Revista da Sociedade Brasileira de Medicina Tropical 47(3):287-294, May-Jun, 2014
http://dx.doi.org/10.1590/0037-8682-0040-2014

Major Article

A complete molecular biology assay for hepatitis C virus detection, quantification and genotyping

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ABSTRACT

Introduction: Molecular biology procedures to detect, genotype and quantify hepatitis C virus (HCV) RNA in clinical samples have been extensively described. Routine commercial methods for each specific purpose (detection, quantification and genotyping) are also available, all of which are typically based on polymerase chain reaction (PCR) targeting the HCV 5' untranslated region (5'UTR). This study was performed to develop and validate a complete serial laboratory assay that combines real-time nested reverse transcription-polymerase chain reaction (RT-PCR) and restriction fragment length polymorphism (RFLP) techniques for the complete molecular analysis of HCV (detection, genotyping and viral load) in clinical samples. **Methods:** Published HCV sequences were compared to select specific primers, probe and restriction enzyme sites. An original real-time nested RT-PCR-RFLP assay was then developed and validated to detect, genotype and quantify HCV in plasma samples. **Results:** The real-time nested RT-PCR data were linear and reproducible for HCV analysis in clinical samples. High correlations (> 0.97) were observed between samples with different viral loads and the corresponding read cycle (Ct - Cycle threshold), and this part of the assay had a wide dynamic range of analysis. Additionally, HCV genotypes 1, 2 and 3 were successfully distinguished using the RFLP method. **Conclusions:** A complete serial molecular assay was developed and validated for HCV detection, quantification and genotyping.

Keywords: Hepatitis C virus. Polymerase chain reaction. Restriction fragment length polymorphism.

INTRODUCTION

Hepatitis C virus (HCV) is recognized as one of the main causes of chronic liver disease worldwide[1]. Hepatitis C virus infection is usually asymptomatic during the acute phase, but more than 80% of patients progress to chronic hepatitis C (CHC). Approximately 130-170 million people worldwide are chronically infected with HCV, and these people are at risk of developing hepatic complications, such as cirrhosis and/or hepatocarcinoma[2].

Hepatitis C virus is an enveloped, single-stranded, positive-sense ribonucleic acid (RNA) virus with a 50nm diameter viral particle and is a member of the Hepacivirus genus of the Flaviviridae family[3]. Its RNA genome encodes a unique polyprotein of approximately 3,000 amino acids[4,5]. The HCV genome is extremely heterogeneous. Published sequence data indicate that the 5' untranslated region (5´UTR) is highly conserved among various HCV isolates and is the target of most HCV molecular biology assays. This region, however, also contains genotypically variable sequences that allow the virus to be classified into six classical genotypes, which were recently updated to seven main genotypes that differ by more than 30% in their nucleotide sequence[6-8]. Hepatitis C virus genotyping is clinically relevant because improved treatment response rates have been observed with genotypes 2 and 3, compared to genotypes 1 and 4[2]. Consequently, patients infected with genotypes 1 or 4 are treated for longer times and with more potent combinations of antivirals than patients with genotypes 2 and 3[9,10].

Chronic hepatitis C is usually associated with mild symptoms. Elevation of aminotransferases, particularly alanine aminotransferase (ALT), is commonly observed, although up to one-third of asymptomatic patients have persistently normal enzymes[11]. Liver histology in CHC generally consists of inflammatory infiltrates and some degree of fibrosis, ranging from minimal expansion of portal tracts to cirrhosis. The fibrotic process, which is driven by liver inflammation, can progress over time, although patients differ greatly in this process depending on several viral and host factors[12]. Cirrhosis occurs in at least 20% of patients with CHC within 20 years[13]. In contrast to other viral chronic infections, hepatitis B virus (HBV) and human immunodeficiency virus (HIV), HCV viral load is not a pivotal factor in monitoring disease progression. However,

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Received 26 February 2014
Accepted 16 June 2014

it is an important prognostic factor for predicting the treatment response[14]. Additionally, monitoring viral load is essential during treatment because this information is necessary for achieving an early virological response (defined as negativity or a more than 2-log decrease in viral load by week 12) and allows clinicians to continuously monitor patient treatment[15].

