

Original Article

Comparison of a novel real-time PCR method (RTA) and Artus RG for the quantification of HBV DNA and HCV RNA

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Abstract

Introduction: The purpose of this study was to evaluate and compare two manual isolation and real-time (RT) polymerase chain reaction (PCR) kits (RTA RT-PCR with RTA isolation kit and Artus RG RT-PCR with QIAamp isolation kit) for molecular diagnosis of hepatitis B virus (HBV) and hepatitis C virus (HCV) infections.

Methodology: The study was conducted on 121 and 54 clinical samples for the detection of HBV DNA and HCV RNA, respectively, with an additional 8 HCV RNA external quality control samples.

Results: Though a high correlation was observed between the two kits for the HBV DNA ($r = 0.955$, $p = 0.001$) and HCV RNA quantifications ($r = 0.828$, $p = 0.001$), discordant results were found in nine of the HBV DNA and in six of the HCV RNA samples. The mean difference between the two systems was found to be 0.4 log IU/mL in Quality Control for Molecular Diagnostics (QCMD) HCV RNA samples by Bland-Altman analysis.

Conclusions: Although there was a high correlation between HBV DNA and HCV RNA tests according to the results of the study, the RTA system requires improvement for the determination of HCV RNA.

Key words: Artus RG; RTA; real-time PCR; spin column; HBV; HCV

J Infect Dev Ctries 2017; 11(7):543-548. doi:10.3855/jidc.8363

(Received 10 March 2016 – Accepted 15 August 2016)

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Introduction

Nucleic acid tests (NATs) have an increasing predominant role in diagnosis and monitoring of viral hepatitis. Real-time polymerase chain reaction (RT-PCR) has become a standard diagnostic method for these types of infections. NATs not only provide an opportunity for early diagnosis, but they also play a substantial role in the determination of response to treatment, the development of resistance, and the duration of treatment [1-5]. Though infections caused by hepatitis B and hepatitis C viruses (HBV, HCV) affect millions of people worldwide and the clinical presentation of infections lead serious health problems, there are numerous satisfactory improvements in diagnosis and treatment [6,7]. Nowadays, there are many commercially available systems for the isolation and amplification of viral nucleic acids. Currently, we use manual spin column-based DNA/RNA isolation kits and amplification-based RT-PCR (Qiagen, Hilden, Germany) systems for the molecular diagnosis of HBV and HCV infections in our laboratory.

In this study, the results of HBV DNA and HCV RNA analyses obtained from the systems used in our laboratory were compared with those obtained from a novel, In Vitro Diagnostics Community European (IVD-CE)-approved RT-PCR system (RTA, Kocaeli, Turkey) for isolation and quantitation of HBV DNA and HCV RNA in terms of correlation and agreement.

Methodology

A total of 121 and 54 serum samples referred to the molecular microbiology laboratory for investigation of HBV DNA and HCV RNA, respectively, were aliquoted and stored at -30°C.

HBV DNA tests were performed within one week, and HCV RNA tests were performed within two weeks following the acceptance of the samples to the laboratory. Extraction of nucleic acids were performed using two different spin column-based methods (Qiagen and RTA), and the quantitation tests were performed with two different RT-PCR methods (Artus and RTA). The same kit was used for the DNA and RNA isolation in the RTA test according to the

manufacturer's recommendations (RTA, Viral Nucleic Acid Isolation kit, Kocaeli, Turkey). Briefly, under highly denaturing conditions, lysis and inactivation of nucleases were accomplished in serum and plasma samples. Carrier RNA was added at the start, serving to enhance the binding affinity of viral nucleic acids to the columns. After lysis was completed, the free viral nucleic acids were transferred into the columns and the silica gel-based membrane captured the viral nucleic acids selectively. In the following consecutive washing steps, contaminants were washed away and pure viral nucleic acids were obtained. Prior isolation of the nucleic acid detection by the Artus test was performed using the QIAamp DNA mini kit and QIAamp Viral RNA kit for HBV and HCV, respectively. These kits use fast spin-column procedures. Nucleic acids bind specifically to the QIAamp silica-gel membrane while contaminants pass through. PCR inhibitors were completely removed in two efficient wash steps, leaving purified nucleic acid to be eluted in the buffer provided by the kit.

The study was approved by the clinical trials ethics committee of Yıldırım Beyazıt University Medical Faculty (86/21.05.2014).

