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## Performance of Molecular Methods for Hepatitis C Virus Diagnosis: Usefulness among Chronic Cases and during the Course of Infection

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### SUMMARY

**Background:** Molecular methods are essential to define hepatitis C virus (HCV) infection. This study was conducted to evaluate the performance of molecular qualitative and quantitative methods for HCV RNA among chronic patients and individuals during the course of HCV infection.

**Methods:** Single serum samples were obtained from 82 HCV infected individuals where six of them donated serial serum samples (n = 52) during the course of HCV infection. Qualitative (in-house RT-nested PCR and COBAS<sup>®</sup> AMPLICOR HCV Test v2.0 and TMA) and quantitative (COBAS<sup>®</sup> AMPLICOR HCV Monitor Test v2.0 and bDNA) techniques were employed.

**Results:** TMA presented the highest rate (87.8%) of HCV detection among qualitative tests and it was the most sensitive for HCV RNA detection during the early and late phases of HCV infection. HCV RNA was quantified among 56 samples and significant correlation was observed between the two assays (r 0.92; p < 0.0001).

**Conclusions:** It is concluded that both quantitative methods can be used among chronic and acute HCV cases, but TMA was the most efficient for HCV qualitative detection among chronic cases and in the early and late phases of HCV infection.

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### KEY WORDS

hepatitis C, molecular diagnosis, performance

### INTRODUCTION

Hepatitis C virus (HCV) is the major cause of chronic liver disease worldwide whose prevalence varies from 2 to 3%, corresponding to 130 - 170 million infected individuals around the world at risk of developing cirrhosis and liver cancer [1]. HCV is a small enveloped virus with a positive-sense, single-stranded RNA genome belonging to the *Flaviridae* Family and *Hepacivirus* genus [2].

Serological and molecular markers are used to diagnose hepatitis C infection and four HCV markers are employed: total anti-HCV antibodies, HCV core antigen levels, HCV RNA levels, and HCV genotypes. Anti-HCV anti-

bodies appear on average 2 - 8 weeks after the acute phase of infection, so the presence of HCV RNA in serum samples is a reliable marker of HCV replication [3]. HCV RNA can be detected or quantified to help doctors to define the phase of HCV infection; however, accurate and precise methods are necessary. Qualitative and quantitative methods for HCV RNA are used to diagnose chronic HCV infection, identify patients who need antiviral therapy, monitor the virological responses to antiviral therapy, and document treatment failure [4-6].

HCV RNA quantification is essential for the management of chronic hepatitis C therapy based on the combination of pegylated interferon (PEG-IFN) and ribavirin (RBV) that is recommended for antiviral therapy management by the Brazilian Ministry of Health [7-9]. Patients infected with HCV with genotype 2 or 3 must

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complete 24 weeks of treatment with the combination of interferon and ribavirin, and patients infected with HCV genotype 4 or 5 must complete 48 weeks of treatment with conventional interferon and ribavirin. Patients infected with HCV genotype 1 should receive pegylated interferon (PEG-IFN) and ribavirin, and complete 48 weeks of treatment, provided it is documented in the presence of early virological response (EVR) at week 12 of treatment, with negative or reduced 2 log (100 fold) of HCV RNA, compared to the pretreatment level [9].

Commercial assays were developed to detect and quantify HCV RNA and most of them presented good performance [6,10-12], but commercial assays are quite expensive for public health settings. Due to this fact, the development of in-house methodologies for HCV RNA detection to minimize the expenses related for monitoring HCV infection is useful, especially during antiviral treatment. In this context, it is essential to evaluate qualitative and quantitative methods for HCV RNA detection in order to establish the most suitable methodologies at different stages of infection, such as before starting treatment and during the course of this procedure.

The present study was assessed to evaluate the performance of methods for HCV RNA detection and quantification for the diagnosis and monitoring of HCV infection.

