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Distribution and molecular characterization of hepatitis C virus (HCV) genotypes in patients with chronic infection from Pernambuco State, Brazil

Mónica V. Alvarado-Mora[a,*], Izolda M. Moura[b], Livia S. Botelho-Lima[a], Raymundo S. Azevedo[c], Edmundo Lopes[d], Flair J. Carrilho[a], João R.R. Pinho[a]

[a] Laboratory of Tropical Gastroenterology and Hepatology, Department of Gastroenterology, School of Medicine, University of São Paulo, São Paulo. SP, Brazil
[b] Central Laboratory of Clinical Hospital, Federal University of Pernambuco (UFPE), Recife, PE, Brazil
[c] Department of Pathology, School of Medicine, University of São Paulo, São Paulo, SP, Brazil
[d] Department of Clinical Medicine, Federal University of Pernambuco (UFPE), Recife, PE, Brazil

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ABSTRACT

Hepatitis C virus (HCV) is a public health problem throughout the world and 3% of the world population is infected with this virus. It is estimated that 3–4 millions individuals are being infected every year. It has been estimated that around 1.5% of Brazilian population is anti-HCV positive and the Northeast region showed the highest prevalence in Brazil. The aim of this study was to characterize HCV genotypes circulating in Pernambuco State (PE), Brazil, located in the Northeast region of the country. This study included 85 anti-HCV positive patients followed up between 2004 and 2011. For genotyping, a 380bp fragment of HCV RNA in the NS5B region was amplified by nested PCR. Phylogenetic analysis was conducted using Bayesian Markov chain Monte Carlo simulation (MCMC) using BEAST v.1.5.3. From 85 samples, 63 (74.1%) positive to NS5B fragment were successfully sequenced. Subtype 1b was the most prevalent in this population (42–66.7%), followed by 3a (16–25.4%), 1a (4–6.3%) and 2b (1–1.6%). Twelve (63.1%) and seven (36.9%) patients with HCV and schistosomiasis were infected with subtypes 1b and 3a, respectively. Brazil is a large country with many different population backgrounds; a large variation in the frequencies of HCV genotypes is predictable throughout its territory. This study reports HCV genotypes from Pernambuco State where subtype 1b was found to be the most prevalent. Phylogenetic analysis suggests the presence of the different HCV strains circulating within this population.

1. Introduction

Hepatitis C virus (HCV) is a blood borne pathogen, recognized as one of the major causes of chronic liver disease worldwide (Shepard et al., 2005). According to the World Health Organization, there are 170 million people infected with HCV, corresponding to 3% of the world population (McHutchison and Bacon, 2004). Actually, it is established that HCV infection is globally important and is a severe health problem that requires extensive and active interventions for the prevention and control. Prospective studies have shown that 80% of cases of acute hepatitis C progress to chronic infection and 10–20% of them develop complications of chronic liver disease such as liver cirrhosis and/or hepatocellular carcinoma (Kiyosawa et al., 1990; Lavanchy (2011); Zhou et al., 2010).

Africa and Asia are the regions of the world with the highest prevalence rates while the low prevalence areas include Australia, North America and Europe. In Latin America, the prevalence of HCV is 1.2% (Te and Jensen, 2010). Nevertheless, this prevalence varies from region to region, from 0.2–0.5% in Chile to 1.7–3.4% in Northeast Brazil (Carrilho and Corrêa, 1998; Soza et al., 2010; Te and Jensen, 2010;). Thus, it is estimated that there are 6.8–8.9 millions anti-HCV positive inhabitants in Latin America and HCV prevalence estimated was 1.5% for the Americas in 2010 (Kershenobich et al., 2011).

HCV is a positive sense single-strand RNA virus with a genome size of 9400 bp. It contains a large open reading frame that encodes a precursor polyprotein of about 3000 amino acids (Suzuki et al., 2007). The virus is classified in the genus *Hepacivirus* of the family *Flaviviridae* (Ishii et al., 1999). Phylogenetic analyses of full-length or partial sequences of HCV strains isolated in various regions of the world has led to the identification of six HCV genotypes (1–6), and a large number of subtypes (WHO, 1999; Simmonds et al., 2005). HCV genotyping is an essential tool for epidemiological studies (Cantaloube et al., 2006; Mora et al., 2010). There is a wide geographical variation considering genotype distribution: genotypes

* Corresponding author at: Depto Gastroenterologia, Faculdade de Medicina, Universidade de São Paulo, Av. Dr. Enéas de Carvalho Aguiar, 500, segundo andar, Prédio IMT 2, São Paulo, SP, Brazil. Tel.: +55 11 3061 8218; fax: +55 11 3064 5932.
E-mail address: monica.viviana@usp.br (M.V. Alvarado-Mora).