Artus HBV RG RT-PCR

HBV DNA isolation was made by using 200 μ L serum samples with QIAamp DNA mini kit; 100 μ L DNA was obtained. Afterwards, amplification was performed in a Rotor Gene 6000 platform (Corbett Research, Sydney, Australia) with an Artus HBV RG RT-PCR kit by using 30 μ L of DNA sample as a template. This method targets a fragment size of 134 base pairs (bp) in the HBV core gene. The declared analytical sensitivity is 3.8 IU/mL (95% detection limit), and the linear range is $1.1\text{--}4\times 10^9$ IU/mL.

RTA HBV RT-PCR

The isolation was performed by spin procedure (RTA, Kocaeli, Turkey), initiating with 500 μ L serum sample and deriving 50 μ L of nucleic acid. Subsequently, by using 10 μ L DNA template, amplification and quantitation were carried out with RTA HBV RT-PCR (RTA, Kocaeli, Turkey) kit in a CFX96 (Bio-Rad, Hercules, USA) instrument. The declared analytical sensitivity of this method is 10 IU/mL, and the linear range is $9.9\text{--}1\times 10^9$ IU/mL. The target region is on the S gene of HBV genome and its length is 104 bp.

Artus HCV RG RT-PCR

An initial 200 μ L of serum was processed for isolation with a QIAamp Viral RNA kit (Qiagen, Hilden, Germany), and a 50 μ L final volume was derived. Afterwards, analysis was performed in a Rotor Gene 6000 platform with the Artus HCV RG RT-PCR kit. The amount of sample RNA used was 30 μ L. This method amplifies a fragment of 240 bp in 5'UTR region with a declared analytical sensitivity of 33.6 IU/mL (detection limit 95%), and a linear range of $65\text{--}1\times 10^6$ IU/mL.

RTA HCV RT-PCR

After the extraction of nucleic acid with an isolation kit (RTA, Kocaeli, Turkey) from 500 μ L of serum sample, analysis was performed with RTA HCV RT-PCR kit in a CFX96 platform. The amount of sample RNA used was 10 μ L. The declared analytical sensitivity of this method is 15 IU/mL, and the linear range is $14\text{--}1\times 10^6$ IU/mL. The target region is on the 5'-UTR at HCV genome, and its length is 76 bp.

The laboratory used in this study is a member of the Quality Control for Molecular Diagnostics (QCMD) system. Two months before the study, eight HCV RNA standard panel samples from QCMD had been evaluated in the laboratory with the Artus system. Aliquots of these samples were re-evaluated by performing concurrent isolations and amplifications with both systems compared during this study.

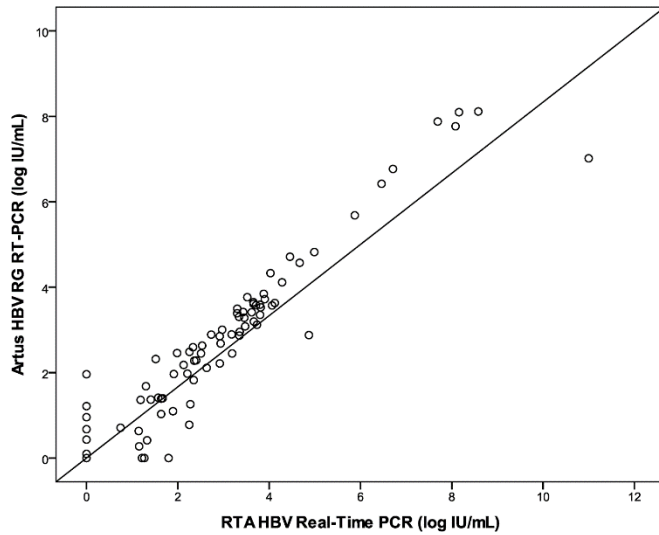
Statistical analysis

Base 10 logarithmic transformations were performed for the HBV DNA and HCV RNA analyses. All statistical analyses were carried out using SPSS version 20.0 (IBM, Armonk, USA). The variables were investigated using histograms, probability plots, and the Kolmogorov-Smirnov test to determine whether to show a normal distribution. Nonparametric tests were used as the logarithms of the HBV DNA and HCV RNA levels that were not normally distributed. The Wilcoxon test was used for the comparison of medians; Spearman correlation and linear regression analysis were used for the calculation of the correlation between the assays. The agreement between the two assays was evaluated with Bland-Altman plots by using MedCalc software (MedCalc Software bvba, Ostend, Belgium). A *p* value of < 0.05 was accepted as statistically significant.

