

Signal to cut-off (S/CO) ratio and detection of HCV genotype 1 by real-time PCR one-step method: is there any direct relationship?

ABSTRACT

Background: Polymerase chain reaction (PCR) methods play an essential role in providing data related to diagnosis, monitoring and treatment of hepatitis C virus (HCV) infection. EIA results are reported as “reactive” or “non reactive” and EIA S/CO ratio may also be reported as “high” or “low.” This study aimed to evaluate the performance of a real-time RT-PCR and assess whether there is relationship between S/CO and PCR results. **Study Design and Methods:** Sera from blood donors were analyzed by Enzyme-Linked Immunosorbent Assay (ELISA) and RT-PCR assay to detect HCV infection. **Results:** The RT-PCR assay to genotypes 1a/b showed an acceptable linear response in serial dilutions. The samples were divided into two groups based on their serological results: group A – S/CO ratio < 3 (60 samples) and group B – S/CO ratio > 3 (41 samples). Viral loads were confirmed positive in group B samples in 90%, and in group A samples were confirmed positive in only 13% by RT-PCR. **Conclusion:** The methodology used was able to detect the presence of RNA-HCV genotype I in 90% of the samples serologically positive in group B. All negative samples were sent to search for other genotypes of HCV (genotypes 2-6) and were confirmed as negative. These data suggests that these negative samples may have HCV RNA viral load below the detection limit of our test (310 IU/mL), or a false positive result in serological test, or spontaneous viral clearance occurred.

Keywords: HCV, HCV genotype 1, real-time PCR, ELISA, RNA extraction.

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INTRODUCTION

The hepatitis C virus (HCV), discovered in 1989, is the major causative agent of hepatitis non-A, non-B. This virus, which belongs to the family *Flaviviridae* and has a genome constituted by a positive single-strand RNA, is transmitted parenterally, showing a high incidence in hemophiliacs hemodialysis patients, injecting drug users, and patients with post-transfusion hepatitis.^{1,2}

Hepatitis C infection has had high prevalence in Brazilian population mainly in the south region.^{3,4} According to literature,⁵ more than half of infected patients (about 70 to 85%) develop chronic hepatitis, some of them (10% to 20%) progressing to cirrhosis and hepatocellular carcinoma in a period of 10 to 15 years. Because hepatitis C has high potential to become chronic, it is considered a serious public health problem, being estimated that there are 170 million people infected worldwide.⁶ Currently, hepatitis C is the leading cause of liver transplants in USA.⁷

Diagnosis of hepatitis C is primarily based on serological tests, with detection of antibodies to HCV by methods based on ELISA⁸ or chemiluminescence.⁹ The HCV antibody is not a neutralizing one and generally is present in patients with viremia; it can be a marker of past infection, because it tends to remain indefinitely in patients who had hepatitis C and progressed to healing.¹⁰ In cases of an inconclusive result in the screening test, complementary tests are necessary. Until recently, this confirmation could only be achieved by recombinant immunoblot assay (RIBA).^{10,11} These techniques (ELISA, chemiluminescence, and immunoblot) have a window-period limitation, which can last 70-82 days, considerably reducing its usefulness for diagnosis of cases of acute HCV infection.⁸ Currently, the PCR test that detects the viral RNA have added many advantages for diagnosis of hepatitis C.^{12,13} This test is useful for: diagnosis of acute infection (seroconversion), diagnosis of infection in patients with negative or undetermined serology tests, evaluate the possibility of false-positive serological results, and also allow quantification of viral RNA.⁴

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Another molecular technique that has been used to differentiate HCV subtypes is genotyping. The molecular characterization of genotypes and sub-genotypes of HCV is particularly important in guiding the response to treatment and disease prognosis.^{5,6,14} There are 11 major genotypes of HCV and 23 subtypes. The prevalence of genotypes of HCV varies according to geographic region. In Brazil, the genotypes 1, 2, and 3 are the most often found.⁶ According to literature, patients infected with genotypes 2 and 3 respond better to treatment than those infected with genotype 1. Genotype 1 patients have a less effective response to treatment and a high viral load.^{15,16} Hepatitis C treatment is based on therapy with interferon- α and Ribavirin. It is a very long treatment with an average duration of 48 to 72 weeks,¹⁷ with a high cost (between U\$ 12,000 and U\$ 15,000 U.S. dollars per patient per year in the U.S). Besides being an expensive and prolonged treatment, it has a successful rate of 45-80% depending on viral genotype,^{17,18} probably because only few HCV genotypes respond quick and completely to this therapy. The fact is that there is a strong relationship between genotype of the virus and effectiveness of treatment increase the importance of genotyping the HCV. Genotyping assess the patient's prognosis providing important information, so that the clinician can tailor the treatment to the case.