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Fig. 1. Geographical localization of Pernambuco state (PE), Brazil.

1, 2 and 3 are more frequent in Europe, North America and Japan; genotype 4 in Central Africa, Egypt and the Middle East; genotype 5 in South Africa; and genotype 6 in Asia. These genotypes differ by 31–34% in their nucleotide sequence and by around 30% in their amino acid sequence. Accurate HCV genotyping can be used for predicting response to anti-viral therapy, as genotypes 1 and 4 are more difficult than genotypes 2 and 3 to respond to interferon and ribavirin (Pawlotsky, 2003).

Pernambuco is a state of Brazil located in the Northeast region of the country. According to the IBGE, there were 8,796,448 people residing in this state (IBGE, 2010). *Mulatos* (African and Portuguese ancestry) and *Mamelucos* (Amerindian and Portuguese ancestry) are more common in the coast of this state and in the small towns, respectively (http://www.ibge.gov.br/estadosat/perfil.php?sigla=pe). The aim of this study was to report the current distribution of HCV genotypes in Pernambuco State, Brazil.

2. Materials and methods

2.1. Study population

This study was carried out in Pernambuco State including 85 HCV positive patients (63 from Recife and 22 from other regions of Pernambuco) collected between 2004 and 2011 in the Central Laboratory of Clinics Hospital, Federal University of Pernambuco (UFPE), Recife, PE, Brazil (Fig. 1). Blood collection was performed by venipuncture and serum was obtained and stored at −20 °C. Eighty-five patients were analyzed, 43 males and 42 females, with age ranging from 26 to 65 years old. Fifty-eight patients were HCV monoinfected patients, 25 also presented the hepatosplenic form of schistosomiasis (HCV/HES) and 2 were HCV/HIV coinfected.

Considering previous treatment, 53 patients were *naïve* and 32 of them have been previously treated with pegylated interferon (PEG-IFN) and ribavirin (Calvaruso and Craxi, 2012). Fibrosis METAVIR stage indexes were obtained from liver biopsies performed in 51/85 patients: *F*0 = 3 (5.88%), *F*1 = 14 (27.4%), *F*2 = 18 (35.3%), *F*3 = 12 (23.5%) and four patients (7.84%) had inconclusive results (Alencar et al., 2008).

2.2. HCV RNA extraction and synthesis of the complementary DNA (cDNA)

HCV-RNA extraction was carried out from 140 μL of serum using QIAamp® Viral RNA Kit (QIAGEN, Valencia, CA, USA), following the manufacturer's instructions. Synthesis of the complementary DNA (cDNA) was made immediately after RNA extraction. Reverse transcriptase reaction was performed using *Moloney Murine Leukemia Virus* Reverse Transcriptase (MMLV-RT) and random primers. The final volume of the reaction was 60 μL with the following concentrations: 50 mM Tris–HCl (pH = 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM each dNTP (10 mM), 450 ng random primers, 30 units RNAse enzyme inhibitor (RNase OUT™) and 300 units MMLV-RT. Samples were submitted to the following temperature cycles: 70 °C for 10 min, 25 °C for 15 min, 37 °C for 60 min and 95 °C for 15 min in a thermocycler (Eppendorf Mastercycler®, Eppendorf, Hamburg, Germany).

2.3. Polymerase chain reaction (PCR)

Polymerase chain reactions (PCR) were carried out in two stages, first and second PCR, to increase its sensitivity. A 380 bp fragment covering the NS5B region was amplified for genotyping analysis using conditions previously described (Enomoto et al., 1990; Sandres-Saune et al., 2003). Reactions were carried out in a final volume of 50 μL. The cDNA (5 μL) was added to 20 mM Tris–HCl (pH = 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP (10 mM), 0.4 pmol/μL of each primer, and 2.5 units *Platinum* Taq DNA polymerase. All the reagents used were from Invitrogen™ Life Technologies, Carlsbad, CA, USA.

