

## Qualitative detection of GB Virus C and Hepatitis C Virus co-infection in cirrhotic patients using a SYBR green multiplex RT-PCR technique

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**Abstract.** GB Virus C (GBV-C) and Hepatitis C Virus (HCV) belong to the Flaviviridae family of viruses and GBV-C is the closest virus to HCV genetically. Possibility of interaction between HCV and GBV-C and its association with other liver diseases are the most important clinical aspects which encourage researchers to develop a technique for detection of these viruses simultaneously. In this study a SYBR Green multiplex real time RT-PCR technique as a new economical and sensitive method was optimized for simultaneous detection of HCV/ GBV-C in cirrhotic patients. After designing two pairs of specific primers for HCV and GBV-C, SYBR Green Real time RT-PCR technique optimization was performed separately for each virus. Then, multiplex PCR was developed. Finally the optimized technique was performed on positive and negative plasma samples. Eighty nine cirrhotic HCV positive plasma samples (29 of genotype 3a and 27 of genotype 1a) were collected from patients before receiving treatment. 14% of genotype 3a and 17.1% of genotype 1a showed HCV/ GBV-C co-infection. As a result, 13.48% of 89 samples had HCV/ GBV-C co-infection that was compatible with other results from all over the world. Data showed no apparent influence of HGV co-infection on the either clinical or virological aspect of HCV infection. Furthermore, with application of multiplex Real time RT-PCR technique, more time and cost could be saved in clinical-research settings.

### INTRODUCTION

GB Virus C (GBV-C) and Hepatitis C Virus (HCV) belong to Flaviviridae family and GBV-C is the closest virus to HCV genetically (Reshetnyak *et al.*, 2008). Although GBV-C is mostly considered as a nonpathogenic and lymphotropic virus, there are however several reports from different countries worldwide on the occurrence of hepatitis by this virus (Reshetnyak *et al.*, 2008; Berzseny *et al.*, 2005). However GBV-C co-infections with HIV, HCV and HBV are more frequent than single infection and therefore are more assessed by researchers than its occurrence as a single infection (Reshetnyak *et al.*, 2008). GBV-C doesn't influence HBV and HCV-related hepatitis (Berzseny *et al.*, 2005). Co-

infection of GBV-C and HIV infection leads to develop an improvement in both morbidity and mortality of HIV-related diseases specially in suppressing of acquired immunodeficiency syndrome (AIDS) (Berzseny *et al.*, 2007). In HCV and HIV co-infected patients, HCV-related liver disease is significantly accelerated including faster development of cirrhosis and hepatocellular carcinoma in these patients (Berzseny *et al.*, 2005; Gibellini *et al.*, 2006). Meanwhile, in patients with triple infection of GBV-C, HCV and HIV a significant reduction in HCV-related liver disease is reported (Berzseny *et al.*, 2007). Common transmission routes explains high rate of GBV-C co-infections with HCV and HIV; transmission through blood and blood products are the main transmission routes for