PCR

PCR depends on the ability to alternately denature (melt) double-stranded DNA molecules and renature (anneal) complementary single strands in a controlled fashion. The second requirement for PCR is the use of synthetic oligonucleotides at least¹⁸⁻²⁰ nucleotides long with a defined sequence. Such synthetic nucleotides can be readily produced with automated instruments based on the standard reaction scheme. A typical PCR procedure begins by heat-denaturation of a DNA sample into single strands. Next, two synthetic oligonucleotides complementary to the 3 ends of the target DNA segment of interest are added in great excess to the denatured DNA, and the temperature is lowered to 50-60° C. These specific oligonucleotides, which are at a very high concentration, will hybridize with their complementary sequences in the DNA sample, whereas the long strands of the sample DNA remain apart because of their low concentration. The hybridized oligonucleotides then serve as primers for DNA chain synthesis in the presence of deoxynucleotides (dNTPs) and a temperature-resistant DNA polymerase, such as that from *Thermus aquaticus* (a bacteria that lives in hot springs). This enzyme, called Taq polymerase, can remain active even after being heated to 95° C and can extend the primers at temperatures up to 72° C. When synthesis is complete, the whole mixture is then heated to 95° C to melt the newly formed DNA duplexes. After temperature is lowered again, another cycle of

synthesis takes place because excess primer is still present. Repeated cycles of melting (heating) and synthesis (cooling) quickly amplify the sequence of interest. At each cycle, the number of sequence copies between the primer sites is doubled; therefore, the desired sequence increases exponentially — about a million-fold after 20 cycles — whereas all other sequences in the original DNA sample remain unamplified.

Use of the PCR technique

The development of DNA segments amplification using PCR opened vast prospects for analysis of genes, diagnosis of genetic diseases, and detection of infectious agents, such as cytomegalovirus, hepatitis B and C viruses, herpes virus simplex, rubella virus, human immunodeficiency virus (HIV), *Chlamydia trachomatis*, *Helicobacter pylori*, *Pneumocystis jirovecii* (formerly *P. carinii*), and *Mycobacterium tuberculosis*.

The advancement of science on the understanding of genes brought into the laboratory routine genetics tools for the diagnosis in molecular level. In the line of genetic diseases, the continuing development of new protocols has enabled the detection of small changes in DNA sequence; for example, in cystic fibrosis.

Other applications especially for the PCR is the cloning of a DNA specific fragment, which may be a gene and knowledge of coding DNA (cDNA) obtained from the molecule of RNA, which allows the study of gene expression.

Finally, PCR has great potential in forensic medicine. Its sensitivity makes it possible to use a very small sample (minimum traces of blood and tissues that could contain the remains of only one cell) and still get a “DNA fingerprint” of the person from which the sample was collected and can therefore make comparisons with those obtained from victims and/or a criminal suspect.¹⁹

Real-time PCR

The ability to monitor real-time PCR has revolutionized the process of DNA and RNA fragment quantification. Real-time PCR makes nucleic acid quantification with greater accuracy and reproducibility, as well as its predictive values during the exponential phase of the reaction. The point that detects the cycle in which the reaction reaches the threshold of exponential phase is called Cycle Threshold (CT). In real-time PCR, each round of amplification leads to the emission of a fluorescent signal and the number of signals per cycle is proportional to the amount of HCV-RNA in the starting sample.²⁰ This allows the accurate and reproducible quantification based on fluorescence.

The issue of fluorescent compounds generates a signal that increases in direct proportion to quantity of PCR product. Therefore, the values of fluorescence are recorded during every cycle and represent the amount of product ampli-

fied. The most used compounds are fluorescent SYBR Green and TaqMan probe.

Real-time PCR requires an instrumentation platform that consists of a thermal cycler, computer, optics for fluorescence excitation and emission collection, and data acquisition and analysis software.

The aim of this work is to evaluate real-time PCR One-Step method to detect hepatitis C virus genotype 1 and try to establish a relationship between S/CO ratio and PCR test results.

MATERIAL AND METHODS

Clinical specimens

From October 2008 to August 2009, blood samples were obtained from 101 blood donors with positive antibody screening for hepatitis C (ELISA – EIA-3; Biomeri ux Co.) and 10 donors with negative screening. All blood samples were negative for other tests mandatory in blood bank screening (ELISA).