## MATERIALS AND METHODS

### Serum Samples Studied

Between April 2004 to July 2009, blood samples were obtained by venipuncture with Vacutainer tubes from 82 individuals referred to the Viral Hepatitis Laboratory at Oswaldo Cruz Foundation. Blood was centrifuged for 5 minutes at 1900 x g and serum was aliquoted in sterile tubes and stored at -20°C until testing. Among 82 individuals that gave serum samples, 82 were chronic HCV patients that donated a single sample and six of them donated serial samples (n = 52) at different periods of time during the course of HCV infection. Among 82 chronic HCV patients, 61 had no history of previous treatment for hepatitis C and 21 had been previously treated. The mean age of the population was 49 years ± 12.1 years (range 20 - 70 years) and 51 (52%) were female. HCV infected individuals who donated serial samples had a mean age of 44.5 years ± 9.8 years (range 38 - 58 years) and only one presented previous history of HCV treatment. Risk factors for these individuals were: sexual contact with HCV infected partner (female, 48 years old), history of tattoo and previous hospitalization (female, 58 years old, the only one with previous history of HCV treatment), unprotected sexual intercourse (female, 44 years old), contact with hospitalized patients (male, 38 years old), hemodialysis (male, 56 years old), and coinfection with HIV (male, 30 years old). No identified risk factors were obtained among 82

chronic HCV patients. This study was approved by the research ethics committee of FIOCRUZ, on July 15, 2008, under the protocol number 433/07.

The diagnosis of chronic hepatitis C was made on the basis of the presence of antibodies to hepatitis C virus (anti-HCV) for more than six months and serum aminotransferase levels above the normal limit for at least 6 months. Anti-HCV antibodies were assayed by a commercial kit (ORTHO HCV 3.0 ELISA Test System with Enhanced SAve, Ortho Clinical Diagnostics, Raritan, NJ, USA) with estimated sensitivity and specificity of more than 99% in immunocompetent patients with detectable HCV RNA (Pawlotsky, 2002) and viremia was detected by reverse transcription-nested polymerase chain reaction (RT-nested PCR).

### Viral RNA extraction, cDNA synthesis and nested PCR

Viral RNA was extracted from serum samples with QIA amp Viral RNA Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions where the input volume of serum was 170 µL and the output was 60 µL.

Reverse transcription was carried out using 10 µL RNA, 20 pmol of random primers and 200 U of SuperScript enzyme Reverse Transcriptase III (Invitrogen, CA, USA) in a final volume of 20 µL at 50°C for 1 hour, followed by 10 minutes incubation at 70°C.

One half of the cDNA was used in a PCR assay that amplified the 5' non coding region (NCR) of HCV. After 4 minutes denaturation at 94°C, DNA was amplified for 30 cycles at 94°C for 30 seconds, 50°C for 60 seconds, 72°C for 1 minute and an additional 7 minutes at 72°C in the last cycle, in a final volume of 25 µL using Platinum Taq polymerase (Invitrogen) in the Perkin Elmer thermal GeneAmp 9600 Thermal Cycler (Perkin-Elmer, CA, USA). The primers used in this first round of PCR were 1B (external anti-sense) 5'GGG TGC AGC GTC TAC GAG ACC 3' (nt 322 - 342) and 2A (external sense) 5' GGC GAC ACT CCR CCA T 3' (nt 18 - 32). Nested PCR was carried out with 1 µL of the first round PCR product for 25 cycles under the same conditions (except that the period of annealing temperature was diminished and 45 seconds were used at 50°C). Internal primers were K15 (inner sense) 5'-ACC ATR RAT CAC TCC CCT GT-3' (nt 17-36) and K16 (inner antisense) 5'-CAA GCA CCC TAT CAG GCA GT-3' (nt 295 - 276), and the Platinum® Taq DNA polymerase (Life Technologies, Invitrogen, Carlsbad, CA, USA) [13]. PCR products were subjected to 1.5% agarose gel electrophoresis followed by ethidium bromide staining to visualize bands of the expected fragment with a length of 278 base pairs.

The PCR assay has proven to be both sensitive and specific for the detection of HCV RNA sequences in serum samples presenting sensitivity of 50 IU/mL as previously described from in-house validation [13]. All PCR assays were carried out according to the recommendations of Kwok and Higuchi [14]. In addition, negative con-

trols (anti-HCV/HCV-RNA negative human serum and DEPC-treated distilled water) and positive controls (human HCV-infected sera) were used in each PCR step to assure the specificity of the assay.