these viruses and sexual and vertical transmission are less important in transmission routes for them (Reshetnyak *et al.*, 2008; Gibellini *et al.*, 2006). A study showed GBV-C RNA in 18.2% of HIV infected individuals and 24.4% of HCV infected individuals (Feucht *et al.*, 1997). Prevalence of GBV-C will fluctuate in particular population but as a proven fact carriage of GBV-C is more frequent in patients on hemodialysis, patients with hemophilia, drug abusers and individuals with unsafe sexual relationships (Reshetnyak *et al.*, 2008; Berzseny *et al.*, 2005). Prevalence of GBV-C in the healthy individuals averages 1.7% (Reshetnyak *et al.*, 2008). Whereas prevalence of GBV-C in high risk groups is more common and significant statistically (Reshetnyak *et al.*, 2008; Berzseny *et al.*, 2005). Diagnosis of GBV-C is not a routine test in clinical laboratories. In research laboratories, applying ELISA to detect Anti-E2 or using RT-PCR to detect GBV-C RNA are methods of diagnosing GBV-C infection (Reshetnyak *et al.*, 2008; Berzseny *et al.*, 2005). Routine tests for diagnosing HCV are serological tests based on detecting HCV specific antibody in serum (Gibellini *et al.*, 2006; De Crignis *et al.*, 2010). However, to have a definite method to detect HCV infection and to monitor treatment process of different genotypes of HCV, variety of nucleic acid tests (NAT) have been developed (Chevalie & Pawlotsky, 2006). Sero-conversion is another reason to distrust serological tests and to use NAT for detecting GBV-C and HCV infections. We can overcome high cost, possibility of false positive results and doubt about sensitivity in low-positive samples by development of a multiplex real time RT-PCR technique to detect HCV and GBV-C genomes simultaneously. Real time PCR techniques include employing specific probes or SYBR Green I dye to detect target genomes (Wittwer *et al.*, 2001). Although probes are more specific, it's hard to design them, they are more expensive and the results are more complicated to interpret comparing to SYBR Green I (Espy *et al.*, 2006; Mackay *et al.*, 2002; Yang *et al.*, 2009). SYBR Green real time PCR is a simple, rapid and economical method. This assay is based on

melting curve analysis and the results are quite easy to interpret and with applying of this method more time and cost could be saved in clinical-research settings (Aldea *et al.*, 2002; Park *et al.*, 2009). This paper describes a SYBR Green multiplex real time RT-PCR technique to detect HCV and GBV-C simultaneously in cirrhotic patients.

## MATERIALS AND METHODS

### *Patients*

A total of 89 cirrhotic HCV positive plasma samples (20 of genotype 3a and 69 of genotype 1a) were collected from patients before receiving treatment. None of these patients were infected with HIV or HBV. Twenty samples were obtained from healthy volunteer blood donors. All of these samples were obtained from the Digestive Disease Research Institute (DDRI), Shariati Hospital, Tehran, Iran. This study was approved by Ethics Committee of Tehran University of Medical Sciences. In addition, a written informed consent was obtained from all the participants before blood sampling.

### *Viral RNA Extraction*

Whole blood samples were collected from HCV positive cirrhotic patients. Extraction of viral nucleic acid was performed using QIAamp Viral RNA Mini Spin kit (Qiagen, Hilden, Germany) and purified RNA stored at -70°C until use.

### *RT-PCR*

Extracted RNA was reverse transcribed. cDNA synthesis was performed in total volume of 20 ml using Expand RT kit upon manufacturer details (Roche Molecular Biochemical, Mannheim, Germany). The master mix was prepared in a 0.5 ml micro tube containing 4ml 5x RT buffer, 2ml DTT (10mM), 2ml dNTP Mix (1mM), 1ml Random Hexamer (20Pmol/ml), 0.5ml RNase inhibitor (20 U), 1ml Expand RT (50U), 9.5ml RNA. Tubes were transferred to the Thermalcycler with following program: 95°C for 5 min and, 42°C for 60 min. Finally, cDNA was stored at -20°C.

### Primers

Highly conserved 5' un-translated region (5' UTR) of HCV and GBV-C were selected as targets for specific primers. The primers were used for amplifying 175bp and 262 bp amplicons within 5' UTR regions of HCV and GBV-C genomes respectively (Table 1).

### SYBER Green Multiplex Real-Time RT-PCR

SYBR Green multiplex real time RT-PCR assay was developed using was performed in 20 µl glass capillary containing 2 µl Fast Start DNA Master SYBR Green I, 2.4 µl of 25mM MgCl<sub>2</sub>, 0.2 µl of each HCV and GBV-C oligonucleotide primers, 12.8 µl H<sub>2</sub>O and 2 µl of cDNA template.

