-West (31.6%) and Northeast (30.4%) and significantly lower in the Southeast region (28.4%, P = 0.03). For the North region, in turn, the lower frequency of genotype 3 (24.7%) was not statistically significant. Genotypes 4 and 5 were found to be rare in the studied population, and all cases were from the Southeast, accounting for 0.3 and 0.2% of all cases, respectively.

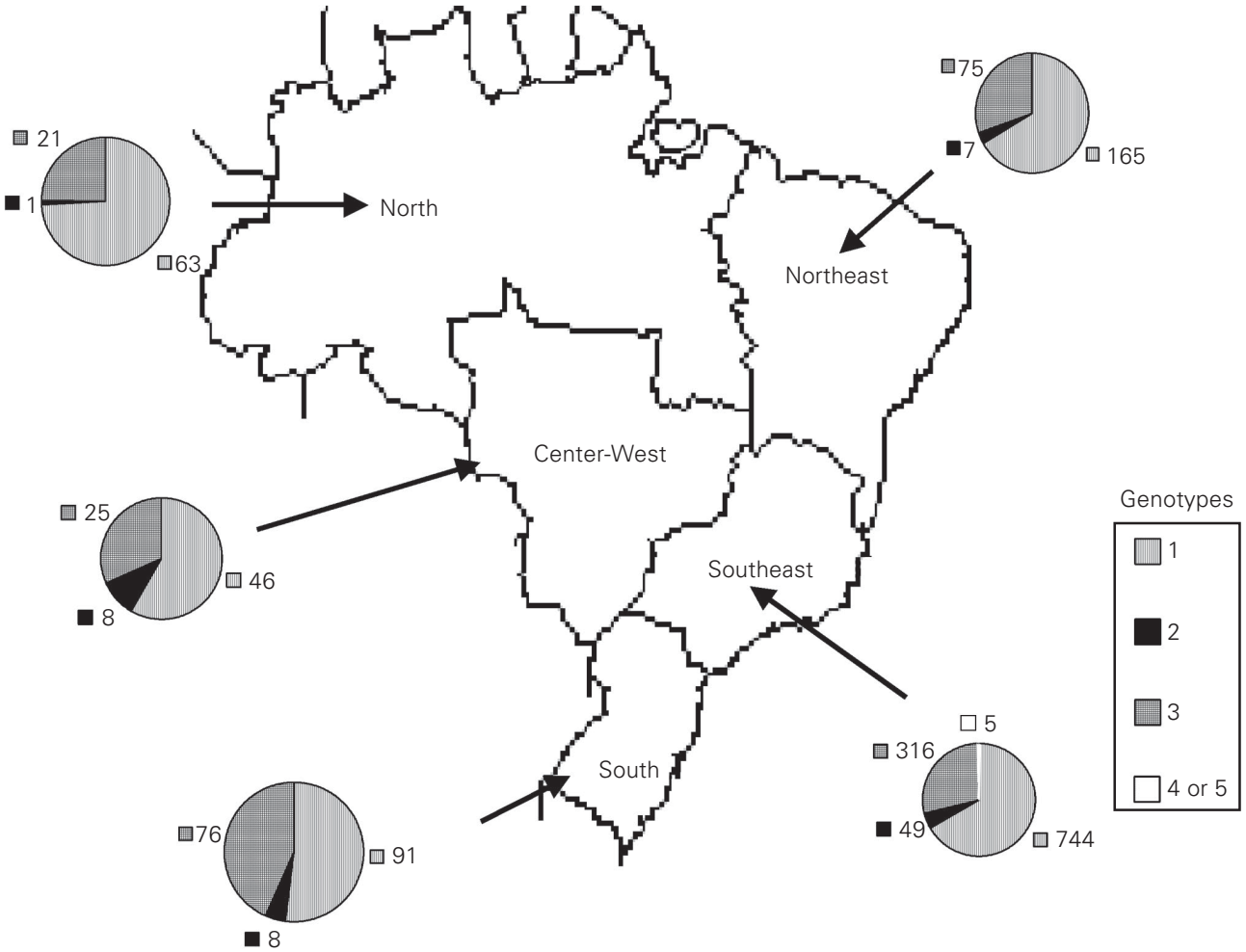

Figure 2. Distribution of hepatitis C virus genotypes in the different Brazilian regions. The absolute number of cases of each genotype found in each region is shown.

To further characterize the distribution of the HCV genotypes in Brazil, only states with a minimum of 8 samples were considered (Table 2). When the States of Acre and Amazonas in the North region were compared, genotype 1 was the most prevalent in both states (78.0 and 64.3%, respectively), followed by genotype 3 (22.0 and 28.6%, respectively), with no significant differences between the two states. Genotype 2 was only observed in Amazonas (7.1%).