Design of primers and probe (FAM – Assay with fluorescent marker/MGB – Minor Groove Binder)

The primers and probe for real-time PCR used in this study were designed in primer express software (Applied Biosystems) based on HCV sequence. The sequences of primers HCV are the following: 5'-CGGGAGAGCCATAGTGGT – 3' (HCV 1F, position: 130-147); 5'-CGCGACCCAACACTACTC – 3' (HCV 1R, position: 256-273). The probe sequence was selected within primer pair HCV 1F and HCV 1R and cited at position 149-169, which was designed to be perfectly complementary to the target sequence in 5'-NCR of HCV genome. The following is the probe sequence: FAM – TGCG-GAACCGGTGAGTACACC – MGB (HCV 1P). Both primers and probe were suspended in DEPC Water and stored at -20° C.

RNA extraction

For preparation of total RNA from human sera, we used the QIAamp Viral RNA kit according to manufacturer's instructions (QIAGEN, Courtaboeuf, France). In brief, 140 µL of serum was incubated with 560 µL of lysis buffer containing chaotropic salts and carrier RNA for 10 min at room temperature. After the addition of 560 µL of ethanol, the precipitated RNA was applied onto a silica-based spin column for purification and was finally eluted with 60 µL of QIAamp elution buffer.

Reverse transcription and amplification

The reverse transcription was performed using a methodology one-step with TaqMan Probes Reaction Mix, containing 0,5 µL Superscript III RT/Platinum Taq Mix; 12,5 µL (2x Reaction Mix); 0,5 µL Forward primer (10 uM); 0,5 µL Reverse

primer (10 µM); 0,1 µL Fluorogenic probe (10 uM); 0,5 µL RNase Out; <10 µL Template (1 pg to 1 µg total RNA) and DEPC-treated water to 20 µL.

Amplification was performed in 20 µL reaction mixture containing TaqMan Universal PCR Master Mix, UNG, DNTPs, a passive reference (6-carboxy-rhodamine; ROX). Standard cycling program was: 15 min hold at 50° C for cDNA synthesis temperature may range from 42-60° C; 2 min hold at 95° C; 40 cycles of: 15 s at 95° C; 30 s at 60° C.

Enzyme Linked Immunosorbent Assay Screening (ELISA)

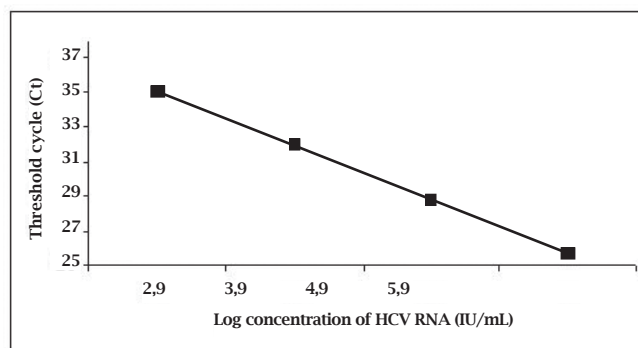
Testing for HCV antibodies was performed with a third generation enzyme immunoassay (EIA-3; Biomeri ux Co.) following the manufacturer's instructions. S/CO ratio (S = sample ratio and CO = Cutoff ratio) was determined for all blood samples and, based on this, we created two groups: group A in which S/CO ratio was < 3, and group B in which S/CO ratio was > 3. This algorithm was developed in order to establish the relationship between S/CO ratio and the probability RT-PCR been positive.²¹

RESULTS

Linear range and limit of detection

Once the standard curve had been calculated, the software automatically quantified the number of HCV RNA, reporting the Ct of the sample to the Ct of the standard curve. The correlation coefficient (R2) of the analysis was above 0.99 for all the experiments, from a commercial standard sample (positive control) with a pre-established number of copies. The standard curve was made using different concentrations (ranging from 310 to 310.000 IU/mL or 2,5 to 5,5 Log) showed in Figure 1.

Figure 1: Standard curve of the HCV RNA concentration (log) in serial dilutions vs. cycle number (Ct). Each dot represents the results of triplicate PCR amplification for each dilution.



In order to compare the reproducibility between our test and a commercial test, we studied 24 samples by another PCR method (Amplicor Monitor HCV RNA assay®, Version 2.0, Roche Diagnostics, Meylan, France). All positive samples were confirmed positive in both methods, showing 100% reproducibility (Figure 2).

In group A samples, from a total of 60 samples, only eight (13%) were positive by real-time PCR test (Figure 3).