The co-amplification was performed as follows: pre-incubation at 95°C for 10 min and followed by 55 cycles as 95°C for 10 s (Denaturation), 57°C for 4 s (Annealing) and 72°C for 8 s (Extension). Single fluorescence detection was performed in each cycle at 72°C to expose positive samples and to reduce dimer primer, despite the fact that such interference was inevitable especially in negative samples. Next step was melting curve temperature that was performed by a gradual increase in temperature (0.1°C/s) up to 95°C. At the end, cooling step was performed as 40°C for 30 s. The LightCycler system (RocheDiagnostics, Mannheim, Germany) using LightCycler 5.3.2 software was used for amplification, data acquisition and analysis. The melting peaks were analyzed to discern HCV and /or GBV-C-specific amplicons. All amplicons derived from clinical samples were run in 2% agarose gel electrophoresis to verify the exact length of amplicons.

### Statistical analyses

Analytical and descriptive statistics were carried out using SPSS 23.0 software (SPSS Inc., Chicago, IL, USA). Descriptive statistics were reported in terms of percent (for categorical) and mean (SD) (for continuous) variables.

## RESULTS

### Optimization

The optimal conditions for SYBR Green multiplex real time RT-PCR were acquired using DNA Master Mix SYBR Green I adjusted by adding 3 mM MgCl<sub>2</sub> and 12.8 µl H<sub>2</sub>O. In addition, concentrations of primers, concentration of cDNA template and primers' annealing temperature were adapted to achieve maximum sensitivity. Specific primer pairs were able to reveal all genotypes of HCV and GBV-C without any relevant interaction with other viral or human sequences.

SYBR Green-based real time distinguishes different amplicons by melting curve analysis (Varga & James, 2006; Kong *et al.*, 2009). Distinctive melting temperature (T<sub>m</sub>) caused by different length and composition of HCV and GBV-C amplicons, particularly reveals the presence of HCV and GBV-C in samples. The melting curves of HCV and GBV-C display a T<sub>m</sub> = 88.3°C and a T<sub>m</sub> = 91.7°C respectively (Fig. 1).

Table 1. HCV and GBV-C Primers

Virus	Primer	Sequence	Amplicon
HCV	Sense	5'-GTGGTCTGCGGAACCGG-3'	175 bp
	Anti-sense	5'-GGGCACTCGCAAGCACCC-3'	
HGV	Sense	5'-GGTCGTAAATCCCGGTCACC-3'	262 bp
	Anti-sense	5'-CCCCTGGTCCTTGTCACCT-3'	

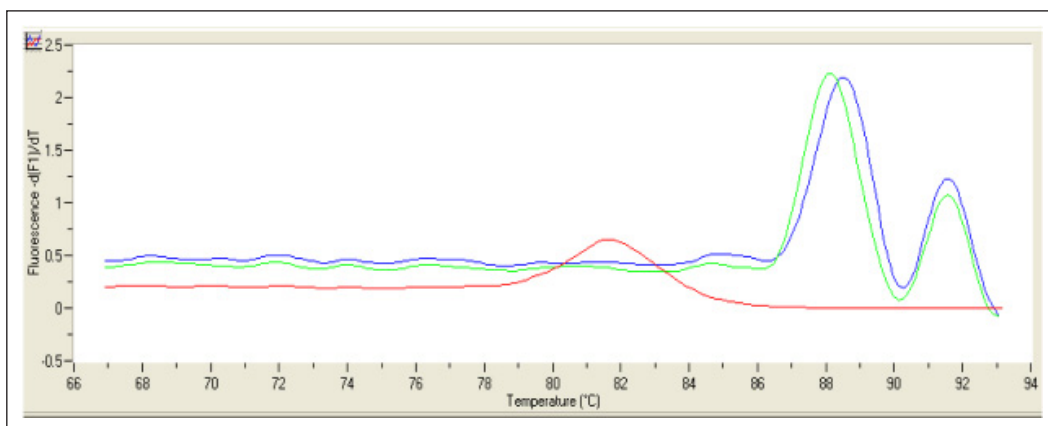


Figure 1. Melting curve analysis of two HCV/GBV-C co-infected samples and a negative sample: the HCV and GBV-C  $T_m$  were stated at 88.3 and 91.7°C, respectively.

