Comparison between the Results of the Real Time PCR Test and Those of the Cobas Amplicor HCV Test, v2.0. In Hepatitis C Virus Infected Egyptian Patients

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Abstract: Purpose: Several assays are used to diagnose HCV infected patients, but measurement of HCV RNA levels has become an integral and increasingly important part of the management of patients with HCV infection to select the appropriate treatment schedule and to estimate the treatment outcome. Real Time PCR test allows for detection of PCR amplification products during the early phase of the reaction & provides a distinct advantage over traditional PCR detection which detects PCR products at the end-point of the reaction which are not as precise as Real-time PCR. The aim of the work is to compare the results of the Real Time PCR test versus that of the Cobas Amplicor HCV test, v2.0. for accurate detection of the HCV virus load in HCV infected patients. Methods: 50 patients were randomly selected from the Outpatient Clinic of Ain Shams University Hospital. Their ages ranged from 30-50 years. They were subjected to screening for antibodies to HCV by ELISA test, RT-PCR test which was done by Automated Amplicor System and the real time PCR test. Results: The results obtained from the real time PCR and the conventional PCR assays were almost parallel to each other. However, variations in the HCV RNA levels had occurred between both tests despite the use of I.U. standard. Conclusion: The real time PCR test is a simple, highly sensitive, specific and reproducible assay for the measurement of serum HCV RNA with a wider detection range and good accuracy of results.

Key words: HCV, PCR, Real-time PCR, Cobas Amplicor HCV test.

INTRODUCTION

Hepatitis C is a major global public health problem and is one of the main causes of cirrhosis and hepatocellular carcinoma. In clinical practice, the usual approach is to test initially for antibodies to HCV (anti-HCV) then to use HCV ribonucleic acid (RNA) detection to document viremia Strader et al., (2004). So, specific diagnosis of HCV infection can be classified into; Serological and molecular diagnosis. The molecular diagnosis classified into qualitative assays and quantitative assays.

Qualitative detection of the HCV RNA by polymerase chain reaction (PCR) is being utilized for confirmation of the serological results (ELISA, RIBA inconclusive results) as well as, for assessing the effectiveness of antiviral therapy, Jungkind (2001) & Pawlotsky (2003). While accurate quantitative assay of HCV RNA in serum is increasingly becoming important because it assess the response to the antiviral therapy during the treatment through monitoring HCV load i.e. amount of circulating virus in serum or plasma which reflects the rate of virion production in the liver and considered the most reliable marker of viral replication, Neumann et al., (1998), Carreno (2002), Castro (2002).

Real-time PCR is a method for precise quantitation of minute amounts of nucleic acids. It allows for detection of PCR amplification products during the early phase of the reaction. Measuring the kinetics of the reaction in the early phase of PCR provides a distinct advantage over traditional PCR detection which uses agarose gels or other post PCR detection methods for detection of PCR products at the final phase i.e. at the end-point of the reaction which is not as precise as Real-time PCR, Bar (2003). Real-time PCR is based on detection and quantitation of a fluorescent reporter. This signal increases in direct proportion to the amount of PCR products in the reaction. By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during exponential phase where the first significant increase in the amount of PCR product correlates to the initial amount of target template, Lee (1999). Theoretically, there is a quantitative relationship between amount of starting target sample and amount of PCR product at any given cycle number. Real-Time PCR detects the accumulation of amplicon during the reaction. The data is then measured at the exponential phase of the PCR reaction. More recently usage of duplex mutation primers for real-time PCR quantification of hepatitis C virus shows high sensitivity, wide linear range and good reproducibility, combined with low cost Qian et al., (2011). Traditional PCR methods use agarose gels or other post PCR detection
methods, which are not as precise. Real-Time PCR makes quantitation of DNA and RNA easier and more precise than the old methods Dorak (2006), due to their very low detection limit and their broad and linear dynamic detection range, Lange & Sarrazin (2010).