Several molecular biology assays have been described for hepatitis C virus-ribonucleic acid (HCV-RNA) detection and quantification. Most commercial methods are polymerase chain reaction (PCR)-based and target the 5'UTR, which is the most conserved region among various genotypes/subtypes[16]. The introduction of real-time PCR-based assays improved both detection and viral load analysis[17,18]. These techniques have a broad dynamic range of quantification, which is well suited to the clinical needs (upper range of quantification: 7-8 log10IU/mL). Additionally, real-time PCR is more sensitive than classical PCR, with limits of detection of 10-15IU/mL. More recently, commercial real-time platforms have become available for the detection and quantification of HCV-RNA: the Cobas TaqMan platform, which can be used together with automated sample preparation with the Cobas AmpliPrep_system (CAP-CTM; Roche Molecular System, Pleasanton, CA), and the Abbott platform (Abbott Diagnostic, Chicago, IL), which uses the m2000RT amplification platform together with the m2000SP device for sample preparation[19].

Molecular methods for genotyping HCV that target various HCV genomic regions have also been described[20,21]. Widely used laboratory procedures include the line probe assay (LiPA) and 5'UTR sequencing[22,23]. Restriction fragment length polymorphism (RFLP) analysis of the 5´UTR of the HCV genome was one of the first assays used in large genotyping studies. In this procedure, a PCR-amplified hepatitis C virus-deoxyribonucleic acid (HCV-DNA) fragment is digested into fragments with restriction enzymes that recognize cleavage sites specific for each genotype[24]. Genotyping based on the amplification of this region has the advantage that it can be performed on PCR amplification products obtained from HCV-RNA detection tests[25]. However, this procedure is not widely used because it involves the use of 5 to 6 different restriction enzymes and is hampered by partial digestions and indeterminate results, making it laborious and time-consuming[26]. This study aimed to develop and validate a complete analytical assay based on nested reverse transcription polymerase chain reaction (RT-PCR) and simpler RFLP methodology for the detection quantification and genotyping of HCV.

METHODS

Sequences and comparative analysis

A total of 1,080 HCV 5´UTR nucleotide sequences (accession numbers EF558854-EF558890, EF564603-EF564609, EF571224-EF571247, AY306229-AY306686, AY309974-AY310119 and AY310921-AY311334) were retrieved using Entrez from the National Center for Biotechnology Information (NCBI, Bethesda, MD). These sequences were obtained from previous HCV genotype prevalence studies with 1,080 different HCV infected patients from different states of the 5 geographic regions from Brazil[27]. Additional 26 reference sequences from different genotypes (according to consensus proposals) were retrieved for comparative analysis[6].

Sequences were edited and aligned with EditSeq and MegAlign (using the Clustal method) programs from the DNAstar package (LaserGene Inc., Madison, WI, USA). Primers and one probe were selected directly from the aligned sequences. The presence of the restriction sites for *Hae III, Hinf* I, *BstN* I, *Rsa* I, *BfuC* I and *BstU* I was determined using Mapdraw program within the DNAstar package (LaserGene Inc., Madison, WI, USA).

Reference and clinical samples

A panel of 57 HCV-positive and HCV-negative plasma samples was obtained from a Brazilian company (ControlLab, Rio de Janeiro - RJ, Brazil). Fifty anti-HCV-negative blood donor samples were obtained from a hospital blood bank (*Hospital das Clínicas de Porto Alegre* - HCPA). A panel of 267 HCV-RNA-positive clinical samples (HCV genotypes 1, 2 and 3) was provided by a molecular diagnostic laboratory (Simbios Biotecnologia, Cachoeirinha, RS, Brazil). All of the plasma samples were collected with ethylenediaminetetraacetic acid (EDTA) in different clinical laboratories and were conserved at -20ºC until analysis.

