

Genotyping hepatitis C virus from hemodialysis patients in Central Brazil by line probe assay and sequence analysis

M.P. Espírito-Santo¹,
M.A.S. Carneiro²,
N.R.S. Reis², A.G. Kozlowski²,
S.A. Teles³, E. Lampe¹,
C.F.T. Yoshida¹
and R.M.B. Martins²

¹Departamento de Virologia, Instituto Oswaldo Cruz, FIOCRUZ,
Rio de Janeiro, RJ, Brasil

²Instituto de Patologia Tropical e Saúde Pública, ³Faculdade de Enfermagem,
Universidade Federal de Goiás, Goiânia, GO, Brasil

Abstract

Correspondence

R.M.B. Martins
Instituto de Patologia Tropical
e Saúde Pública
Universidade Federal de Goiás
74605-050 Goiânia, GO
Brasil
Fax: +55-62-3521-1839
E-mail: rbringel@terra.com.br

Research supported by CNPq
(No. 620023/2004-0).

Received September 23, 2005
Accepted May 23, 2006

The present study examined the distribution of hepatitis C virus (HCV) genotypes and subtypes in a hemodialysis population in Goiás State, Central Brazil, and evaluated the efficiency of two genotyping methods: line probe assay (LiPA) based on the 5' noncoding region and nucleotide sequencing of the nonstructural 5B (NS5B) region of the genome. A total of 1095 sera were tested for HCV RNA by RT-nested PCR of the 5' noncoding region. The LiPA assay was able to genotype all 131 HCV RNA-positive samples. Genotypes 1 (92.4%) and 3 (7.6%) were found. Subtype 1a (65.7%) was the most prevalent, followed by subtypes 1b (26.7%) and 3a (7.6%). Direct nucleotide sequencing of 340 bp from the NS5B region was performed in 106 samples. The phylogenetic tree showed that 98 sequences (92.4%) were classified as genotype 1, subtypes 1a (72.6%) and 1b (19.8%), and 8 sequences (7.6%) as subtype 3a. The two genotyping methods gave concordant results within HCV genotypes and subtypes in 100 and 96.2% of cases, respectively. Only four samples presented discrepant results, with LiPA not distinguishing subtypes 1a and 1b. Therefore, HCV genotype 1 (subtype 1a) is predominant in hemodialysis patients in Central Brazil. By using sequence analysis of the NS5B region as a reference standard method for HCV genotyping, we found that LiPA was efficient at the genotype level, although some discrepant results were observed at the subtype level (sensitivity of 96.1% for subtype 1a and 95.2% for subtype 1b). Thus, analysis of the NS5B region permitted better discrimination between HCV subtypes, as required in epidemiological investigations.

Key words

- Hepatitis C
- Hepatitis C virus
- Genotypes
- Hemodialysis

Hepatitis C virus (HCV) is a well-known agent of liver disease, including cirrhosis and hepatocellular carcinoma, with an estimated 170 million persons being infected with this agent around the world (1). Patients

undergoing hemodialysis are at high risk of acquiring this blood-borne pathogen, since HCV is efficiently transmitted by the parenteral route. In addition, infected patients have an increased tendency to develop chronic

hepatitis and to be also a potential reservoir for its transmission, possibly contributing to the nosocomial spread of HCV in dialysis centers and explaining the high prevalence of HCV infection among hemodialysis patients (2).

HCV has a positive-sense, single-stranded RNA genome that presents a high mutation rate (1.44×10^{-3} substitutions per site per year). On the basis of the similarity of nucleotide sequences, HCV is classified into six major genetic groups designated genotypes (1 to 6), each comprising multiple subtypes (designated a, b, c, etc.). These genotypes have distinct geographical distributions. Although HCV genotypes 1, 2, and 3 appear to have a worldwide distribution, their relative prevalence varies from one geographic area to another. HCV genotype 4 is found in the Middle East and North Africa, and genotypes 5 and 6 in South Africa and Asia, respectively (3).

Genotype determination is a relevant predictive parameter of the response to antiviral treatment since genotype 1 is associated with a lower sustained virologic response (40-45%) compared to genotypes 2 and 3, whose sustained virologic response is 70-80% with peginterferon and ribavirin combination therapy for 48 and 24 weeks, respectively (1). Furthermore, the genotyping of HCV isolates is a useful tool in molecular studies carried out to establish the source of outbreaks in hemodialysis centers and other nosocomial settings (4,5).