An example of the traditional PCR tests is Cobas Amplicor test version 2.0 which is made by Roche Molecular Systems Poljak et al., (1997). The Cobas Amplicor HCV Test version 2.0 detects the presence of HCV RNA in serum and plasma which provides an accurate estimate of the rate of virion production in the liver, i.e. the level of viral replication Neumann et al., (1998). It is the first HCV RNA assay that reports the analytical sensitivity in International Units (IU/mL) as defined by the WHO International Standard for HCV RNA for Nucleic Acid amplification technology (NAT) assays, Dale et al., (2002).

MATERIALS AND METHODS

A total number of 50 patients (42 males and 8 females) were included in this work. They were randomly selected from the Outpatient Clinic of Ain Shams University Hospital between the periods of June 2006 to September 2006. Their ages ranged from 30-50 years.
- All cases were submitted to the following:
  - Full history taking: name, age, sex, marital status, history of blood transfusion, dental therapy, intravenous drug abuse and history of previous admission in fever hospital.
  - Complete general and local examination.
  - Screening for antibodies to HCV at Ain Shams University Clinical Pathology Department using ORTHO 3rd ELISA test (ORTHO Clinical Diagnostic, Inc., a Johnson & Johnson Company, Raitan, New Jersey 08869, USA).
  - The RT-PCR test which was done by Automated Amplicor System (version 2.0) at Ain Shams Specialized Hospital supplied by Roche Diagnostic’s detection (Roche Diagnostics, Branchburg, New Jersey, USA).
  - The real time PCR test which was done by Stratagene Mx3000P instrument (Stratagene Mx3000PQPCR Systems, La Jolla, CA 92037, USA) in Clinical Pathology Department in Ain Shams University Hospital.

The COBAS Amplicor HCV test v. 2.0 is Based on Five Major Processes:
1- Sample preparation through RNA extraction and utilization of HCV internal control (IC). The HCV internal control RNA is introduced into each specimen with the lysis reagent and serves as an extraction and amplification control for each independently processed specimen.
2- Reverse transcription of the target RNA to generate complementary DNA (cDNA). The processed specimens are added to the amplify reaction mixture in amplification tube (A-tubes) in which both reverse transcription and PCR amplification occur. The downstream or antisense primer is biotinylated at the 5’ end, the upstream or sense primer is not biotinylated. The reaction mixture is heated to allow the downstream primer to anneal specifically to the HCV target RNA and the HCV internal control target RNA. In the presence of Mn+2 and excess deoxynucleotide triphosphates (dNTPs).
3- PCR amplification of target cDNA using HCV specific complementary primers as well as amplification of internal control. The HCV internal control has been added in the COBAS Amplicor HCV test, v 2.0 to permit the identification of processed specimens containing substance that may interfere with PCR amplification. The HCV internal control is an RNA transcript with primer binding regions identical to those of the HCV target sequence, a randomized internal sequence of similar length and base composition as the HCV target sequence, and a unique probe binding region that differentiates the HCV internal control from target amplicon. These features were selected to ensure equivalent amplification of the HCV internal control and the HCV target RNA.
4- Hybridization of the amplified products to oligonucleotide probes specific to the target. Following PCR amplification, the analyzer automatically adds denaturation solution to the A-tubes to chemically denature the HCV amplicon and the HCV internal control amplicon to form single-stranded DNA. An oligonucleotide probe specific for HCV or HCV internal control is added to the individual. The biotin labeled HCV and HCV internal control amplicon are hybridized to the target-specific oligonucleotide probes bound to the magnetic particles. This hybridization of amplicon to the target-specific probes increases the overall specificity of the test.
5- Detection of the probe bound amplified products. Following the hybridization reaction, the COBAS Amplicor analyzer washes the unbound material and then adds Avidin-horseradish peroxidase conjugate which binds the biotin-labeled amplicon hybridized to the target-specific oligonucleotide probes, then washing step occurs. After that, a substrate solution containing hydrogen peroxide and tetramethylbenzidine (TMB) is added to form a colored complex measured at wave length 660 nm by COBAS Amplicor photometer.
The real time PCR test was done, by Stratagene Mx3000P instrument, using Brilliant HCV QRT-PCR reagents that constitute a ready-to-use system for the detection of HCV RNA using polymerase chain reaction (PCR) in Stratagene’s Mx3000P quantitative real-time PCR system. Brilliant HCV QRT-PCR kit contains reagents and enzymes for the reverse transcription and specific amplification of a specific region of the HCV genome in fluorescence detector (FAM). Brilliant HCV QRT-PCR kit contains a second heterologous amplification system to identify possible PCR inhibition. This is detected as an Internal Positive Control (IPC) in fluorescence detector (HEX). External positive controls (HCV Standards IU/ml) are supplied which allow the determination of the pathogen load using standard curve (Absolute Quantitation). The 2X master mix contains an optimized RT-PCR buffer, MgCl2, nucleotides (GAUC), Taq DNA polymerase, and stabilizers. In addition, reverse transcriptase in combination with RNase block is provided in a separate tube. A passive reference dye (ROX) is provided in a separate tube.