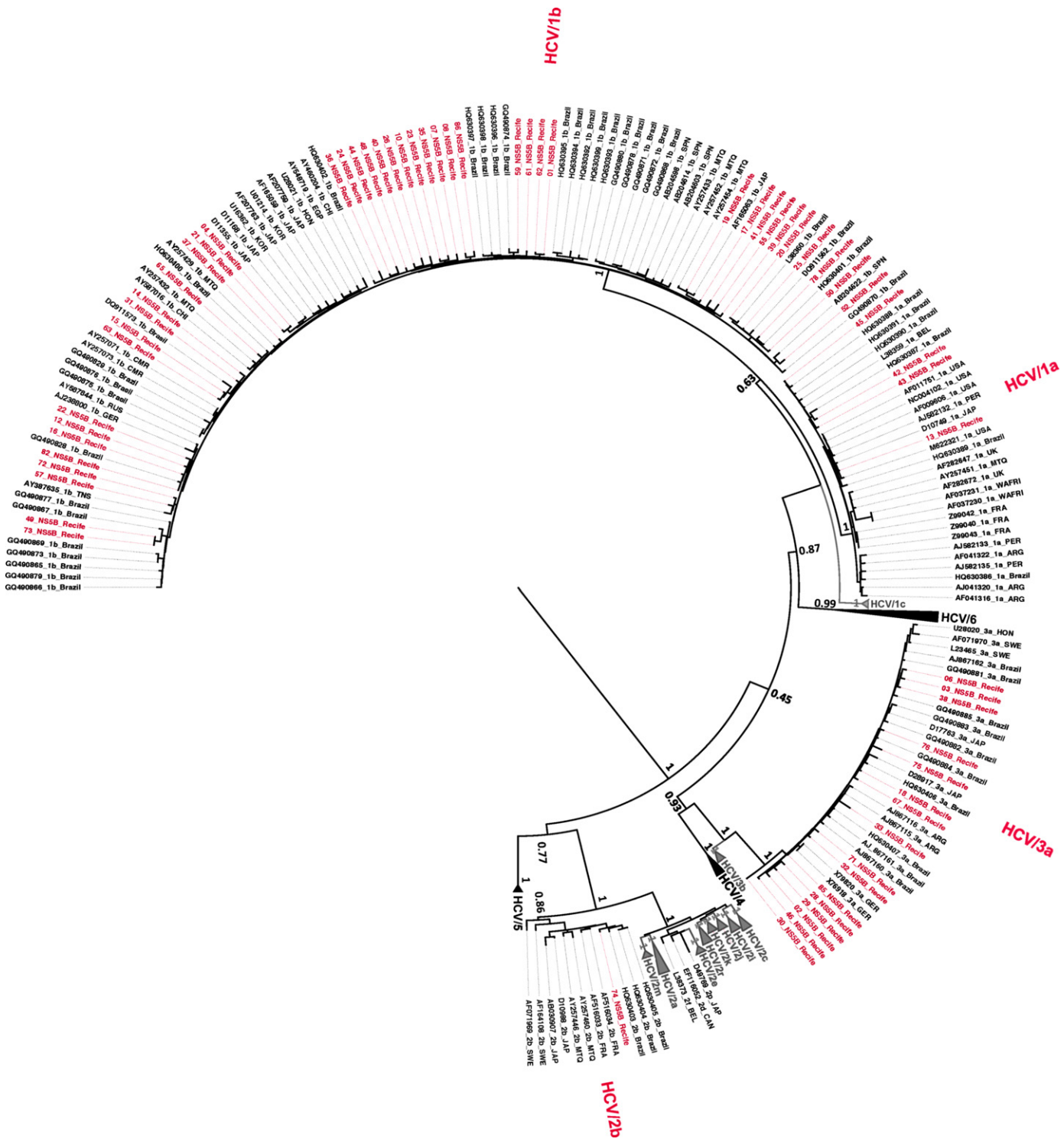

Fig. 2. The maximum clade credibility (MCC) tree was estimated by Bayesian analysis of 244 HCV NS5B sequences. The posterior probabilities of the key nodes are depicted above the respective nodes. The samples obtained from Pernambuco state ($n = 63$, red taxes) were analyzed together other HCV strain from the Genbank. The collapsed clades correspond to the other HCV subtypes. (For interpretation of references to color in this figure legend, the reader is referred to the web version of this article.)