#### **Transcription Mediated Amplification (TMA)**

RNA from serum samples was extracted, amplified and detected using VERSANT HCV RNA qualitative assay (Siemens Medical Solutions Diagnostics, Tarrytown, NY, USA), following the manufacturer's instructions. VERSANT HCV RNA qualitative assay is a target amplification-based nucleic acid probe test that detects HCV RNA in human plasma and serum that has an input volume of 500  $\mu$ L. VERSANT HCV RNA qualitative assay utilizes Transcription Mediated Amplification (TMA) to amplify conserved regions within the 5'-NC region of the HCV genome. Monitoring of target capture and amplification is achieved by adding an internal control RNA to each sample. The target RNA is then amplified with an isothermal TMA process that requires the addition of primers, reverse transcriptase, and T7 RNA polymerase. Detection of the amplified product is based on hybrid protection and the dual kinetic assays [15,16]. Each tube produces a chemiluminescent signal that is read as relative light units (RLU). Data are reported both as calculated RLU and as signal-to-cutoff ratios. When the signal-to-cutoff ratio is  $> 1$ , the specimen is considered reactive or as having detectable HCV RNA. There are two possible outcomes of this assay; HCV RNA is either detected or not detected. Maximum throughput of HCV TMA in one run is 91 reportable specimens. A total of nine calibrators and controls are required. The test has a detection limit of 9.6 IU/mL as reported by the manufacturer.

#### **Qualitative PCR**

HCV PCR is a semiautomated assay. RNA from serum samples was extracted, amplified, and detected using COBAS<sup>®</sup> AMPLICOR HCV Test v2.0 (Roche Diagnostics, France) following the manufacturer's instructions with an input volume of 200  $\mu$ L. This test is based on five major processes: sample preparation, reverse transcription of RNA targets to produce complementary DNA (cDNA), PCR amplification of target cDNA using specific primers complementary to HCV, hybridization of amplified products with specific oligonucleotide probes(s) target(s), and detection of amplified products and connected to the probe by colorimetric determination. After manual extraction of HCV RNA from serum or plasma, subsequent steps, including reverse transcription, DNA amplification, and detection, are performed on the Cobas instrument. RNA detection is achieved by hybridizing the denatured, biotinylated PCR products to complementary HCV DNA and to internal control probes. There are three possible outcomes for this test: HCV RNA detected, HCV RNA not detected, and gray zone (i.e., indeterminate). The Amplicor Cobas analyzer automatically interprets specimens with weak signals (A660 range of 0.15 to 1.0) as gray zone. Specimens

whose signals fall within this range are to be retested in duplicate. On retesting, the final result is considered to be HCV RNA positive if all three tests yield an A660 equal to or greater than 0.15. The result is HCV RNA not detected if the A660 value of any one of the three tests is less than 0.15 and the corresponding internal control is amplified and detected [17]. Maximum throughput for a single run is 22 reportable specimens. The test has a detection limit of 50 IU/mL as reported by the manufacturer.

#### **Quantitative PCR**

RNA from serum samples was extracted, amplified, and detected using COBAS<sup>®</sup> AMPLICOR HCV MONITOR Test, v2.0 (Roche Diagnostics, France) following the manufacturer's instructions with an input volume of 100  $\mu$ L. HCV RNA from the specimen was extracted by lysis and ethanol precipitation. A quantitation standard of known copy number was added to each specimen to permit accurate quantitation. Reverse transcription and PCR amplification of both HCV target RNA and the internal HCV quantitation standard, followed by colorimetric detection, were done with use of the COBAS Amplicor Analyzer (Roche Diagnostics). Results were reported in copies per milliliter. More recent production lots of the Amplicor 2.0 test kits are standardized against the WHO International Standard for HCV RNA, generating reporting ranges in international units per milliliter, and they include the manufacturer's conversion of data (to copies per milliliter) that is specific to the kit lot. Detection range of this assay is 600 - 700,000 IU/mL, with test kit-specific approximate conversion to 1,500 - 2,125,000 copies/mL as reported by manufacturer. Maximum throughput for a single run is 21 reportable specimens.