The principal of TaqMan chemistry for QPCR applications includes a third oligonucleotide in the reaction known as the probe. A fluorescent dye, typically FAM, is attached to the 5’ end of the probe and a quencher. As long as the two molecules (reporter and quencher) are maintained in close proximity, the fluorescence from the reporter is quenched and no fluorescence is detected at the reporter dye’s emission wavelength. The probe is designed to anneal to one strand of the target sequence just slightly downstream of one of the primers. As the polymerase extends that primer, it will encounter the 5’ end of the probe. Taq DNA polymerase has 5’–3’ nuclease activity, so when Taq DNA polymerase encounters the probe it displaces and degrades the 5’ end, releasing free reporter dye into solution. Following the separation of reporter dye and quencher, fluorescence can be detected from the reporter dye. The fluorescence intensity increases proportionally with each amplification cycle in response to the increased amplicon concentration. This allows quantification of the template to be based on the fluorescent signal during the exponential phase of amplification, before limiting reagents, accumulation of inhibitors, or inactivation of the polymerase have started to have an effect on the efficiency of amplification.

Reference Range of Conventional PCR and Real Time PCR:

The conventional PCR reference range considers the result within 600-250000 IU/ml weak viremia, that within 250000-2.5 million IU/ml moderate viremia and that within 2.5 million- 5 million IU/ml high viremia. The real time PCR considers results less than 100,000 IU/ml low viremia, that within 100,000-1 million IU/ml moderate viremia and that more than 1 million IU/ml high viremia.

Results:

Table (1) shows the results of Real time PCR and conventional PCR results.

Figure (1) Study the correlation between the real time PCR results and conventional PCR results and revealed that there was a strong positive correlation between conventional PCR and Real time PCR with r=0.60  P<0.001 (with a strong association).

Table 1: The results of Real time PCR& conventional PCR results.

<table>
<thead>
<tr>
<th>Case number</th>
<th>Conventional (COBAS) PCR results (IU/ml)</th>
<th>Real time PCR results (IU/ml)</th>
<th>Case number</th>
<th>Conventional (COBAS) PCR results (IU/ml)</th>
<th>Real time PCR results (IU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>521,000</td>
<td>26</td>
<td>327,000</td>
<td>750,000</td>
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<tr>
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<td>1,050,000</td>
<td>798,000</td>
<td>27</td>
<td>52,500</td>
<td>40,200</td>
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<td>305,000</td>
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<td>91,400</td>
<td>165,000</td>
</tr>
<tr>
<td>4</td>
<td>473,000</td>
<td>1,250,000</td>
<td>29</td>
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</tr>
<tr>
<td>5</td>
<td>328,000</td>
<td>175,000</td>
<td>30</td>
<td>431,000</td>
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<tr>
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<tr>
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<td>41</td>
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<td>49</td>
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<td>177,000</td>
<td>499,000</td>
<td>50</td>
<td>131,000</td>
<td>43,500</td>
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</table>
Table (2) shows the comparison between the results of Real time PCR and conventional PCR which revealed that the Real time PCR agreed with conventional PCR in 26 cases (52% of all cases) and differed with conventional PCR in 24 cases in which 20 cases (40% of all cases) are overestimated i.e. Real time PCR results > conventional PCR results while 4 cases (8% of all cases) are underestimated Real time PCR results < conventional PCR results.