RNA extraction

RNA was purified from 100µL of plasma using a silica RNA extraction method that has been previously described[28]. Briefly, 100µL of plasma was added to 900µL of lysis buffer in a micro-tube and incubated at room temperature for 10 min. Then, 20µL of the silica particle suspension was added to each micro-tube and centrifuged at 10,000rpm for 30 sec. The pellet was washed twice with washing solution, twice with 75% ethanol and once with acetone. After the last centrifugation step, supernatant was removed, and the pellet was dried at 56-60°C for 15 min. RNA was eluted with 50µl of elution buffer, and the tube was incubated at 65°C for 5 min.

RT-PCR and nested real-time PCR assay

Reverse transcription and the first round of PCR amplification was performed in a 20µL reaction volume using 14.5µL of a mastermix solution with a final concentration of 75mM KCl, 50mM Tris-HCl, pH 8.3, 3mM $MgCl_2$, 2.5mM DTT, 1mM dNTPs, 2.0µM of the primers, 24U of MMLV-RT (Life Technologies, Carlsbad, CA, USA), 4U RnaseOut (Life Technologies, Carlsbad, CA, USA), 1U Taq DNA polymerase (Cenbiot Enzimas, Porto Alegre, RS, Brazil) and 5µL of extracted RNA. Reverse transcription polymerase chain reaction amplification was performed for 30 min at 37°C followed by 15 cycles of the following temperatures and times: 94°C for 30 sec, 60°C for 30 sec and 72°C for 60 sec. A nested real-time PCR (second amplification stage) was performed in a 30µL reaction volume using 28.6µL of a mastermix solution with a final concentration of 50mM KCl, 10mM Tris-HCl, pH 8.3, 1.5mM $MgCl_2$, 1mM dNTPs, 0.25µM of the primers, and 0.125µM of the probe. The PCR amplification was performed for 35 cycles of 94°C for 15 sec and 60°C for 60 sec. Positive and negative samples were added as controls in all detection steps. Quantitative analysis was conducted using

standard samples with pre-defined viral loads. Hepatitis C virus concentrations were log_{10}-transformed for analysis. The linear range was examined by plotting the data and comparing them to a line of equality. Correlation coefficient calculations and linear regression analyses were performed on scatter plots of log-transformed HCV RNA levels using Microsoft Excel (Microsoft Corp., Redmond, WA, USA).

5'UTR genotyping

Genotyping of all HCV-positive RNA samples was performed using an RFLP procedure adapted from the original method[24]. Briefly, three restriction enzymes were used in two separate digests to cleave each nested PCR product. First, the HCV-amplified fragment was digested with *Hae* III alone in the appropriate buffer and conditions (New England BioLabs, Ipswich, MA, USA). The fragment was also digested with *Hinf* I and *BstN* I simultaneously in appropriate buffer and conditions (New England BioLabs, Ipswich, MA, USA). Some samples were digested with three other enzymes: *Rsa* I (Promega, Fitchburg, WI, USA), *BfuC* I and *BstU* I (New England BioLabs, Ipswich, MA, USA). Single digests were prepared with the respective enzyme and the appropriate buffer and conditions: *Rsa* I (Promega, Fitchburg, WI, USA), *Bfu*C I and *Bst*U I (New England BioLabs, Ipswich, MA, USA). Digested products were separated by electrophoresis on a 12.5% polyacrylamide gel and visualized after rapid silver staining. Banding patterns of the various HCV genotypes were deduced from those previously established by analysis of the 5'UTR sequences obtained from gene databases. Genotypes were then identified according to previous findings[6].

Confirmation of RFLP analysis by sequencing

PCR products were sequenced to confirm the results. Forward and reverse sequencing reactions were performed using template DNA, inner primers and BigDye Terminator v3.1 Cycle Sequencing reagent (Applied Biosystems Inc., Norwalk, CT, USA). Sequencing was performed using the thermocycler Veriti 96 (Applied Biosystems Inc., Norwalk, CT, USA) with an initial denaturation step at 95ºC for 3 min followed by 40 cycles of 95ºC for 10 sec and 60ºC for 240 sec. Samples were purified using an ethanol/EDTA/sodium acetate protocol, and DNA products were injected in the automated DNA sequencing ABI 3130 XL Genetic Analyzer (Applied Biosystems Inc., Norwalk, CT, USA). Sequence data were collected and quality analysis was performed using the Sequencing Analysis v.5.3.1 software by evaluating the main technical parameters as raw data, electropherograms and quality values of sequenced bases (Applied Biosystems Inc., Norwalk, CT, USA). The nucleotide sequences from the same amplicon (performed with sense and antisense primers) were edited and assembled using SeqMan software (DNAStar, Madison, WI, USA). Nucleotide sequences were aligned using the MegAlign program (DNAStar, Madison, WI, USA), and genotypes were deduced using the phylogenetic analysis protocols in this software. Additionally, data obtained were compared with sequences available in GenBank database (www.ncbi.nlm.nih.gov).