Results

Of the 121 clinical samples, 3 were excluded from the HBV DNA study because an inhibition was observed with RTA PCR analysis and the amount of the

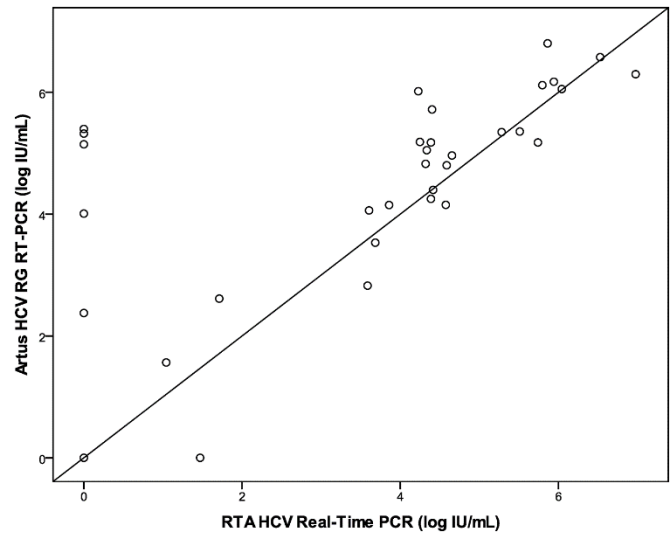
Figure 1. Correlation of HBV DNA levels in clinical samples measured by the Artus RG and RTA assays ($r = 0.955$, $p = 0.001$).



sample was inadequate to reiterate the study. A total of 118 samples were evaluated. The median logarithmic level of HBV DNA with the RTA assay (1.73; interquartile range [IQR]: 0 - 3.46) was found to be significantly higher than that with the Artus assay (1.41; IQR: 0–3.29; $p = 0.001$). As shown in Figure 1, Spearman correlation analysis revealed a significantly positive correlation between HBV DNA log/mL levels of the two tests ($r = 0.955$, $p = 0.001$). Over one log difference was determined in nine of the samples (7.6%). Six of the nine discordant results determined to be HBV DNA positive with the Artus HBV RG RT-PCR test were found to be negative with the RTA HBV RT-PCR test (mean quantitation value: 4.0×10^1 IU/mL). The remaining three samples determined to be positive with the RTA assay were found to be negative with the Artus assay (mean quantitation value: 2.6×10^1 IU/mL).

Two of the three samples that had inhibition in the RTA systems from the HCV RNA study were re-tested, and one of them was excluded because of the

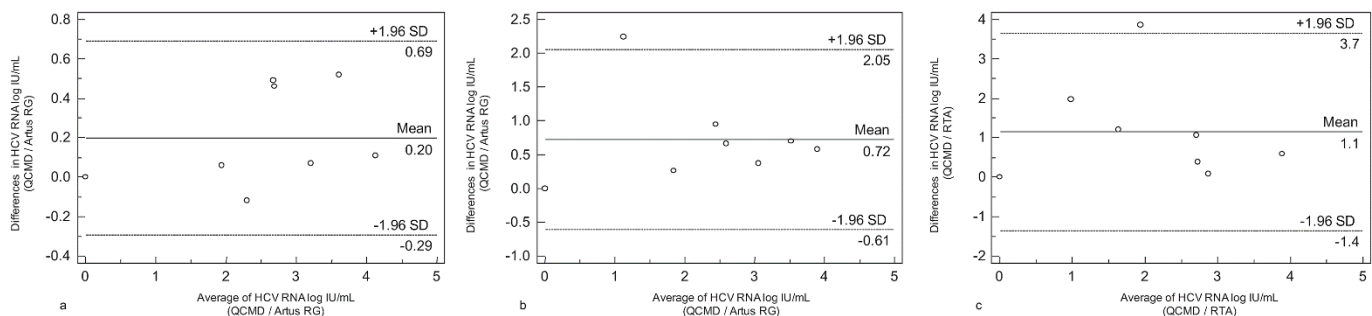
Figure 2. Correlation of HCV RNA levels in clinical samples measured by the Artus RG and RTA assays ($r = 0.828$, $p = 0.001$).



inadequate amount of sample. A total of 53 samples were included to the study. The median logarithmic level of HCV RNA quantitated by the Artus assay (3.53; IQR: 0–5.18) was significantly higher than that of the RTA assay (1.04; IQR: 0–4.41) ($p = 0.005$). As shown in Figure 2, Spearman correlation analysis showed significantly positive correlation between HCV RNA log/mL levels of the two tests ($r = 0.828$, $p = 0.001$). Five samples (9.4%) determined to be positive with Artus HCV RG RT-PCR test (mean quantitation value: 6.1×10^5 IU/mL) were found to be negative with the second system. Also, one sample determined to be negative with the Artus HCV RG RT-PCR test was found to be positive with the RTA HCV RT-PCR (5.6×10^2 IU/mL) test.