#### *Sensitivity and Specificity of the Assay*

Specificity of this assay was assured by meticulous selection of primer pairs. Moreover, specificity of assay was confirmed by examining available HCV/GBV-C negative controls comprising HIV-1, HBV, HSV-1 and HSV-2. In addition of these controls, three HCV/GBV-C negative samples obtained from healthy individuals were examined which no cross-reactivity was observed with these controls and samples.

To determine detection limit of our assay, serial dilutions was used as follows: for HCV a sample with definite viral load (10<sup>5</sup> IU/ml) was reverse transcribed and from cDNA endpoint dilutions were prepared. The experiments were performed in duplicates from 10<sup>5</sup> IU/ml to 25 IU/ml. The lowest dilution which could be detected by optimized assay considered as detection sensitivity limit which was determined 50 IU/ml for HCV in this experiment (Table 2).

For GBV-C due to lack of standard plasma dilution, a set of dilutions (1/2, 1/10, 1/20 and 1/100) were prepared from a GBV-C positive control in duplicates which the developed assay could detect 1/20 dilution as detection sensitivity limit for GBV-C. Finally, a set of dilutions (1/2, 1/10, 1/20 and 1/100) were prepared from our double positive control (HCV/GBV-C positive control) and our assay could detect 1/100 dilution of HCV and 1/20 dilution for GBV-C as the lowest dilutions which could be detected by our optimized assay (Table 3 and Fig. 2).

#### *Performing the Assay on Clinical Samples*

In order to evaluate SYBR Green multiplex real time RT-PCR assay on patients' plasma samples, we collected 89 non cirrhotic HCV positive plasma samples (20 of genotype 3a and 69 of genotype 1a), a double HCV/GBV-C positive as positive control and 20 plasma samples from healthy individuals as negative control. The positive control was previously tested and verified by RT Nested-PCR assay represented by HCV and GBV-C specific primers and all of 89 HCV positive plasma samples were previously proven to be positive by serological tests. In addition to positive and negative controls a non-template control (NTC) was examined by our assay. The results demonstrated that 13.48% of 89 HCV positive samples (17.1% for genotype

Table 2. Analytical Sensitivity of the Assay Using EndPoint Dilution of HCV

HCV, IU/ml	Replicates (detected/replicates)
100000	200
10/10	10/10
10000	100
10/10	10/10
1000	50
10/10	5/10
500	25
10/10	0/10

Table 3. Analytical Sensitivity of the Assay Using EndPoint Dilution of HCV/GBV-C

HCV, IU/ml	Detected/Replicates	GBV-C, IU/ml	Detected/Replicates
1/2	10/10	1/2	10/10
1/10	10/10	1/10	10/10
1/20	10/10	1/20	5/10
1/100	5/10	1/200	0/10
1/200	0/10	1/200	0/10

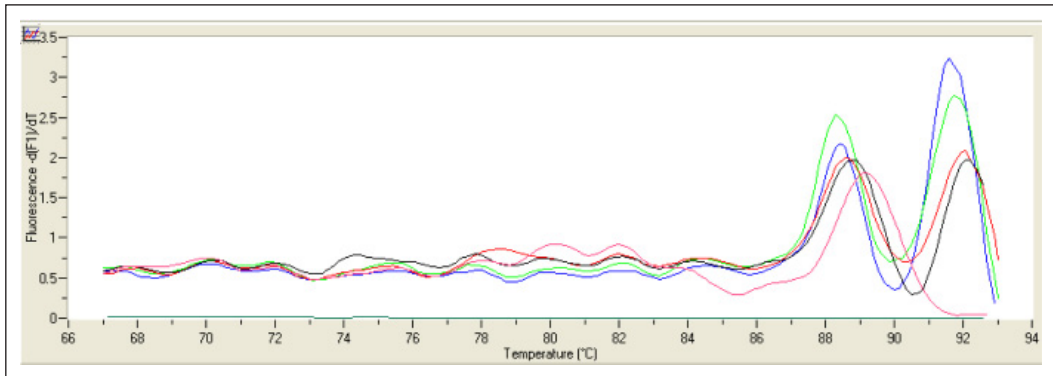


Figure 2. Evaluation of analytical sensitivity for HCV/GBV-C positive controls using melting curve analysis.