#### **Branched DNA**

RNA from serum samples was extracted, amplified and detected using VERSANT<sup>®</sup> HCV RNA 3.0 Assay (Siemens Medical Solutions Diagnostics, Tarrytown, NY, USA) following the manufacturer's instructions. The bDNA test is based on hybridization of virus-specific RNA oligonucleotides to the synthetic 5' NC region and highly conserved central region of the gene of HCV RNA immobilized on the surface of the plate. Synthetic bDNA molecules amplified and multiple copies of an alkaline phosphatase-linked probe are hybridized to the immobilized complex. The complex is incubated with a chemo-luminescent substrate and light emission is measured, the signal is proportional to the level of nucleic acid target. The test values recorded between 3,200 copies/mL and 40 million copies/mL as reported by the manufacturer.

#### **Genotyping**

Positive samples obtained by in-house RT nested PCR were genotyped by restriction fragment length polymorphism (RFLP) according to Driesel et al. [18], and genotypes were determined according to Simmonds' clas-

sification [19]. Briefly, restriction digestions were carried out for 3 hours after adjustment with 10X enzyme reaction buffer as appropriate. Reactions were carried out at 37°C in the presence of 5 units of *Ava*II and 10 units of *Rsa*I. Digestion products were visualized under ultraviolet light after 3% TBE agarose gel electrophoresis (BMA, Rockland, ME, USA) in 1X Tris borate buffer containing 0.5 µg/mL ethidium bromide. Samples previously genotyped at the Laboratory of Viral Hepatitis (IOC/FIOCRUZ) were used as positive controls for genotypes 1 and 3. This RFLP assay properly allowed distinguishing between genotypes 1, 2, and 3 in previous HCV isolates from Brazil [20,21].

### Data analysis

Data are expressed as mean, median, and ranges. Mean virus titers were compared using paired Student's *t* test and analysis of variance, and median virus titers were compared using nonparametric test (Mann-Whitney U test) and when sample size was too short and for variables which did not pass normality test (Kolmogorov-Smirnov test). Spearman's correlation was used to compare matched sample results. Categorical comparisons were made with Fisher's exact test. The two-tailed *p* value < 0.05 was considered to be statistically significant. Statistical analysis, including multiple logistic regression analysis, was performed using GraphPad InStat version 3.01 (GraphPad Software, San Diego, CA, USA) and MedCalc for Windows, version 8.0.1.0 (MedCalc Software, Mariakerke, Belgium).

## RESULTS

### Comparison of HCV RNA qualitative methods

Qualitative HCV RNA assays (TMA, COBAS® AMPLICOR HCV Test and in-house RT-nested PCR) were used to test 82 serum samples and 70 (85.4%) were concordant. Forty-nine (59.7%) and 10 (12.2%) were positive and negative, respectively, in the 3 assays. TMA appeared to be the most sensitive qualitative methods, since 72 (87.8%) samples were HCV RNA positive while COBAS® AMPLICOR HCV Test was the less sensitive detecting HCV RNA among 53 samples (54.1%). All 60 reactive samples by the in-house nested RT-PCR, were also positive for TMA but 11 of them were not reactive for COBAS® AMPLICOR HCV Test (Table 1).

A concordance of qualitative results between Cobas Amplicor HCV Test and in-house RT-nested PCR was 81.7% [kappa value (*k*) of 0.644] and between TMA and in-house RT-nested PCR 85.4% (*k* of 0.700), while between TMA and Cobas Amplicor HCV Test it was 76.8% (*k* of 0.571). Among 27 discrepant samples, 11 samples (12.2%) were positive by in-house RT-nested PCR and negative by Cobas Amplicor HCV Test, 12 (12.2%) samples were negative by in-house RT-nested PCR and positive by TMA, and 4 (12.2%) samples were negative by in-house RT-nested PCR and positive by

both methods: Cobas Amplicor HCV Test and TMA. For the 11 sera presenting HCV RNA, positive by in-house RT-nested PCR and negative results for Cobas Amplicor HCV, no evidence of the presence of an inhibitor was found since the ODs with the internal standard in the Cobas Amplicor HCV Test were as high as those obtained after coamplification with the other specimens, including the positive and negative controls of the kit run in the same experiment.