For the eight HCV RNA reference samples, an agreement was observed between the expected and the obtained values from two tests and is shown in Figure 3 (a, b, c). The mean difference of HCV RNA levels in the first evaluation by the Artus RG RT-PCR assay and maximal difference were 0.20 (-0.29, 0.69) and 0.52

Figure 3. Bland-Altman analysis between the expected values and quantitated by Artus RG and RTA assays in QCMD HCV RNA reference samples. a: first quantitation by Artus; b: second quantitation by Artus; c: quantitation by RTA



IU/mL, respectively (with 95% CI) (Figure 3a). In the second evaluation of the Artus RG RT-PCR assay, the mean difference value of HCV RNA levels and maximal difference were 0.72 (-0.61, 2.05) and 2.24 IU/mL, respectively (with 95% CI) (Figure 3b). The mean difference value of QCMD HCV RNA levels by the RTA RT-PCR assay and maximal difference were 1.1 (-1.4, 3.7) and 3.87 IU/mL, respectively (with 95% CI) (Figure 3c).

The agreement analysis of HCV RNA levels quantitated by the Artus RG and RTA assays in eight reference samples is shown in Figure 4. The mean and maximal difference value were 0.4 (-2.3, 3.2) and 3.2 IU/mL, respectively (95% CI).

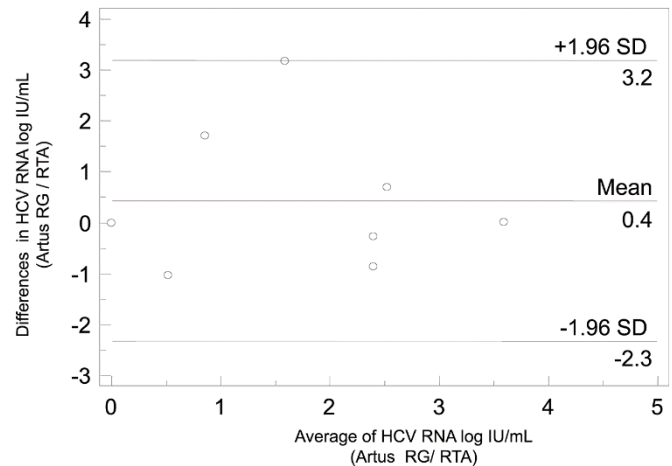
Discussion

Molecular methods used to detect and/or analyze the nucleic acids of microorganisms have an important role in current diagnosis of infectious diseases. Generally, the specificities of molecular tests are high and they have many advantages over other methods [4].

In this study, the processes and the results of two commercial manual isolation and RT-PCR systems were compared in patient samples referred to our molecular microbiology laboratory to evaluate for HBV DNA and HCV RNA. Both methods are IVD-CE licensed.

The kinetic studies of HBV DNA and HCV RNA are useful tools for clinicians in assessment of treatment duration, evaluation of the response or resistance to the antiviral therapy, and preparation of guidelines. Although there are several quantitative assays available for monitoring the response to treatment, it is essential to use the most appropriate method [1,2,5]. The key components that the safety of molecular tests are based on are accurate detection and quantitation. However, some steps of the protocols used require intensive effort and they have some risks. RT-PCR is used as a reasonably standardized diagnostic tool for a large number of pathogenic viruses, and substitutes other virological diagnostic methods because of its high specificity and wide dynamic range [8]. It is recommended to use standardized methods in clinical practice for the efficient monitoring and control of HBV infections. The lower limit of the detection is recommended to be at least 10 IU/mL to determine the viral rebound early. Also, the method must be able to quantitatively detect all the HBV genotypes in equal accuracy [9]. In this study, a high correlation was seen between the two test methods in terms of accuracy and quantification (Figure 1), but differences over one log were observed in nine of the patients (7.6%). As a

Figure 4. Bland-Altman analysis of the HCV RNA levels measured with the Artus RG and RTA assays in QCMD reference samples.



difference over one log has a crucial importance for the determination of response to treatment and occurrence of resistance in early periods, this observation should not be ignored [5].