In four Northeastern states, genotype 1 was by far the most prevalent, ranging from 60.7% in Pernambuco to 82.1% in Alagoas; the lower frequency of genotype 1 in Pernambuco was statistically significant (P = 0.045). The frequency of genotype 3 was lower in Alagoas (17.9%) and higher in Pernambuco (36.9%, P = 0.02). Genotype 2 was found in Pernambuco (2.4%) and Maranhão (10.5%), with the frequency in the latter state being statistically higher (P = 0.017) compared with the other Northeastern states.

Table 2. Distribution of HCV genotypes in different Brazilian states.

	Genotype					
	1	2	3	4	5	Total
North region						
Acre	46 (78.0%)	0	13 (22.0%)	0	0	59
Amazonas	9 (64.3%)	1 (7.1%)	4 (28.6%)	0	0	14
Others	8 (66.7%)	0	4 (33.3%)	0	0	12
Northeast region						
Alagoas	23 (82.1%)	0	5 (17.9%)	0	0	28
Maranhão	26 (68.4%)	4 (10.5%)	8 (21.0%)	0	0	38
Paraíba	26 (74.3%)	0	9 (25.7%)	0	0	35
Pernambuco	74 (60.7%)	3 (2.4%)	45 (36.9%)	0	0	122
Others	9 (64.3%)	0	5 (35.7%)	0	0	14
Center West region						
Goiás	21 (60.0%)	1 (2.9%)	13 (37.1%)	0	0	35
Mato Grosso	17 (54.8%)	8 (25.8%)	6 (19.4%)	0	0	31
Others	7 (53.9%)	0	6 (46.1%)	0	0	13
Southeast region						
Espírito Santo	7 (77.8%)	0	2 (22.2%)	0	0	9
Minas Gerais	21 (75.0%)	2 (7.1%)	5 (17.9%)	0	0	28
Rio de Janeiro	109 (79.1%)	1 (4.7%)	38 (16.3%)	0	0	234
São Paulo	525 (62.5%)	39 (4.6%)	271 (32.2%)	3 (0.4%)	2 (0.2%)	840
South region						
Paraná	82 (52.6%)	9 (5.7%)	65 (41.7%)	0	0	156
Santa Catarina	6 (50.0)%	0	6 (50.0%)	0	0	12
Rio Grande do Sul	3 (37.5%)	5 (62.5%)	0	0	0	8

In the Center-West, samples from only two (Goiás and Mato Grosso) of the four states in this region were assessed. Again, HCV genotype 1 was the most frequent in these states (60.0 and 54.8%, respectively). Genotype 2, in turn, was found at unexpectedly high frequency (25.8%) in Mato Grosso State, while genotype 3 was found in 6 (19.3%) samples. In Goiás, genotype 2 was found in only one subject (2.9%), but genotype 3 was found in 13 (37.1%) samples. The frequency of genotype 2 was higher in Mato Grosso than in Goiás (P = 0.009) and Brazil as a whole (P = 0.00004).

In the Southeast region, genotype 1 was the most frequent, ranging from 62.5% in São Paulo to 75.0% in Minas Gerais, 77.8% in Espírito Santo and up to 79.0% in Rio de Janeiro; the difference between São Paulo and the other states was statistically significant (P = 0.000001). Genotype 3 was more frequent in São Paulo State (32.3%) than in the other three states (Rio de Janeiro - 16.3%, Minas Gerais - 17.9%, and Espírito Santo - 22.2%). Genotype 2 was found in São Paulo, Rio de Janeiro and Minas Gerais at frequencies of 4.6, 4.7 and 7.1%, respectively. Genotypes 4 and 5 were found only in São Paulo at frequencies of 0.4 and 0.2%, respectively.

In the South region, genotype 1 was the most frequent in the State of Paraná (52.6%). In Santa Catarina, genotypes 1 and 3 were present at the same frequencies (50%). Contrary to all the other states analyzed, genotype 3 was the most frequent among samples from Rio Grande do Sul (62.5%), the southernmost state in Brazil, but this difference was not statistically significant.

Discussion

The study of viral diversity provides a better understanding of the origins and dynamics of viral infection. Genetic variants of HCV are known to be widely spread around

the world. Genotypes 1, 2 and 3 are found on all continents, but in some geographical areas, such as Africa and Southeast Asia, viral isolates are highly divergent and particular genotypes or subtypes are predominant (13-16). These data suggest the existence of a long-term endemic infection in these areas and some investigators have hypothesized that HCV might have originated in such places (17). Pybus et al. (18) found significant differences in the epidemic behavior of different HCV genotypes. Genotypes 1 and 3 rapidly spread before blood donor screening methods were adopted, while infections with genotypes 4 and 6 followed a pattern of community-acquired diseases by a variety of undefined social and domestic routes.

HCV genotypes 1, 2, 3, 4, and 5 were found among the present samples. The pattern of their distribution is similar to those found in Europe where the most prevalent genotypes (1 and 3) have an epidemiological behavior typical of viral strains that have spread exponentially in recent years, probably through blood transfusions.