This difference between the conventional PCR and real time PCR was statistically significant P<0.05 with Wilcoxon Signed Ranks Test Z=3.27 P =0.001.

<table>
<thead>
<tr>
<th>PCR</th>
<th>Real time PCR</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low</td>
<td>Moderate</td>
</tr>
<tr>
<td>Weak</td>
<td>10</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>43.5%</td>
<td>56.5%</td>
</tr>
<tr>
<td>Moderate</td>
<td>4</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>14.8%</td>
<td>59.3%</td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>28.0%</td>
<td>58.0%</td>
</tr>
</tbody>
</table>

Fig. 1: The correlation between Cobas Amplicor PCR and Real time PCR.

**Statistical Analysis:**

The data were coded, entered and processed on an IBM-PC compatible computer using SPSS (version 11). The level P < 0.05 was considered the cut-off value for significance.

Correlation analysis: assessing the strength of association between two variables. The correlation coefficient denoted symbolically $r$, defines the strength and direction of the linear relationship between two variables.

The Wilcoxon signed-rank test considered information about both the sign of the differences and the magnitude of the differences between pairs.

**Discussion:**

Hepatitis C virus is the main causative agent of non-A non-B viral hepatitis worldwide, Enomoto et al., (2001). Also it is the main causative agent of chronic viral hepatitis, Castelain et al.,(2004). Approximately 170 million individuals are chronically infected with hepatitis C virus worldwide, Beld et al., (2003). Infection with HCV also often progress to liver cirrhosis and hepatocellular carcinoma over several years, Enomoto et al., (2001).

Several assays are used to diagnose HCV infected patients. They can be divided into 4 main categories: serological tests that detect the presence of the anti HCV antibodies (ELISA and RIBA), assays that detect presence or absence of HCV RNA genome in patient plasma or serum (qualitative HCV RNA test), assessment of the quantity of the HCV RNA in the blood (quantitative HCV RNA test) and assays that determine the genetic nature of the HCV or HCV genotyping, Barrera (2000). Although the routine diagnosis of HCV
infection is currently based on the detection of antibodies to HCV, measurement of HCV RNA levels has become an integral and increasingly important part of the management of patients with HCV infection to select the appropriate treatment schedule and to estimate the treatment outcome. It is most beneficial in measurement of the viral load at the start of therapy and after 12 weeks of treatment to decide the usefulness of further treatment, and demonstration of the presence or absence of HCV RNA at the end of treatment.

The most commonly used assays to quantify HCV RNA are based on PCR or Branched DNA assay (bDNA) techniques, Desombere (2005). In the present study, we measured the HCV RNA levels using a conventional PCR-based quantitative assay produced by Roche Diagnostics and known as “Complete Bioanalytical System” or COBAS amplicor HCV monitor assay and another real time PCR-based quantitative assay using taqman probe chemistry. After that, the results of both assays were compared. The comparison of results revealed that there was a positive correlation between conventional PCR and real time PCR results with correlation coefficient \( r = 0.60 \) and \( P<0.001 \).

Our results are in accordance with a study done by Enomoto and colleagues (2001). They compared the results of 50 HCV infected patients by the use of both real time PCR as a quantitative assay for HCV RNA measurement and amplicor monitor as a conventional PCR test for HCV RNA determination. They found that the results obtained by using the real time PCR quantitative assay were significantly correlated with those obtained by the conventional assay (correlation coefficient; \( r \) was 0.8 between both tests and with \( P \) value was \( <0.0001 \)).

Similarly, Castelain and colleagues (2004), also evaluated the results of 173 cases infected by HCV using the real time PCR technique (Taqman chemistry) in comparison to those of the conventional PCR using the quantitative amplicor monitor HCV RNA version 2.0 (Roche Diagnostics). They estimated detection limit for both assays as 550 IU/ml and 600 IU/ml for real time PCR and amplicor monitor respectively. According to their study, the correlation coefficient was statistically significant between the real time PCR and the conventional PCR with \( P<0.0001 \) with very good agreement between the results of both tests.