### Comparison of HCV RNA quantitative methods

Median HCV RNA titer derived from the use of the bDNA assay was 9,044 IU/mL, range from 928 to 3,181,220 IU/mL. Using the PCR-based assay, the median virus titer was 70,000 IU/mL ranging from 851 to more than 700,000 IU/mL. Most of the samples presented less than 500,000 IU/mL either for bDNA (71/82) and COBAS® AMPLICOR HCV MONITOR (59/82). Twenty samples (24.4%) could not be titrated by both quantitative methods, six samples were titrated by COBAS® AMPLICOR HCV MONITOR (mean viral load of 3,168 IU/mL) and not detected by bDNA. Fifty-six samples were quantified by both methods and significant correlation between the two assays were found (*r* 0.92; *p* < 0.0001), despite higher titers found with COBAS® AMPLICOR HCV MONITOR compared to those found with the bDNA assay (mean of the COBAS® AMPLICOR HCV MONITOR of 378,140 IU/mL and mean of bDNA equal to 304,108 IU/mL) (Figure 1).

A good agreement was observed between the bDNA test and COBAS® AMPLICOR HCV MONITOR, with a kappa value equal to 0.873. On the other hand, qualitative results by RT-nested PCR were related closer to COBAS® AMPLICOR HCV MONITOR results (kappa = 0.891) than bDNA (kappa = 0.810).

### HCV qualitative and quantitative methods in relation to HCV Genotypes

In the present study, 50 presented genotype 1 and 10 presented genotype 3.

The median viral load was 133,337 copies/mL for genotype 1 and 64,708 copies/mL for genotype 3 using bDNA. Using COBAS® AMPLICOR HCV MONITOR, the median viral load was 343,500 copies/mL for genotype 1 and 377,000 copies/mL for genotype 3. Among unclassified genotyped samples, two HCV positive samples by bDNA presented a median value of 8,083.5 copies/mL while three HCV positive samples by COBAS® AMPLICOR HCV MONITOR showed a median value of 3,000 copies/mL (Table 2).

All reactive samples by the in-house RT-nested PCR were genotyped, while 4 positive samples by COBAS® AMPLICOR HCV Test and 12 positive samples by TMA were not genotyped, since the genotype method used in the present study was developed for in-house RT-nested PCR.

**Table 1. Performance of HCV RNA qualitative molecular assays.**

In-house RT-Nested PCR*	COBAS® AMPLICOR HCV Test		TMA	
	Positive N = 53	Negative N = 29	Positive N = 72	Negative N = 10
Positive N = 60	49	11	60	0
Negative N = 22	4	18	12	10

\*Since HCV genotype was determined by RFLP analysis, only HCV RNA samples detected by in-house RT-nested PCR could be genotyped. From 60 HCV RNA reactive samples, 50 were genotype 1 and 10 were genotype 3. Among 11 discordant samples (HCV negative by Cobas Test and positive by in-house PCR), 2 were genotype 3 and 9 were genotype 1.

**Table 2. HCV RNA viral load according HCVgenotypes in the population studied.**

Genotype	N	HCV RNA viral load*	
		COBAS® AMPLICOR HCV Monitor	bDNA
All types	60	346,000	114,990
1	50	343,500	133,337
3	10	377,000	64,708
Unclassified	22	3,000	8,083

\*Expressed as median of IU/mL.

**Table 3. Frequency of HCV reactive serum samples obtained from anti-HCV reactive individuals who donated the samples during different periods after onset of symptoms.**

Period (weeks) of sample collection (n = 52)	RT- nested PCR n	Qualitative PCR* n	TMA n	bDNA n	Quantitative PCR+ n
W <sub>0</sub> - W <sub>12</sub> (n = 14)	7	9	13	6	7
W <sub>13</sub> - W <sub>24</sub> (n = 10)	5	6	7	4	5
> W <sub>24</sub> *(n = 28)	7	10	14	4	5
Total	19	25	34	14	17

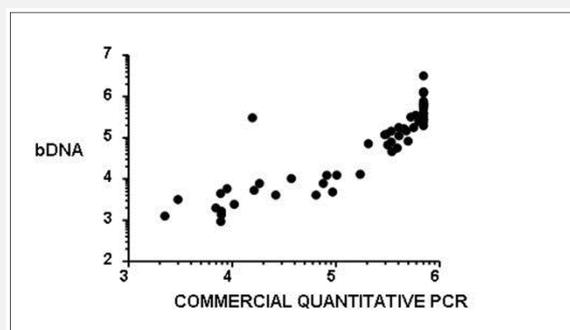
\*COBAS® AMPLICOR HCV Test v2.0, +COBAS® AMPLICOR HCV MONITOR Test v2.0, W - weeks after onset of symptoms, \* - up to week 290<sup>th</sup>.