HBV DNA results from the RTA method were found to be significantly higher when compared to the results of the Artus method. Isolation systems and the variability in the amount of samples can be the explanation for this situation. Viral nucleic acid isolation is the key step before amplification and quantification. The performance of efficient nucleic acid isolation and amplification step can be improved, and successful results can be obtained even from the samples that contain very small amounts of nucleic acids [10]. When compared to automated systems, manual isolation systems are both time consuming and quite open to contamination. In addition, they can be highly affected by technical problems originating from users [11]. Nevertheless, these systems are still available in laboratories that perform few tests with experienced technicians, considering the costs.

Both the methods used for isolation in this study were spin column-based manual methods. However, while the Qiagen method was initiated with 200 μ L of sample and derived 100 μ L of nucleic acids, in the RTA method, these amounts were 500 μ L and 50 μ L, respectively. Some possible effects of the sample amount on the sensitivity of tests were reported previously [12]. The amount of initial sample and the duration of the process are the main factors for the preference of the method, as the small amount of sample allows for re-testing when needed, and the short duration decreases the risk of contamination. In our study, sample volumes were used according to the manufacturer's instructions; therefore, comparisons

could not be performed by using equal amounts. Mean isolation time per sample in the Qiagen systems is approximately 20 minutes for the DNA kit and 30 minutes for the RNA kit. In RTA systems, the same kit is used for both DNA and RNA isolation, and the duration of the process is approximately 40 minutes. Consequently, it can be concluded that the Qiagen system is superior to RTA in terms of isolation time.

The importance of the regular usage of the same method for monitoring patients who underwent anti-viral treatment is frequently emphasized, as the results obtained from different viral load detection systems can be variable. In monitoring patients, the comparison of the results obtained from different methods is not recommended, because of the significant logarithmic differences between different test results [13,14]. Accordingly, in case of a method change, comparative studies should be conducted and clinicians should be informed.

Though a high correlation was observed between the HCV RNA results of the two methods, quantities derived from the Artus systems were significantly higher than those from the RTA systems (Figure 2). Also, five patients (9.4%) found to be positive with the Artus systems were found to be negative with RTA systems. Conversely, a sample from a patient who had previous low-level positive results for anti-HCV was found to be 10^2 IU/mL with the RTA system and negative with the Artus kit.

Taking these discordant results into account, we decided to compare the two systems by using some samples with known contents. For that purpose, eight QCMD HCV RNA quality control samples that remained from the first analysis by the Artus systems done in our laboratory two months prior were employed. The mean difference found between the expected and obtained results was 0.2 log IU/mL. In the current study, the re-test of the same samples revealed a mean difference of 0.72 log IU/mL. It was thought that this increase of difference can be attributed to the storage of samples at -30°C for two months. On the other hand, the mean difference between the expected values and those obtained by the RTA systems was found to be 1.1 log IU/mL (Figure 3c). Based on these observations, the Artus systems can be said to detect the HCV RNA more accurately than the RTA systems.

The RTA isolation and RT-PCR system, which is developed in Turkey, is a molecular test system with an IVD-CE certificate. Based on the results of our study, although there was a high correlation between HBV DNA and HCV RNA tests, the RTA system requires improvement for the determination of HCV RNA.

One of the limitations of this study was that the HBV detection performances were evaluated in only clinical samples. But because a high correlation was observed between the two methods, this can be ignored. Another limitation was that genotype evaluation had not been performed neither for HBV nor for HCV samples. This can also be partially ignored, because, according to the current data, genotype D of HBV is highly dominant in our country [15]. As for HCV, however, this factor may contribute to the observed differences between the results because, though genotype 1b is prevalent in Turkey, some other genotypes have also been emerging recently [16,17].

Conclusions

Although there was a high correlation between HBV DNA and HCV RNA tests based on the results of the study, the RTA system is in need of improvement for the determination of HCV RNA. To our best knowledge, this is the first study that evaluated the RTA manual isolation and RT-PCR systems. The results of such comparative studies will be useful to detect and overcome the imperfections of the novel diagnostic systems before it comes into routine use, and may contribute to improving them.

Acknowledgements

RTA isolation and RTA RT-PCR kits employed in this study were provided by RTA Laboratories, Turkey.

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Conflict of interests: No conflict of interests is declared.