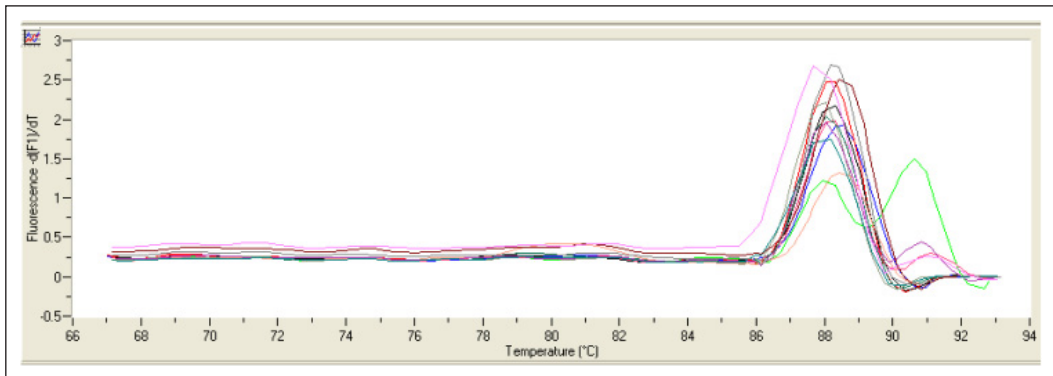


Figure 3. A typical melting curve analysis of three HCV/GBV-C positive plasma samples and a HCV/GBV-C positive control beside some HCV positive but GBV-C negative plasma samples.

1a and 14% for genotype 3a) were GBV-C positive as well. On the other hand, all of 89 patients' samples were HCV positive, positive control was HCV/GBV-C positive and negative controls and NTC were HCV/GBV-C negative showing the specificity of the multiplex assay (Fig. 3).

Patients infected with different genotypes of HCV and GBV-C was detected by our technique thus illustrating the good pliability of oligonucleotide primers. The melting curve analysis revealed credible identification of HCV and GBV-C amplicons with distinct  $T_m$  with values of 88.3°C for HCV and 91.7°C

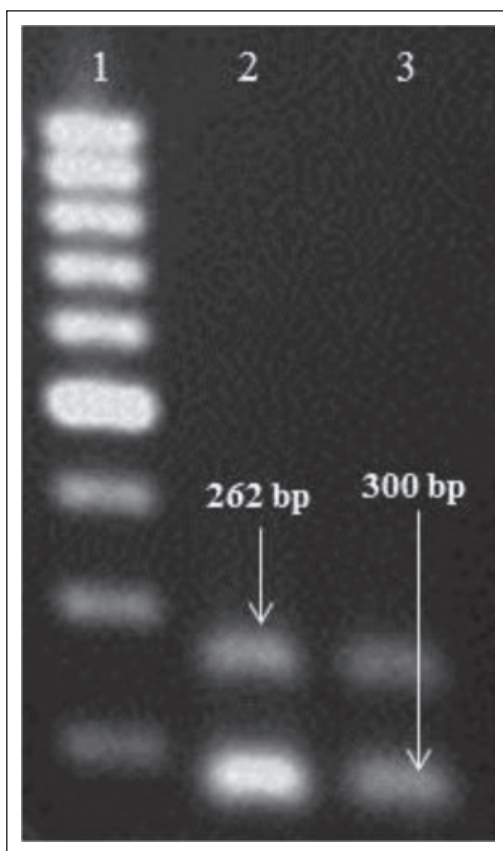


Figure 4. Agarose gel electrophoresis analysis of HCV/GBV-C co-amplified samples. Lane 1 represents molecular markers. Lane 2 and 3 demonstrate a co-amplification of two HCV/GBV-C positive samples.

for GBV-C. Anyhow, 1 melting temperature variations caused by different fragment internal sequences were occasionally observed. Agarose gel electrophoresis confirmed certified the exact length of HCV and GBV-C amplicons: a specific band of 175 bp for HCV and a specific band of 262 bp for GBV-C (Fig. 4). All of HGV positive samples were sequenced. The results were confirmed obtained data by the technique.