Ethical considerations

This project was approved by the Ethical Committee of the Universidade Luterana do Brasil (Canoas, RS, Brazil).

RESULTS

Real-time nested RT-PCR primers and probe

All 5´UTR HCV sequences obtained from GenBank were aligned using PrimerSelect software (Lasergene Inc., Madison, WI, USA). Four primers and one probe were designed to accommodate all HCV types **(Table 1)**. These oligonucleotides were used to develop and standardize a molecular method to detect, quantify and genotype HCV. The complete procedure consists of reverse transcription and a nested PCR. Reverse transcription and the first amplification were performed in the same reaction tube. The second amplification was performed using TaqMan targeting all HCV genotypes.

RFLP patterns and HCV genotyping

A total of 1,080 NCBI sequences were evaluated for genotype-specific RFLP patterns. The differentiation of the three main genotypes (1, 2, 3) present in Brazil could theoretically be accomplished using a double digest with restriction enzymes *Bst*N I and *Hinf* I. The banding patterns of Genotype 3 samples consist of a 165bp fragment, while almost all genotype 1 samples (98.1%) contain a 117bp fragment and the majority of the genotype 2 samples (94.5%) have a 221bp fragment **(Table 2)**. To further differentiate HCV genotypes 4 and 5 (which present the same patterns as genotypes 3 and 2, respectively) and to differentiate a significant proportion (5.5%) of the genotype 2 samples that have a 117bp-fragment, the restriction enzyme *Hae* III was selected because it was informative for all of these discriminations. **Table 1** shows the predicted patterns using *BstN* I and *Hinf* I double digestion and *Hae* III single digestion for the HCV Brazilian sequences. Based on these results, the following workflow for genotyping HCV was defined: *BstN* I + *Hinf* I double digestion and *Hae* III single digestion in a first round for all samples. Samples with indeterminate patterns in the first round would be secondarily digested with *Rsa* I, *Bfu*C I and *Bst*U I.

TABLE 1 - Primer and probe nucleotide sequences.

Sequences (5' - 3')
1st amplification primers
CCCCTGTGAGGAACTWCTGTCTTCACGC
AGGTTTAGGATTTGTGCTCAT
2nd amplification primers
GAAAGCGYCTAGCCATGGCGTTAG
ACGGTCTACGAGACCTCCCGGGGC
Probe
CACCCTATCAGGCAGTACCACAAGGCC*

*This probe was labeled with the fluorophore FAM and the quencher TAMRA. **FAM:** fluorescein amidite**; TAMRA:** tetramethylrhodamine.

TABLE 2 - Restriction fragment sizes and frequency of HCV restriction patterns found in the samples of the different genotypes, originated from the NCBI sequences.