**Performance evaluation of molecular techniques for detection and quantification of HCV during the course of infection**

Six individuals provided consecutive serum samples during the first to 290<sup>th</sup> week after the onset of symptoms (presence of jaundice, pale faces, or dark urine) as reported by the patient and the frequency of positive results were displayed on Table 3. Using TMA, a high number of HCV RNA samples were obtained from the first to 12<sup>th</sup> week and following 24<sup>th</sup> week after the onset of symptoms. The bDNA technique was the most ef-

ficient for samples obtained during the 13<sup>th</sup> to 24<sup>th</sup> week after onset of symptoms while COBAS® AMPLICOR HCV MONITOR presented the same efficiency during all the period studied (Table 3).

Molecular methods were used for HCV RNA detection and quantification among consecutive sera samples obtained from six individuals. The first was a female aged 58 years presenting a previous history of tattoos, hospitalization, and HCV genotype 1, who donated samples at the 28<sup>th</sup>, 32<sup>nd</sup>, 36<sup>th</sup>, 72<sup>nd</sup>, and 85<sup>th</sup> week after the onset of symptoms. This patient had reactive samples by all



**Figure 1.** Viral load (log<sub>10</sub> IU/mL) of samples from HCV infected individuals presenting concordant results among branched DNA and COBAS<sup>®</sup> AMPLICOR HCV MONITOR Test v2.0.

methods at the 28<sup>th</sup> and 32<sup>nd</sup> weeks. Using COBAS<sup>®</sup> AMPLICOR HCV MONITOR, the viral load was 589,000 IU/mL at the 28<sup>th</sup> week, 284,000 IU/mL at the 32<sup>nd</sup> week, and 2,508 IU/mL at the 36<sup>th</sup> week, whereas using the bDNA, the viral load was 352,557 IU/mL at the 28<sup>th</sup> week and 55,796 IU/mL at the 32<sup>nd</sup> week. Using the three qualitative methods, reactive samples were obtained until the 85<sup>th</sup> week. This patient initiated HCV treatments with PEG-interferon and ribavirin following the 32<sup>nd</sup> week after onset of the symptoms.

The second patient was a female aged 48 years with sexual contact with an HCV positive partner. She donated serum samples at the 3<sup>rd</sup>, 4<sup>th</sup>, 7<sup>th</sup>, 10<sup>th</sup>, 12<sup>th</sup>, 16<sup>th</sup>, 20<sup>th</sup>, 21<sup>st</sup>, 24<sup>th</sup>, and 28<sup>th</sup> weeks after onset of symptoms, presented HCV genotype 1 and did not receive HCV treatment during the period of this study.

TMA was the most efficient technique since it detects HCV RNA during the whole period studied, except at the 7<sup>th</sup> week. Using the in-house RT-nested PCR and COBAS<sup>®</sup> AMPLICOR HCV MONITOR tests, reactive samples were obtained at the 12<sup>th</sup> week (viral load = 3,200 IU/mL), 20<sup>th</sup> week (2,207 IU/mL), 24<sup>th</sup> week (9,480 IU/mL), and 28<sup>th</sup> week (418 IU/mL). Using the COBAS<sup>®</sup> AMPLICOR HCV Test, reactive results were obtained in the weeks cited above and also at the 16<sup>th</sup> week. With bDNA, reactive samples were obtained at the 12<sup>th</sup> (1,197 IU/mL) and 24<sup>th</sup> (3,805 IU/mL) weeks. The third patient was a female subject aged 44 years with a previous history of sexual contact with an individual of unknown HCV status. This patient donated serum samples at the 13<sup>th</sup>, 50<sup>th</sup>, 63<sup>rd</sup>, 72<sup>nd</sup>, 85<sup>th</sup>, 96<sup>th</sup>, 113<sup>th</sup>, 129<sup>th</sup>, 144<sup>th</sup> weeks and presented negative results in all molecular tests, except by TMA at week 144.