The first data about HCV genotypes in Brazil came from Rio de Janeiro. In this state, we presently found genotypes 1, 2 and 3 at frequencies similar to those previously reported (19-22), except for genotype 2. In another study involving intravenous drug users from Rio de Janeiro, genotype 2 was found at a higher frequency (23).

In São Paulo State, we found genotypes 1, 2, 3, 4, and 5. These data are comparable to those previously published for São Paulo (24,25), except for the fact that we found two genotype 5 samples, possibly because we analyzed a larger population and/or included samples from outside São Paulo City.

Previous data from Minas Gerais were obtained for hemophiliacs (26) or patients under hemodialysis (23). We confirmed the predominance of genotype 1 in this state, but we also detected genotypes 2 and 3. In Espírito Santo, we found genotypes 1 and 3, in agreement with reports regarding cirrhotic patients in the same state (23).

Among the Northeastern states, published data are available only for Rio Grande do Norte (21), Ceará (23), and Bahia (27). We analyzed a small number of samples from these states and obtained comparable results. Among the other four Northeastern states analyzed here, genotype 1 was by far the most prevalent and genotype 3 was present at a rather high frequency. Genotype 2 was more frequent in Maranhão State.

In the Center-West, genotype 1 was also the most frequent, and genotype 2 was found at an unexpected high frequency in the State of Mato Grosso. Previous data available from Mato Grosso were obtained for 10 patients under hemodialysis, all of them infected with genotype 1 (23). Further studies should be carried out to determine the prevalence of genotypes in this state and to investigate the reasons for such unexpected high prevalence of genotype 2.

In the South, genotype 3 was found at percentages always higher than 40.4%, confirming that it is particularly common in this region, as previously shown by Krug et al. (28).

Cases of mixed HCV genotypes were not identified in our study. Infections with mixed HCV genotypes have not been reported frequently. Five HCV co-infections were identified in 205 samples from Spain using LiPA methodology, but only 2 cases were confirmed by sequencing (29). A low percentage of multiple infections with different HCV types was identified in 213 chronically infected Italian patients by restriction fragment length polymorphism, but only 4 of the 23 supposedly mixed cases were confirmed by sequencing (30). Few cases of HCV genotype co-infection have been reported using type-specific amplification and DNA immunoassays (31). A high incidence of multiple-genotype infection (17%) among children with chronic post-transfusion hepatitis C was determined by RT-PCR with type-specific primers (32). Mixed HCV genotype infec-

tions appear to be a rare event and their detection seems to be related to the technique used for genotyping. DNA sequencing cannot certify the presence of mixed infections, especially if one of the genotypes constituting the mixture is present in a very low percentage.

The distribution of HCV genotypes found in the present study was quite similar to that detected in many European countries. We speculate that the HCV circulating in Brazil has been introduced recently, after the arrival of the European immigrants, when blood transfusion practices became more common and no test to detect HCV was available. It is noteworthy that no HCV genotype seems to be specifically related to South America, while HCV genotype F (subtype *adw4*) is specific for the New World (33).

Sequencing the HCV 5'UTR has proved effective in discriminating the major genotypes, despite some mistyping in the annotation of subtypes (34,35). Sequencing other viral regions is indicated for more accurate subtyping and molecular epidemiological studies (36), but this would require re-amplification of all samples after RNA detection with different pairs of primers. Other studies with different viral genomic regions should be carried out to better understand the dynamics of HCV infection in Brazil, since most available data come from 5'UTR analysis. In the present study, we did not classify the viral isolates down to subtype level due to the known limitations in distinguishing different HCV subtypes by 5'UTR analysis. In some isolates, only one or two minor nucleotide changes distinguish subtypes, e.g., an adenine to guanine substitution between subtypes 1a and 1b. The relative failure in subtyping the genotype 2 samples at the 5'UTR by LiPA methodology has already been reported (37). Nevertheless, classifying HCV at the genotype level has been shown to be sufficient for clinical prognosis and treatment orientation (3).

Other investigators have sequenced the Amplicor HCV amplicon (a fragment of the 5'UTR) for genotyping and, on the basis of i) the clinical significance of genotype determination, ii) the overall gain in efficiency, iii) the shorter turnaround time, iv) the inclusion of contamination controls, and v) the low rate of test failure, concluded that 5'UTR sequencing has significant advantages for HCV genotyping over other methods based on the *NS5* gene and other techniques (34).

In conclusion, the present data indicate that the distribution of HCV genotypes in Brazil can be very variable between different regions and even different states from the same region. The need for careful epidemiological surveys throughout Brazil in different population groups is reinforced since knowing the frequency and distribution of the genotypes would provide key information for understanding the spread of HCV as well as to furnish subsides for treatment guidelines.

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