Genotype	Predicted fragment lengths with *BstN* I + *Hinf* I and *Hae* III		Number	Percentage	Banding patterns
1	221/45	208/58	1	0.14	G
	163/58/45	160/58/48	1	0.14	-
	158/63/45	208/58	9	1.29	-
	158/63/45	266	1	0.14	-
	117/63/45/41	208/58	662	95.11	A
	117/63/45/41	160/58/48	6	0.86	I
	117/63/45/41	266	7	1.01	-
	117/63/45/32/9	208/58	1	0.14	-
	117/60/45/41/3	208/58	3	0.43	-
	117/57/45/41/6	208/58	1	0.14	-
	117/47/45/41/16	208/58	1	0.14	-
	117/108/41	208/58	1	0.14	-
	117/104/45	208/58	1	0.14	-
	69/63/48/45/41	208/58	1	0.14	-
2	221/45	208/58	12	21.82	G
	221/45	160/58/48	20	36.36	C
	221/45	104/58/56/48	19	34.55	E
	221/45	152/58/56	1	1.82	-
	117/63/45/41	114/104/48	1	1.82	-
	117/63/45/41	104/58/56/48	1	1.82	F
	117/104/35	104/58/56/48	1	1.82	-
	117/63/45/41	152/58/56	-	D	
	117/63/45/41	160/58/48	-	I	
3	165/56/45	218/48	2	0.61	-
	165/56/45	208/58	66	20.25	H
	165/56/45	160/58/48	252	77.30	B
	165/56/45	132/76/58	1	0.31	-
	165/45/34/22	160/58/48	1	0.31	-
	165/101	208/58	2	0.61	-
	165/101	160/58/48	1	0.31	-
	165/101	266	1	0.31	-
4	165/56/45	208/58	1	100.0	H
5	221/45	208/58	1	100.0	G

HCV: hepatitis C virus; **NCBI:** National Center for Biotechnology Information; **BstN I + Hinf I and Hae III:** restriction enzymes.

HCV detection and quantification validation tests

First, an HCV-positive (genotype 1) standard sample at a concentration of 10,000,000 IU/mL was diluted ten-fold to 1IU/mL, and the complete procedure (RNA extraction, RT-PCR and nested real-time PCR assay) was performed on all dilutions. Real-time PCR results obtained from the dilutions were linear and reproducible. High correlations (> 0.97) were observed between samples with different viral loads, and the

corresponding cycle threshold (Ct) had a wide dynamic range of analysis across the entire spectrum of clinically relevant viral loads (100 to 10,000,000IU/mL). Additionally, reproducibility was tested using four different HCV RNA genotype 1 samples (A = 6,000,000IU/mL, B = 30,000IU/mL, C = 3,000IU/mL, D = 1,500IU/mL) in 20 runs on different days. The total coefficients of variation of the cycle threshold (Ct) were 8.77% for sample A (mean Ct = 10.32 ± 0.90), 3.72% for sample B (mean Ct 18.36 ± 0.68), 3.96% for sample C (mean Ct = 21.72 ± 0.86) and 3.67% (mean Ct = 22.81 ±0.84).

The limit of detection was estimated using the Probit test by evaluating nine replicates of seven diluted HCV RNA samples ranging from 51 to 3,300IU/mL. The assay sensitivities were 1,500IU/mL for 95% positive repetitions and 500IU/mL for 50% positive repetitions. Specificity was tested using hepatitis C-negative blood donors, and no false-positive results were observed in a total of 50 anti-HCV-negative samples.

The procedure was then used to analyze the 57 plasma samples obtained from an inter-laboratorial program (ControlLab). In total, 36 samples tested positive and 21 negative, with 100% of agreement with the results reported by other laboratories. In the viral load analysis of the 36 HCV-positive samples, values ranging from 1,350 (log 3.13) to 5,250,000 (log 6.72) IU/mL were obtained. These data correlated well with the values reported by the ControlLab program. Linear regression of this comparison returned an intercept of -0.09 and a slope of 0.94. The overall correlation coefficient was 0.91 **(Figure 1)**.

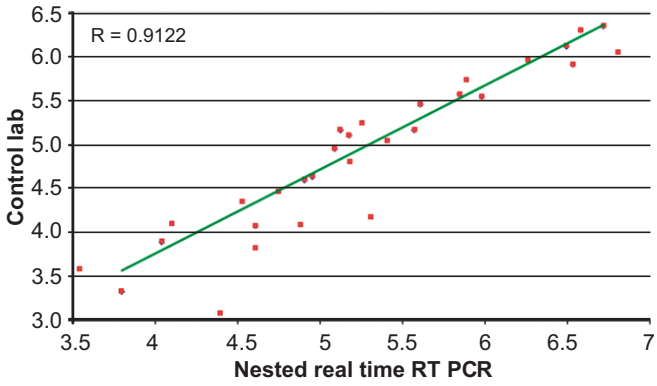

FIGURE 1 - The relationship between HCV RNA viral load levels given by ControlLab and obtained by nested real-time RT-PCR. HCV RNA: hepatitis C virus ribonucleic acid; **RT-PCR:** Reverse transcription polymerase chain reaction.