The fourth patient was a male aged 56 years with a previous history of hemodialysis and donated serum samples from weeks 0, 1, 4, 7, 8, 10, 12, 16, and 20. This individual presented HCV genotype 1 and did not receive treatment during the period of the study. Reactive

HCV samples were obtained during all the period using TMA, while using COBAS<sup>®</sup> AMPLICOR HCV Test only sample obtained at first week was negative. Using COBAS<sup>®</sup> AMPLICOR HCV MONITOR and in-house nested RT-PCR, reactive samples were obtained at weeks 0, 7, 8, 10, 12, 16 and 20 while using bDNA, reactive samples were obtained from the 7<sup>th</sup> to 20<sup>th</sup> weeks. The fifth patient was a male aged 36 years and HIV positive. This individual did not receive HCV treatment during the period of this study and presented HCV genotype 1. This individual donated serum samples on weeks 8, 14, 25, 32, 48, 64, 73, 83, 112, 146, 161, 176, 179, and 209. Reactive HCV samples were obtained by the COBAS<sup>®</sup> AMPLICOR HCV Test, in-house RT-nested PCR, bDNA, and TMA from weeks 8 to 32. The same was observed using COBAS<sup>®</sup> AMPLICOR HCV Monitor, except at the 25<sup>th</sup> week. Using TMA, it was also possible to detect HCV at weeks 48, 161, 176, and 179 while COBAS<sup>®</sup> AMPLICOR HCV Test also allows HCV detection at weeks 112 and 176.

The sixth patient was a male aged 30 years with previous contact with a familiar who was hospitalized. This individual donated serum samples at the 12<sup>th</sup>, 19<sup>th</sup>, 23<sup>rd</sup>, 28<sup>th</sup>, 32<sup>nd</sup> weeks and no reactive HCV results were obtained using COBAS<sup>®</sup> AMPLICOR HCV Monitor, bDNA, and in-house RT-nested PCR during all this period. Using the COBAS<sup>®</sup> AMPLICOR HCV Test, HCV was detected at the 12<sup>th</sup> week and using TMA at weeks 12 and 32.

## DISCUSSION

Molecular methods for HCV diagnosis are important to identify ongoing and resolved infection and to determine and evaluate antiviral treatment. For these reasons, these methods should be specific, sensitive, reproducible, and accurate [22]. In the present study, five molec-

ular techniques were evaluated for HCV RNA detection and quantification. An overall concordance was observed among qualitative HCV RNA methods, however TMA was the most sensitive technique to identify active infection. Low levels of HCV RNA can be detected using TMA which can be very useful among patients with inhibitory compounds in their serum, such as dialysis patients [15] or at the end of the treatment [23]. As reported by Morishima et al. [24], a positive TMA result among negative PCR samples may indicate the presence of low levels of HCV RNA. On the other hand, because patients with positive TMA results may achieve SVR, management decisions during therapy should not be based on a single positive TMA test result.

In-house RT-nested PCR was able to detect HCV RNA in 60/82 samples and presented a concordance of 85.4% to TMA, the most sensitive method for qualitative detection. These results showed the potential of an in-house technique optimized in this study to identify HCV infected individuals presenting good performance compared to commercial methods. Optimization of nested PCR could increase the sensitivity of the method, since magnesium concentration, cycling conditions, and DNA concentration are essential to increase the sensitivity of the method. In-house RT-nested PCR has also been used for HCV RNA detection among different HCV exposure categories of individuals, such as drug users [20,21,25], or other blood compartments, such as platelets [13,26] with high sensitivity and specificity. In the present study, some modifications were done in the standard protocol to improve the efficiency of the technique. Since this technique presents lower cost than TMA, it should be employed in laboratories with difficult access to high cost techniques.