Also our results were in agreement with the study done by Sarrazin et al., (2008), who made also a comparison between both real time PCR and COBAS amplicor monitor version 2.0 tests, they showed a good correlation between the results of both techniques with a correlation coefficient; \( r \) was \( 0.96 \) (among the different HCV genotypes).

In the present study also we found that the results of real time PCR agreed with those of conventional PCR in 26 cases (52% of all cases) and differed from them in 24 cases. From these 24 cases, 20 cases (i.e. 40% of all cases) are overestimated and 4 cases (i.e. 8% of all cases) are underestimated by real time PCR. This difference between the conventional PCR and real time PCR was statistically significant with \( P<0.05 \).

On the other hand, Sarrazin and colleagues (2008), observed a group of cases that were underestimated by the real time PCR assay in comparison to the conventional PCR. In their opinion, the reasons of this under quantification were unknown and could be explained by general mismatch of primers or the taqman probe or suboptimal binding of the oligonucleotide structure.

Fortunately, Halfon and his team (2006), presented interesting data concerning this issue as they observed significant differences in the real time HCV taqman chemistry results versus amplicor monitor test version 2.0 (the conventional PCR) results (\( P<0.05 \)) with the mean of the real time PCR (taqman assay) results was lower than that of the conventional PCR (COBAS) results. They suggested that the HCV RNA results vary according to the method used, their study evaluated for the first time four widely available assays used frequently to measure the HCV RNA levels in the clinical practice which are: Versant HCV RNA 3.0 (Bayer), real time PCR (Taqman, Roche), LCx HCV RNA (Abbott), and the COBAS amplicor monitor test version 2 (Roche). The main advantage of these tests is that each has been standardized. Moreover the National Institute for Biological Standards and Controls and the World Health Organization developed and certified a uniform standard for measuring the HCV RNA levels in international units (IU/ml) Pawlotsky (2002), to make quantitation of the HCV RNA comparable among the different assays, but despite that discrepancies may occur when the patient is monitored by different assays. In addition Halfon and colleagues (2006) also observed some false positive and false negative results as well as variation of the HCV RNA levels of more than 1 to 2 log unit. They proposed that these differences have many explanations. One is related to the variable nature of these assays with reference to the I.U. standard. Thus, before the introduction of the I.U. standard, 1 I.U. turned out to be equivalent, for example, to 2.4 copies/ml for the amplicor assay, and to 5.4 mEq/ml by the Versant HCV RNA 3.0, Saldanha (1999). Another difference among these assays is the lower and the upper limit for quantitation of the HCV RNA level as the I.U. standard does not address this issue.

According to both team works of Germer (2005) and Konnick (2005), the real time PCR technology has the advantage of online detection of HCV RNA serum level with a broader dynamic range than other conventional PCR methods which enables this technique to measure HCV RNA levels ranging from very low to extremely high concentrations and therefore, the real time PCR is considered the technique of choice for highly sensitive quantification of HCV RNA target.
This opinion is supported by several studies Castelain et al. (2004), Sábató et al. (2007), Forman et al. (2008) who all showed a good agreement between the results of both tests. They concluded that the real time PCR assay was considered a rapid, accurate, and reproducible method with a high dynamic range for determination of HCV viral loads in the clinic laboratory settings. This conclusion was in accordance with that of Enomoto and colleagues (2001), who showed that the sensitivity of the real time PCR method was the highest and also goes with Chevaliez and colleagues (2009), who concluded from their work that real-time PCR system for HCV RNA quantification is sensitive, specific, and precise.

In summary, the present study had shown that the results obtained from the real time PCR and the conventional PCR assays were almost parallel to each other. However, variations in the HCV RNA levels had occurred between both tests despite the use of I.U. standard. With the sensitivity of the real time PCR was the highest as it measures the RNA levels in the exponential phase where exact doubling of the viral RNA is accumulating at every cycle of the reaction so, the reaction is very specific and precise and therefore more accurate results are expected to be found. On the other hand, the conventional PCR method measures the RNA levels in the plateau phase of the reaction where the RNA amplification is more affected by reaction kinetics so, the data would not truly represent the initial amounts of the RNA material and results will not be very accurate or precise.

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REFERENCES