Out of 41 positive samples with S/CO ratio > 3, 37 samples (90%) were confirmed positive by RT-PCR test in group B, while four samples were negative (Figure 4).

Figure 2: Comparison between Real Time RT-PCR vs. Amplicor Roche.

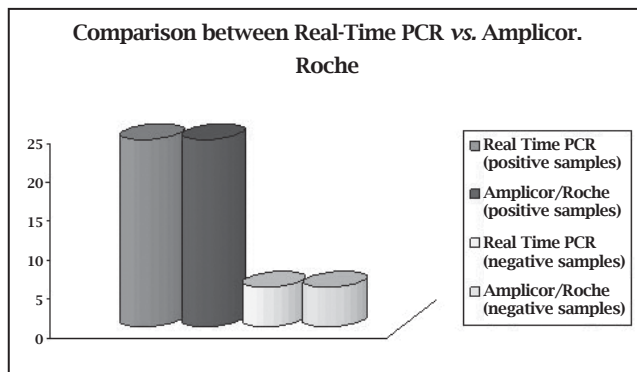


Figure 3: Samples with S/CO ratio < 3.

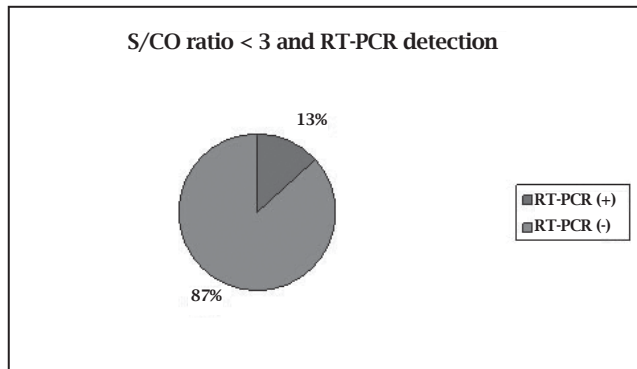
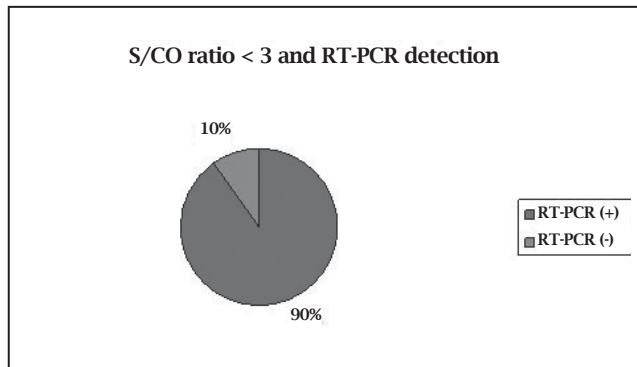


Figure 4: Samples with S/CO ratio > 3.



DISCUSSION

Although the risk of transfusion-transmitted HCV has progressively decreased during the past few decades, thanks to introduction of several preventive measures such as careful selection of blood donors, enhanced donor questioning for eligibility and refinement in blood donation screening, and stimulation to donor fidelity, the implementation of NAT together with the existing serological assays can reduce the residual risk of recipient's infection by shortening the window period (temporal gap spanning from the time of infection to seroconversion). During this period, an infected donor may harbor large amounts of infection viral particles in absence of serological markers and/or signs and symptoms of an ongoing infection. Due to the relatively long seronegative window period for HCV infection, about 82 days if using the third generation antibody test available, in this study we showed the importance of testing for viral RNA, which enables detection 10 days after infection and leads to safe transfusions.

Polymerase chain reaction (PCR) is a powerful tool for detection of minimal amounts of nucleic acids. Due to the exponential amplification of the target sequence, it has exquisite sensitivity. Few copies of any transcript can be readily detected, even against a high background of unrelated nucleic acid. The establishment of PCR-based detection methods has provided the basis for rapid and reliable detection of viral nucleic acids in the clinical setting. It was shown that monitoring virus load and kinetics of virus proliferation have prognostic relevance for the course of disease and clinical outcome.²²⁻²⁷ The availability of quantitative virus detection tests is therefore of paramount importance for the clinical management of virus infections.