In Brazil, a country of continental dimensions, the distribution of HCV genotypes and subtypes among hemodialysis patients has not been well documented. The few studies carried out in São Paulo (6,7), Tocantins (8) and Recife (9) have shown that genotype 1 (subtype 1a) was the predominant one in those patients. However, the genotyping methods employed in those studies, which were based on 5' noncoding (5'NC) region analyses, did not permit the correct identification of HCV subtypes (10-

17). To our knowledge, the present study is the first to analyze Brazilian HCV samples using two genotyping methods: line probe assay (LiPA, based on the 5'NC region) and the nucleotide sequencing analysis of the nonstructural 5B (NS5B) region, which has been used as a reference method for accurate genotyping (10-17). Thus, our objective was to determine which HCV genotypes and subtypes occur in hemodialysis patients in Central Brazil and also to compare the genotyping efficiency of LiPA and direct sequencing of the NS5B region.

The study was carried out in all dialysis units (N = 15) in Goiás State. Between April and December 2002, blood samples were collected from all chronic hemodialysis patients (N = 1095). The study protocol was approved by the Ethics Committee of the Federal University of Goiás, and written informed consent was obtained from each patient.

All samples were submitted to RNA extraction, reverse transcription, and a nested PCR with primers complementary to the conserved area of the 5'NC region of HCV, essentially as described by Ginabreda et al. (18). PCR products were loaded onto 1.5% agarose gel electrophoresis and visualized by staining with ethidium bromide under ultraviolet light. HCV genotyping was determined in all HCV-RNA-positive samples. A line probe assay (Inno-LiPA HCV II, Innogenetics, Ghent, Belgium) was used to determine the genotype in the amplicons of the 5'NC region according to the procedure described by the manufacturer.

Genotyping by sequence analysis was performed after nested RT-PCR for the NS5B region using the primers and PCR conditions described by Sandres-Sauné et al. (17). The nested RT-PCR products were purified using the QIAquick gel extraction kit (Qiagen - GmbH, Hilden, Germany) and submitted to a direct nucleotide sequencing reaction in both directions using a Big Dye Terminator kit (version 3.1, Applied Biosystems, Foster

City, CA, USA) and analyzed with the ABI 3730 automated DNA sequencer. The sequence from nucleotide 8279 to nucleotide 8619 was used for analysis and subtyping was performed by phylogenetic analysis using reference sequences retrieved from GenBank. The DNA alignments were generated with the Clustal X program (19). The phylogenetic tree was constructed with Mega 2.1 software (20) using the neighbor-joining method and the Kimura-two parameter and its reliability was assessed by bootstrap resampling (1000 pseudo-replicas). To avoid cross-contamination between samples, standard precautions were used in all manipulations. Separate areas were used for reagents, samples and manipulation of amplified products.

The presence of HCV RNA was detected in 131 samples by RT-nested PCR of the 5'NC region. The LiPA assay was able to genotype all 131 HCV RNA-positive samples. Among them, 92.4% were of genotype 1, subtypes 1a (65.7%) and 1b (26.7%). The remaining samples (7.6%) belonged to genotype 3, subtype 3a (Table 1).

For sequence analysis, 106 samples could be amplified and sequenced in the NS5B region. Using phylogenetic tree analysis of the NS5B region (Figure 1), 98 sequences (92.4%) were classified as genotype 1, subtype 1a (72.6%) and 1b (19.8%). All genotype 3 sequences (N = 8; 7.6%) were grouped inside the clad of subtype 3a of the phylogenetic tree (Figure 1 and Table 1).

The two methods were 100% concordant at the genotype level and the subtypes determined by the two methods were concordant in 96.2% of cases (102/106). Only four samples showed discrepant results. Sequence analysis of the NS5B region revealed that three samples, which were identified as subtype 1b by LiPA, were of subtype 1a. Conversely, one sample (subtype 1a by LiPA) was identified as 1b by sequence analysis of the NS5B region. Using the sequence analysis of this region as a reference method for

HCV subtyping, the sensitivity of the LiPA was 96.1% (74/77) for subtype 1a and 95.2% (20/21) for subtype 1b.