2.4. HCV NS5B sequencing

Amplified cDNA was purified using ChargeSwitch® PCR Clean-Up Kit. Sequencing was performed in an Applied Biosystems® 3500 Genetic Analyzer (Life Technologies, Foster City, CA, USA) using dideoxynucleoside triphosphates (ddNTPs) containing fluorescent markers (*Big Dye® Terminator v3.1 Cycle Sequencing Ready Reaction kit* – Life Technology's, Foster City, CA, USA). The quality of each electropherogram was evaluated using the Phred-Phrap software (Ewing and Green, 1998; Ewing et al., 1998) and consensus sequences were obtained by alignment of both sequenced strands (sense and antisense) using CAP3 software available at the web page *Electropherogram quality analysis* (http://asparagin.cenargen.embrapa.br/phph/).

2.5. Phylogenetic analysis

The HCV sequences were genotyped by phylogenetic reconstructions using reference NS5B sequences from each HCV subtype obtained from GenBank ($n = 224$). The sequences were aligned using Muscle software (Edgar, 2004) and edited in the SE-AL program (available at http://tree.bio.ed.ac.uk/software/seal/). The parameters of the evolutionary model of DNA substitution were estimated by MODELTEST v.3.7 (Posada and Crandall, 1998). Phylogenetic analyses were conducted using the Markov Chain Monte Carlo (MCMC) simulation implemented in BEAST v.1.5.3 (Drummond and Rambaut, 2007) by both relaxed uncorrelated $\log_{normal}$ and relaxed uncorrelated exponential molecular clock using (GTR+G+I) model of nucleotide substitution. Twenty million generations were run to obtain the convergence of parameters. The maximum clade credibility (MCC) tree was obtained from summarizing the substitution trees and then it was removed 10% of burn-in using Tree Annotator v.1.5.3 (Drummond and Rambaut, 2007). Phylogenetic trees were visualized and midpoint rooted in FigTree v1.2.2 (http://tree.bio.ed.ac.uk/software/figtree).

2.6. Statistical analyses

Statistical analyses were performed using Minitab Software v.15. The χ^2 test for linear trend ($\alpha = 0.05$) was used to examine the distribution of HCV subtypes in patients according to age group, sex, geographical origin, previous treatment and presence of HES. Results were considered statistically significant when $p < 0.05$.

3. Results

We obtained 63 (74.1%) PCR positive samples that were successfully sequenced. To analyze the distribution of HCV genotypes in this population, phylogenetic trees were reconstructed with the obtained NS5B region sequences. Subtype 1b was the most frequent in this population (42–66.7%), followed by 3a (16–25.4%), 1a (4–6.3%) and 2b (1–1.6%) (Fig. 2). All sequences were deposed in GenBank (accession numbers: JQ323440–JQ323502).

When the capital city was compared with the other regions, we found the same distribution: 45 positive samples were from Recife [1b (30–66.7%), 3a (12–26.7%), 1a (2–4.4%) and 2b (1–2.2%)] and 18 positive samples were from other small towns [1b (12–66.7%), 3a (4–22.2%) and 1a (2–11.1%)]. Also, among the 19 PCR positive patients that presented hepatosplenic form of the schistosomiasis (HCV/HES) (ten from Recife and nine from the other small towns), twelve (63.2%) were infected with subtype 1b and seven (36.8%) with subtype 3a, respectively. Finally, the only one subtype 2b was found in a HCV/HIV patient.

Statistical analyses were carried out to analyze the distribution of HCV subtypes in the different demographic and clinical variables. Statically significance was not found when genotypes distribution was analyzed according to sex ($p = 0.745$), presence of HCV/HES ($p = 0.130$), origin (Recife vs. others) ($p = 0.842$) and previous antiviral treatment (treatment-*naïve* and previously treated patients – non-responders) ($p = 0.487$).