Most assays described for the detection of viral DNA or RNA is based on the use of hydrolysis probes, also referred to as TaqMan. The probes bind to the target strand between PCR primers. They are dually labeled with a fluorescence reporter dye attached to the 5'-end, a quencher dye, attached to the 3'-end. When the reporter molecule on TaqMan probe is stimulated, an appropriate light source to emit fluorescence, the energy is transferred to the quencher, thereby suppressing the emission of fluorescence by the reporter.²⁸ During PCR, when DNA polymerase extends the primers, the hybridized probes are split by the enzyme 5' exonuclease activity and the corresponding quencher and reporter molecules are separated. The energy transferred to the quencher molecule is thus abrogated, and the reporter starts emitting fluorescence, which can be measured at the end of each extension step.²⁹ In well established assays, there is a linear correlation between the number of released reporter molecules and the number of amplicons synthesized during each PCR cycle. This correlation serves as a basis for calculation of initial copy number of target transcript.

This method shows several advantages, such as fast execution and reduced risk of contamination, due to the lack of post-PCR processing steps. TaqMan technology requires specific conditions for the choice of primers and probe: They should have a T_m difference of 10°C , the absence of guanosine at the 5' amino probe sequence and an amplification length ideally under 100bp. The amplicon length, together with the restrictive conditions of primers and probe designs, does not facilitate the assay use with RNA viruses, in particular, due to their genetic variability. The development of DNA probes with conjugated minor groove binder (MGB) groups has allowed the use of shorter probes in hybridization-based assays. Interestingly, the design of an array within the rare conserved region of 5'-UTR of HCV was facilitated by the use of a 3'-MGB probe.

In our laboratory, the TaqMan quantitative HCV assay showed linearity over a range of $3,1 \times 10^2$ to $3,1 \times 10^5$ IU/mL. Thus, serum samples with a viral load higher than 310.000 IU/mL have to be diluted. The detection limit of this test can vary as noted in other publications that showed an estimated limit at 550 IU/mL^{30,31} because different methods are used for determining RNA concentration.³²⁻³⁵

Trani *et al.*³⁶ also used the technique of one-step real-time PCR with MGB probe to detect avian influenza viruses. The linear correlation (R²) of that study was 0.99, the same found in our study, showing high sensitivity of test. Arruda *et al.*,³⁷ even using a different technology, SYBR Green technique, to evaluate patients with HTLV I/II infection, made a standard curve using serial dilutions of plasmid in concentrations from 10^0 to 10^5 , getting $R^2 = 0,89$.

Wang Lu-Nan *et al.*,³⁸ through technology Roche Cobas Amplicor method, evaluated R² for detection of HCV and was able to detect a range of 600 to 850,000 IU/mL, unlike the data presented by us, demonstrating that the technique used by our laboratory was more sensitive in detecting low levels of viral load.

EIA results are reported as "reactive" or "nonreactive" and EIA signal to cutoff (S/CO) ratio may also be reported as "high" or "low." EIA S/CO ratio is a comparison of the optical density of the patient's positive EIA result to the optical density of the laboratory's positive EIA control. If the ratio is high > 3.0 using the most widely employed diagnostic kits, ELISA, the positive predictive value (that the patient truly has HCV antibody in the blood) of the patient's result is high, still emphasize the importance of confirming these results using PCR or RIBA.

In our results, we could observe that 90% of the group B samples (S/CO ratio > 3) was positive in the PCR test, confirming the serological tests, but four samples did not confirm the results obtained by ELISA; in contrast with group A, in sixty samples of group B (S/CO ratio < 3) only 8 were confirmed as positive by RT-PCR. The reason for the difference in results between the techniques is not clear. We

can suggest that these patients have low viral load in which was not possible to detect the RNA, as our test showed the detection limit of 310 IU/mL. Moreover, there are data from literature showing that some patients, who developed anti-HCV, had clearance of viral RNA during acute infection.^{39,40} However, this subject need to be more explored and studied. On the other hand, our service use a safety margin of 10% on cut-off, meaning that the samples negative by RT-PCR and positive by ELISA, could be within this margin, confirming the data obtained by RT-PCR.

In other studies in Brazil, samples repeatedly positive to EIA-2 with S/CO ratio > 3 were associated with 100% of true-positive results and have around 92% of positivity for HCV RNA by RT-PCR.⁴¹

Hence, through our study, we conclude that the One-Step methodology was easy to apply, adding agility to the PCR methods with reliable results, and we can state that there is a strong positive relationship between S/CO and the results presented by real-time PCR in HCV detection.

DISCLOSURE

All the authors declared no competing interests, and the work content represents the views of co-authors, and that neither the author nor the main co-authors submitted manuscripts duplicate or overlapping in another publication, and that the article mentioned in the text as personal communications are approved by the person referenced.

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