The distribution of HCV genotypes and subtypes found in the present study was similar to those previously reported in Brazilian hemodialysis patients (6-9), who showed a predominance of HCV subtype 1a. These data suggest that subtype 1a is more likely to disseminate in the hemodialysis environment or could be more adapted to the immunosuppression of these patients (7).

In order to determine HCV genotypes and subtypes, the choice of the genome region to be analyzed is crucial. This region must present genotype-specific and subtype-specific motifs. Additionally it must be highly conserved to be detected by most of the assays based on nucleic acid amplification. Several assays were developed to identify HCV genotypes and subtypes from the 5'NC region because this region is readily amplified by PCR. On the other hand, this region does not contain sufficient information for the recognition of all different types and subtypes. Thus, in the present study we chose the NS5B region for sequence analysis since this region appears to be much more accurate for identifying variations in the nucleotide sequences (10-17).

LiPA is a reverse hybridization assay based on variations of the highly conserved 5'NC region. In the present study, all 131 HCV RNA-positive samples were genotyped by this method. Of these, 25 (19.1%) could

Table 1. Distribution of the hepatitis C virus subtypes isolated from hemodialysis patients in Central Brazil using the line probe assay and nonstructural 5B (NS5B) sequencing.

Method	Subtypes			Total
	1a	1b	3a	
Line probe assay	86 (65.7%)	35 (26.7%)	10 (7.6%)	131 (100%)
NS5B sequencing	77 (72.6%)	21 (19.8%)	8 (7.6%)	106 (100%)

Twenty-five samples could not be genotyped by the NS5B sequencing method because of failures in the amplification step.

not be genotyped by NS5B sequence analysis due to failure of the amplification procedure despite successive attempts to amplify cDNA with the NS5B primers. Although these primers are thought to bind highly conserved sequences (17), primer-target mismatching within the NS5B region is still likely because of inherently high sequence variability. Despite this inability to amplify all HCV RNA-positive samples, NS5B sequence analysis was used here as a reference method for genotyping accuracy.

Other studies (10,12,14) have reported that genotyping methods based on the 5'NC region using LiPA and NS5B region by sequence analysis show high rates (98.6 to 100%) of concordance at the genotype level. In the present study, discrepant results at the subtype level were obtained for four of the 106 samples successfully typed by both methods. Three samples identified as 1a by NS5B sequencing were subtyped as 1b by LiPA. Another sample was subtyped as 1b by sequencing but was identified as 1a by LiPA. The inability of LiPA to identify the correct subtype has been reported by others, especially regarding subtypes 1a and 1b (10,12, 14,16). These subtypes have been frequently mistyped, mostly because subtyping in the 5'NC region is based on a single-base change

at position 99 (adenine to guanine) (10).

Although the limitation of the 5'NC region for HCV typing is not crucial for clinical practice, especially in the management of antiviral therapy, the NS5B sequencing appears to be more useful for epidemiological investigations. Phylogenetic analysis of this region could be useful to identify the route of HCV transmission, e.g., in the detection of HCV subtype(s) implicated in nosocomial transmission (4,15).

The present study demonstrated that HCV subtype 1a is the most prevalent among hemodialysis patients in Central Brazil by means of NS5B sequence analysis and LiPA assay. In addition, both methods provide similar HCV genotyping results at the genotype level, but the method involving direct sequencing of the NS5B region is preferable for the precise identification of HCV subtypes that may suggest nosocomial transmission at dialysis centers or for epidemiological studies.

Acknowledgments

We are grateful to the sequencing group of the PDTIS program of Fundação Oswaldo Cruz for performing the DNA sequencing.