4. Discussion

In Brazil, the main risk factors of HCV infection are (i) individuals who received blood transfusions before 1993 and (ii) injecting drug users under 30 years (Toledo et al., 2005; Silva et al., 1995). A recently study shows that the overall result for anti-HCV prevalence in all capital cities from Brazilian states was 1.38% (95% CI 1.12–1.64%). Particularly, HCV prevalence from state capitals of the Northeast region ranged from 0.4 to 1.0% (http://www.aids.gov.br/publicacao/2011/estudo_de_prevalencia_de_base_populacional_das_infeccoes_pelos_virus_das_hepatites_b). These results place this Brazilian region as a low HCV prevalence area. Others studies were carried out to evaluate HCV in particular groups from Pernambuco state: among hemodialysis and schistosomiasis patients, HCV frequency was 8.4% (Mello Lde et al., 2007) and 11.9% (Silva et al., 2008), respectively.

In Latin America, genotype 1 is the most prevalent (Mendez-Sanchez et al., 2010). Nonetheless, differences in distribution of genotypes of HCV have been observed within our continent, particularly in the Caribbean region, where genotypes 2 and 4 have a major frequency, differing from the rest of Latin America (Jimenez-Mendez et al., 2010; Martial et al., 2004). In Brazil, Campiotto et al., 2005 reported that genotype 1 was the most frequently genotype. Particularly, in the North region, genotype 1 was the most prevalent in Amazonas and Acre states (78.0% and 64.3%, respectively), followed by genotype 3. In Pernambuco, genotype 1 was the most prevalent (60.7%), followed by genotypes 3 (36.9%) and 2 (2.4%). Also, in HCV/HIV patients from this state, the most common HCV subtypes were 1b (45%), 3 (33%) and 1a (22%) and the results of multiple logistic regression confirms the variable blood transfusion was a remaining risk factor for HCV (Carvalho et al., 2009). When compare with other states from the Northeast region of Brazil, for example, Piauí, it was observed a homogeneous distribution between genotypes 1 (50.0%) and 3a (49.0%) (Veras et al., 2009). In Rondônia state, located in the Northwestern region in Brazil, it was found 50% of presence of subtype 1b, followed by 1a (27.2%), 2b (13.6%) and 3a (9.0%) (Vieira et al., 2011).

Subtype 1b is mainly found among old people who have a previous history of blood transfusion and is related with a higher rate of chronic active hepatitis or cirrhosis and low response to treatment with Peg-interferon plus Ribavirin compared with genotypes 2 and 3 (Pawlotsky, 2003; Mora et al., 2010). Furthermore, molecular epidemiology studies have shown that HCV genotype 3, subtype 3a (HCV-3a) is significantly associated with transmission through injecting drug use in industrialized countries (Pawlotsky et al., 1995; Bourliere et al., 2002). HCV-3a apparently emerged originally and diversified in Asia (potentially South- East Asia, and the Indian subcontinent), where multiple other subtypes of HCV genotype 3 have been described (Lole et al., 2003). Recently, it was reported that there was a significant increase in cocaine consumption among students in the State capitals of the Northeast of Brazil (Salvador, Bahia; Recife, Pernambuco; and Fortaleza, Ceará) (Duailibi et al., 2008). Also, it was determined 6.9% of prevalence rates of drug use among 4230 high school students from Pernambuco State (Carvalho et al., 2011). Since we not have data about injecting drug use in these patients, we cannot perfom more inferences about this, but probably this practice in the population may be is associated with significant prevalence of the subtype 3a found in this state.

Schistosomiasis is a parasitic infection that also affects the liver and is considered a public health problem in many different Brazilian regions (SVS, 2005). It has been previously described that it might alter the evolution of viral hepatitis (Aquino et al., 2000), especially for hepatitis B. It has also been previously described that this infection was associated with a higher prevalence of anti-HCV and it might be associated with a different distribution of HCV genotypes, but the distribution of HCV genotypes was not different from the overall group.

5. Conclusions

In conclusion, this study reports a high frequency of subtype 1b and 3a among HCV patients from Pernambuco State. Our results also shows that HCV infection in this region derives from different

HCV lineages that are circulating also around the world and not to a particular strain that was associated to most infections in this area. More studies are needed to allow a better understanding of the dynamics of the HCV epidemic in this state to development of an effective prevention policy.

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