The other qualitative method evaluated, COBAS<sup>®</sup> AMPLICOR HCV Test, presented an intermediate efficiency when compared to the other 2 qualitative methods in the transversal study. Some studies have reported higher values of concordance between the TMA and COBAS<sup>®</sup> AMPLICOR HCV Test [22,27] than in the present study. One possible explanation for this difference was the difference in the study population. In the previous studies, only patients with HCV genotype 1 or patients with different anti-HCV profiles were evaluated, but in the present study only anti-HCV positive patients presenting HCV genotypes 1 and 3 were evaluated.

Accurate HCV RNA quantification is important in order to include HCV infected individuals to antiviral treatment as well as to evaluate the response to the treatment [28]. Therefore, it is always important to validate and compare different methods in use. A low viral load (less than  $2 \times 10^6$  RNA copies/ml) is a strong predictor of a sustained response to therapy. Therefore, a high sensitive method for HCV RNA quantification is essential. In the present study, both methods could detect from 928 IU/mL showing their ability to detect low viral load. Furthermore, a good correlation was observed among quantitative methods as reported before [22,29,

30]. Although COBAS<sup>®</sup> AMPLICOR HCV Monitor gave a higher viral load than bDNA, the linear range of this technique is low, and the sample should be diluted to measure viral load. This procedure can increase the variability of sample results at higher virus loads.

Samples obtained from individuals during the course of HCV infection also demonstrated that TMA was the most sensitive technique, since positive results were obtained up to 144 weeks after onset of symptoms. Moreover, a good correlation was observed among COBAS<sup>®</sup> AMPLICOR HCV Monitor and bDNA showing the applicability of both techniques. In-house RT-PCR was also efficient during the follow up and demonstrates that this technique can be employed in these situations. Individuals that did not present a reduced 2 log (100 fold) HCV RNA at week 12 of treatment or had detectable HCV RNA at week 24 of treatment will not continue antiviral treatment as determined by the Brazilian HCV treatment guidelines [9]. In this way, both quantitative methods and TMA were more appropriate for monitoring the course of infection as seen in the prospective study.

In the prospective study, Cobas test showed higher sensitivity than in-house PCR probably due to low viral load of some samples along the study, since 5 of 6 samples detected by the Cobas Test and not detected by the in-house PCR were also not detected by quantitative methods. These quantitative methods presented a dynamic range of 1,500 - 2,125,000 copies/mL by the Cobas Monitor and 3,200 copies/mL and 40 million copies/mL by bDNA. Samples detected by the Cobas Test could detect viral loads below these limits. On the other hand, the high sensitivity of the in-house PCR in the transversal study could be due to HCV genomic variability; however, it is not possible to confirm this hypothesis, since sequence analysis was not conducted in the samples detected by in-house PCR and not detected by Cobas Test. Regarding the HCV genotype, all samples included in the prospective study belong to genotype 1 while HCV genotype 1 and 3 samples were included in the transversal study. The Cobas test did not detect 2 of 10 HCV genotype 3 samples, all of them were detected by in-house PCR in transversal study. One limitation of this study was the inclusion of COBAS<sup>®</sup> AMPLICOR HCV Monitor not actually done and which needs redilution in the case of samples with viral load above 700,000 IU/ml. Nonetheless, it is an assay widely used and has been used as a standard in the past. Moreover, in the present study, the samples were not rediluted to minimize the effects of this procedure. On the other hand, this study allows the optimization of in-house nested RT-PCR and the evaluation of different molecular techniques for HCV detection and quantification during the course of HCV infection.

An important finding of the present study was the optimization of in-house RT-PCR for HCV detection. This technique presented good efficiency and low cost and can be used to identify HCV infection cases during the course of HCV infection. This study also gives some in-

formation about different molecular methods for HCV detection and quantification in new HCV cases and during the course of HCV infection.

It was concluded that TMA was the most sensitive technique for HCV detection and presented good concordance to in-house nested RT-PCR. The latter presented low cost and good efficiency and can also be employed for HCV detection, especially in laboratories in developing countries. Both methods, COBAS® AMPLICOR HCV Monitor and bDNA, presented good concordance for HCV quantification.

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#### Declaration of Interest:

Authors declare no conflict of interest regarding this study.

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