HCV genotyping validation test

To validate the genotyping procedure, we performed a blind study comparing RFLP analysis and nucleotide sequencing for 20 random clinical samples. RFLP results with the *Hinf* I + *BstN* I double digest and the *Hae* III digest revealed nine samples with the pattern predicted for genotype 1 (A), nine samples with the predicted pattern for genotype 3 (B), one sample with the primary predicted pattern for genotype 2 (C) and one sample with an unpredicted pattern (D) (**Figure 2**). All 19 samples from genotypes 1, 2 and 3 had exacting the same genotyping results with 5´UTR sequencing. The banding pattern of the remaining sample was not previously observed. Digests with the three other restriction enzymes (*Rsa* I, *Bfu*C I and *Bst*U I) were performed, and the banding patterns were consistent with genotype 2. The sequencing result and the subsequent phylogenetic analysis revealed similarity to genotype 2c (**Figure 3**).

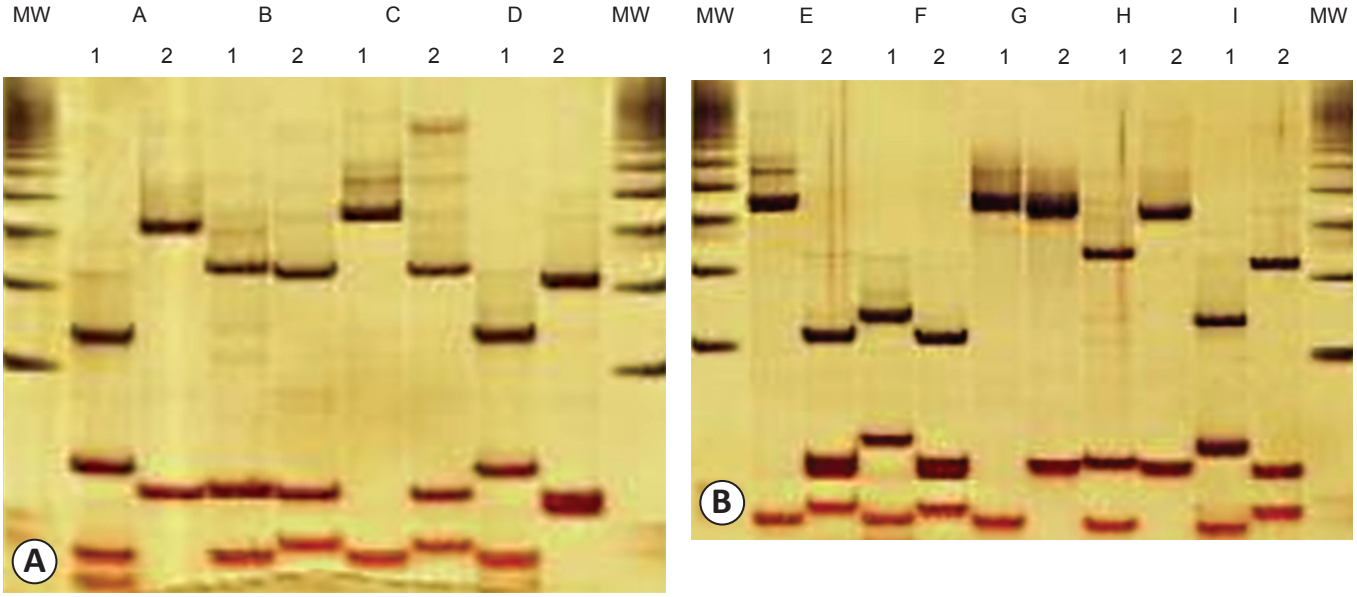

FIGURE 2 - Silver-stained polyacrylamide gels showing the different banding patterns obtained in the validation of the methodology (A) and in the analysis of the 804 clinical samples (B). The numbers indicate the digestion system (1 = *Hinf* I + *Bst*N I and 2 = *Hae* III), and the letters indicate the banding patterns (A, B, C, D, E, F, G, H and I). The 50-bp molecular weight is also shown.
MW: molecular weight.