## DISCUSSION

Present study depicts the progress in the development of SYBR multiplex Green real time RT-PCR for simultaneous detection of HCV and GBV-C genomes in plasma samples.

The multiplex RT-PCR was performed using specific primers in order to reverse transcribe HCV and GBV-C RNA genomes into cDNA and to detect all genotypes of these viruses concurrently. Furthermore, the optimization of this assay focused on compatibility of primers including regulation of target temperature during annealing segment of amplification and applying the primers' optimum concentration, optimizing magnesium chloride concentration and optimizing the concentration of cDNA template. By using intercalating fluorescence dyes like SYBR Green I and LightCycler system, the accumulation of amplicons during real time PCR can be monitored over the reaction step by step (Fan *et al.*, 2007; Richards *et al.*, 2004). Since SYBR Green binds to every possible double-stranded DNA, SYBR Green-based real time PCR differentiates target amplicons by melting curve analysis (Beuret 2004; Tam *et al.*, 2009). Our assay identified HCV and GBV-C genomes through detection the  $T_m$  of HCV and GBV-C melting curves which were 88.3°C and 91.7°C respectively. Although slight variation in melting curves occurred in consequence of sequence alterability in some samples, the melting curve analysis could distinguish HCV and GBV-C specific amplicons.

Even though conventional multiplex RT-PCR are sensitive methods, they are labor intensive and time consuming methods because using agarose gel electrophoresis is a necessary post-PCR step to discriminate target amplicons (Defoort *et al.*, 2000). In contrast, SYBR Green real time RT-PCR grants several utilities such as reduction in cycle time and so elevating the speed, obviation of post amplification electrophoresis, high sensitivity, and lower possibility of product contamination and proportionately simple and economical technical possibility (Watzinger *et al.*, 2006). On the other hand, utilizing specific probes can improve the specificity yet it's more expensive and paraphrasing the results could be much complicated (Liu *et al.*, 2008; Martinez *et al.*, 2008). Albeit SYBR Green multiplex real time RT-PCR is a qualitative assay, using plasma samples with known

viral load or reference positive controls can give an approaching indication of its sensitivity (Adami *et al.*, 2004). Practically, the analytical sensitivity of our assay was concluded at 50 IU/ml for HCV and the assay could detect 1/100 dilution for HCV and 1/20 dilution for GBV-C of our positive control.

After development and optimization of SYBR Green multiplex real time RT-PCR assay, we applied this technique on 89 cirrhotic HCV positive patients' samples (20 of genotype 3a and 69 of genotype 1a) which 14% of genotype 3a, 17.1% of genotype 1a had HCV/GBV-C co-infection. We applied this technique on sample infected with genotypes 1a and 3a of HCV because these are the most frequent HCV genotypes in IRAN (Keyvani *et al.*, 2007; Samimi-Rad *et al.*, 2004; Zali *et al.*, 2000). Although our sample size was not big enough to infer the results as a reference for occurrence of HCV/GBV-C co-infection in Iranian population, our results are comparable to other results reported from other countries worldwide (Kupfer *et al.*, 2005; Yang *et al.*, 2006). Accordingly more researches should be settled to achieve the real amount of GBV-C occurrence in Iranian HCV positive patients.

### CONCLUSION

In conclusion, we described a SYBR Green multiplex real time RT-PCR assay as a simple, rapid, sensitive, specific and relatively inexpensive technique to identify two viruses from the same family simultaneously. This technique is easier and faster than conventional PCR and is less expensive and less confusing comparing to probe-based real time PCR. This technique can be applied on HCV positive samples and even on HIV positive samples. With applying this technique on expanded sample size, it will be possible to achieve findings like real occurrence of HCV/GBV-C co-infection in Iranian population and even occurrence of HCV/GBV-C co-infection in HIV positive patients and analyze the clinical aspects of the interaction between HIV, HCV and GBV-C.

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### Declaration of interest

The authors report that they have no conflict of interest.

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