References

1. Heathcote J, Main J. Treatment of hepatitis C. *J Viral Hepat* 2005; 12: 223-235.
2. Meyers CM, Seeff LB, Stehman-Breen CO, Hoofnagle JH. Hepatitis C and renal disease: an update. *Am J Kidney Dis* 2003; 42: 631-657.
3. Simmonds P. Genetic diversity and evolution of hepatitis C virus - 15 years on. *J Gen Virol* 2004; 85: 3173-3188.
4. Abacioglu YH, Bacaksiz F, Bahar IH, Simmonds P. Molecular evidence of nosocomial transmission of hepatitis C virus in a haemodialysis unit. *Eur J Clin Microbiol Infect Dis* 2000; 19: 182-186.
5. Kokubo S, Horii T, Yonekawa O, Ozawa N, Mukaide M. A phylogenetic-tree analysis elucidating nosocomial transmission of hepatitis C virus in a haemodialysis unit. *J Viral Hepat* 2002; 9: 450-454.
6. Moreira R, Pinho JR, Fares J, Oba IT, Cardoso MR, Saraceni CP, et al. Prospective study of hepatitis C virus infection in hemodialysis patients by monthly analysis of HCV RNA and antibodies. *Can J Microbiol* 2003; 49: 503-507.
7. Perez RM, Ferraz ML, Figueiredo MS, Contado D, Koide S, Ferreira AP, et al. Unexpected distribution of hepatitis C virus genotypes in patients on hemodialysis and kidney transplant recipients. *J Med Virol* 2003; 69: 489-494.
8. Souza KP, Luz JA, Teles SA, Carneiro MA, Oliveira LA, Gomes AS, et al. Hepatitis B and C in the hemodialysis unit of Tocantins, Brazil: serological and molecular profiles. *Mem Inst Oswaldo Cruz* 2003; 98: 599-603.
9. Albuquerque AC, Coelho MR, Lopes EP, Lemos MF, Moreira RC. Prevalence and risk factors of hepatitis C virus infection in hemodialysis patients from one center in Recife, Brazil. *Mem Inst Oswaldo Cruz* 2005; 100: 467-470.
10. Chen Z, Weck KE. Hepatitis C virus genotyping: interrogation of the 5' untranslated region cannot accurately distinguish genotypes 1a

- and 1b. *J Clin Microbiol* 2002; 40: 3127-3134.
11. Germer JJ, Majewski DW, Rosser M, Thompson A, Mitchell PS, Smith TF, et al. Evaluation of the TRUGENE HCV 5'NC genotyping kit with the new GeneLibrarian module 3.1.2 for genotyping of hepatitis C virus from clinical specimens. *J Clin Microbiol* 2003; 41: 4855-4857.
 12. Halfon P, Trimoulet P, Bourliere M, Khiri H, De Ledinghen V, Couzigou P, et al. Hepatitis C virus genotyping based on 5' noncoding sequence analysis (Trugene). *J Clin Microbiol* 2001; 39: 1771-1773.
 13. Laperche S, Lunel F, Izopet J, Alain S, Deny P, Duverlie G, et al. Comparison of hepatitis C virus NS5b and 5' noncoding gene sequencing methods in a multicenter study. *J Clin Microbiol* 2005; 43: 733-739.
 14. Nolte FS, Green AM, Fiebelkorn KR, Caliendo AM, Sturchio C, Grunwald A, et al. Clinical evaluation of two methods for genotyping hepatitis C virus based on analysis of the 5' noncoding region. *J Clin Microbiol* 2003; 41: 1558-1564.
 15. Othman SB, Trabelsi A, Monnet A, Bouzgarrou N, Grattard F, Beyou A, et al. Evaluation of a prototype HCV NS5b assay for typing strains of hepatitis C virus isolated from Tunisian haemodialysis patients. *J Virol Methods* 2004; 119: 177-181.
 16. Roque-Afonso AM, Ferey MP, Poveda JD, Marchadier E, Dussaix E. Performance of TRUGENE hepatitis C virus 5' noncoding genotyping kit, a new CLIP sequencing-based assay for hepatitis C virus genotype determination. *J Viral Hepat* 2002; 9: 385-389.
 17. Sandres-Sauné K, Deny P, Pasquier C, Thibaut V, Duverlie G, Izopet J. Determining hepatitis C genotype by analyzing the sequence of the NS5b region. *J Virol Methods* 2003; 109: 187-193.
 18. Ginabreda MG, Yoshida CF, Niel C. Genomic characterization of Brazilian hepatitis C virus genotypes 1a and 1b. *Braz J Med Biol Res* 1997; 30: 339-345.
 19. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The CLUSTAL-X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 1997; 25: 4876-4882.
 20. Kumar S, Tamura K, Jakobsen IB, Nei M. MEGA2: molecular evolutionary genetics analysis software. *Bioinformatics* 2001; 17: 1244-1245.