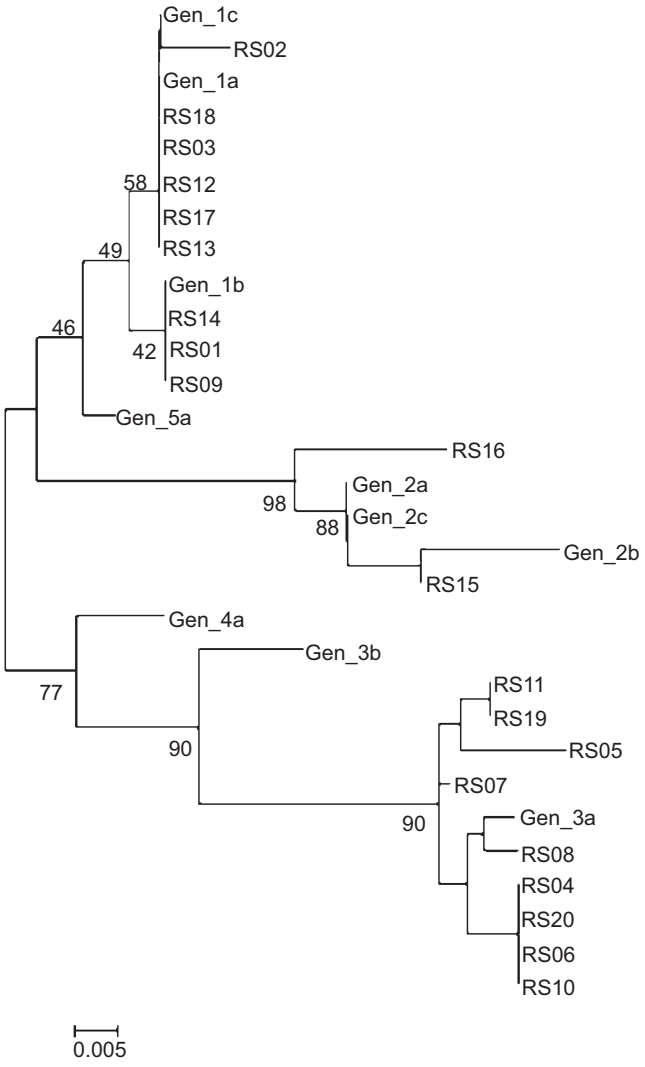

FIGURE 3 - Phylogenetic tree with the 5′UTR HCV sequences of the reference genotypes (1a, 1b, 1c, 2a, 2b, 2c, 3a, 3b, 4a and 5a) and 20 randomly selected HCV-positive plasma samples.

RS: randomly selected; **HCV:** hepatitis C virus; **5' UTR HCV:** HCV 5' untranslated region HCV.

Analysis of clinical samples

A total of 267 consecutive HCV-positive samples were tested using the nested real-time RT-PCR protocol in a routine laboratory. The mean viral load was 6,316,241 (log 6.32)IU/mL (standard deviation of log 0.80), ranging from 514 (log 2.71) to 93,850,240 (log 7.97)IU/mL. All samples were genotyped using the RFLP method and were classified according to the banding pattern **(Table 2)**. Patterns A, B, C, E, F, G, H and I were identified in 155 (58.1%), 96 (36.0%), 9 (3.4%), 1 (0.4%), 1 (0.4%), 1 (0.4%), 2 (0.7%) and 2 (0.7%) samples, respectively (**Figure 2**). Banding pattern D was not observed in any sample. A total of four randomly selected samples representing the three main banding patterns (A, B and C) and all samples presenting patterns E, F, G, H and I were sequenced and confirmed to be of the expected genotypes (A and I genotype 1, C, E, F and G genotype 2, B and H genotype 3). In this group of samples, 157 (58.8%) were from genotype 1, 12 (4.5%) from genotype 2 and 98 (36.7%) from genotype 3. Genotypes 4, 5 and 6 were not found.

DISCUSSION

Hepatitis C virus detection, quantification and genotyping are the crucial tests required to define and monitor the treatment of hepatitis C. Consequently, the introduction of assays with good analytical performance, high-throughput capacity and low cost for these three analyses will benefit both laboratories and patients. Studies have described the development and validation of *in-house* and commercial real-time PCR assays for HCV detection and quantification[17,18]. We developed a new procedure based on a nested PCR approach. Although this method is based on two rounds of PCR amplification, the results obtained by real-time PCR were linear and reproducible, similar to other *in-house* or commercial real-time techniques previously described. The use of RT-PCR amplification with a limited number of cycles (in a first round) followed by a real-time PCR with a higher number of cycles (in a second round) allowed the precise quantification of clinical samples. Further, this real-time nested RT-PCR assay demonstrated similar sensitivity and specificity values compared to other methods[29,30]. According to the analyses of 57 samples from an inter-laboratorial program, the correlation with other techniques was also very good. This assay could be useful in detecting and quantifying HCV in plasma samples.

Several methods have been used for assessing HCV genotypes in the clinical laboratories. The gold standard technique for genotyping HCV involves sequencing one or more genes in the HCV genome (mainly the 5′UTR, core, E1, NS3 and NS5) and comparing these sequences to the established genotypes by computer analysis. This approach is considered too expensive and time-consuming for large-scale diagnostics and has not been widely used in the majority of clinical laboratories[26]. Commercial genotyping tests, such as the Invader assay, Trugene 5′NC and the INNO-LiPA HCV II test are available; however, they all require an initial PCR amplification step[26].

Reverse transcription polymerase chain reaction analysis of the 5′UTR of the HCV genome was the first genotyping method used for large-scale epidemiological studies[20,24] and became the preferred method of routine HCV genotyping in clinical laboratories for some years. However, its use declined because of the difficulty involved in performing the necessary restriction analyses. The methodology was considered laborious, time-consuming and difficult to evaluate (primarily when the banding patterns are visualized in ethidium bromide gels)[26]. In the present study, we propose a simplified procedure for HCV genotyping using only three restriction enzymes in two digests. The analysis of the 1,080 available HCV sequences from different Brazilian regions demonstrated that only four RFLP banding patterns (A, B, C and E), characteristic of the three main genotypes (1, 2 and 3), would be obtained in the great majority of the HCVs present in Brazil. Samples with different banding patterns could be either confirmed using a more complete set of

restriction enzymes or submitted to sequencing. This procedure could also identify genotypes 4 and 5 (patterns H and G, respectively), which are rarely found in Brazil[27]. Concordance between this RFLP procedure and direct nucleotide sequencing was observed in the analysis of all HCV-RNA positive samples. Further analysis of clinical samples demonstrated a total of eight possible banding patterns (A, B, C, D, E, F, G H and I), which are easily visualized in polyacrylamide gels. The most frequent banding patterns are characteristic of specific genotypes (A - genotype 1; B - Genotype 3; C, D, E and F - genotype 2). Only a few exceptions (banding patterns G, H and I presented in less than 3% of the positive samples) must be analyzed using a more informative technique (e.g., sequencing). This RFLP procedure is certainly easier and less laborious than the original method[20,24]. The banding patterns are also clear, and the identification is easy to perform in a routine service (**Figure 2**).

Finally, the complete molecular biology assay was successfully applied for the analysis of 267 HCV positive plasma samples in a clinical laboratory setting. The whole *in-house* procedure could be performed completely in less than 48 hours, including the determination of viral load and genotype in HCV-positive plasma samples. Moreover, this approach eliminates the need for sequencing or hybridization with specific probes after PCR amplification, eliminating the costs of expensive automated sequencers and/or hybridization platforms.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FINANCIAL SUPPORT

This work was supported by the project titled 'Development of Molecular Test Kits to Detect and Quantify Viral Agents – Hepatitis B and C and HIV – Based on the Real-Time PCR Technology, sponsored by FINEP and Simbios Biotecnologia (FINEP/FULBRA/LDM - *Simbios Biotecnologia* - 0.1.07.0102.00).

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