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Original Article

Development of a SYBR green real time multiplex RT-PCR technique for simultaneous detection of HCV and GBV-C Co-infection in plasma samples

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Abstract

Background: Accumulative research is in progress to clarify clinical aspects of GBV-C. The possibility of interaction between HCV and GBV-C as well as its consequence on development of liver diseases is the most important clinical aspect which encourages researchers to develop a rapid and cost effective technique for simultaneous detection of both viruses.

Methods: In this study, a SYBR Green real time multiplex RT-PCR technique as a new economical and sensitive method was designed and validated for simultaneous detection of HCV/GBV-C in HCV positive plasma samples. SYBR green real time RT-PCR technique optimization was performed separately for each virus. Multiplex PCR was established next. Standard sera with known concentrations of HCV RNA and dual HCV/GBV-C positive control samples along with negative control samples were used to validate the assay.

Results and Conclusions: Fifty six non cirrhotic HCV positive plasma samples [29 of genotype 3a and 27 of genotype 1a] were collected from patients before receiving treatment. 20.6% of genotype 3a and 18.7% of genotype 1a showed HCV/GBV-C co-infection. As a result, 19.6% of 56 samples had HCV/GBV-C co-infection that was compatible with other results from all over the world. SYBR Green real time multiplex RT-PCR technique can be used to detect HCV/GBV-C co-infection in plasma samples. Furthermore, with application of this method more time and cost could be saved in clinical-research settings.

Keywords: HCV, GBV-C, SYBR Green real time, rapid detection, multiplex RT- PCR

Introduction

GBV-C, or hepatitis G virus (HGV) and hepatitis C virus (HCV) belong to *Flaviviridae* family of viruses 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 reports from different countries worldwide on occurrence of hepatitis by this virus [Berzsenyi et al., 2005; Reshetnyak et al., 2008]. However GBV-C co-infections with HIV, HCV and HBV are more frequent than single infection

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and therefore are more assessed by researchers than its single infection [Reshetnyak et al., 2008]. Combination of GBV-C and HIV infection helps in developing an improvement in both morbidity and mortality of HIV-related diseases specially postponing the occurrence of acquired immunodeficiency syndrome (AIDS) [Berzsenyi 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 (HCC) in these patients [Berzsenyi et al., 2007; Berzsenyi et al., 2005; Gibellini et al., 2006]. Meanwhile, in patients with triple infections of GBV-C, HCV and HIV, a significant reduction in HCV-related liver disease is reported [Berzsenyi et al., 2007].

Common transmission routes explains high rate of GBV-C co-infections with HCV and HIV; transmission through blood and blood products is the main transmission route for these viruses but sexual and vertical transmission are less important in their transmission [Berzsenyi et al., 2007; Berzsenyi et al., 2005; Gibellini et al., 2006; Reshetnyak et al., 2008]. 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 fluctuates in particular patient populations but as a proven fact carriage of GBV-C is more frequent in patients on hemodialysis, hemophiliacs, drug abusers and individuals with unsafe sexual relationships [Berzsenyi et al., 2005; Reshetnyak et al., 2008]. Several studies have attempted to show the rate of GBV-C RNA in blood donors worldwide including 4.5% in Caucasians, 3.4% in Asians, 17.2% in Africans and average of 4.8% of all cases [Reshetnyak et al., 2008]. 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 statistically significant [Berzsenyi et al., 2005; Reshetnyak et al., 2008].

Diagnosis of GBV-C is not a routine test in clinical laboratories. In research laboratories, ELISA to detect Anti-E2 or RT-PCR to detect GBV-C RNA isamong methods of choice in diagnosing GBV-C infection [Berzsenyi et al., 2005; Reshetnyak et al., 2008]. Routine tests for diagnosing HCV are serological tests based on detecting HCV specific antibody in serum [De Crignis et al., ; Gibellini et al., 2006]. However, to have a more sensitive method to detect HCV infection and to monitor treatment process of different genotypes of HCV, a variety of nucleic acid tests (NATs) have been developed [Chevaliez and Pawlotsky, 2006]. Seroconversion is another reason to distrust serological tests and to use NAT for detecting GBV-C and HCV infections. However, high cost, possibility of false positive results and doubt about sensitivity in low-positive samples are weaknesses of NATs [Espy et al., 2006] that can overcome by development of a real time multiplex RT-PCR technique to detect HCV and GBV-C genomes simultaneously. Real time PCR techniques include employing specific probes or SYBR Green I fluorochrome dye to detect target genomes [Wittwer et al., 2001]. Although probes are more specific, they are difficult to design, more expensive and the results are more complicated to interpret comparing to SYBR Green I [Mackay et al., 2002; Wittwer et al., 2001; 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. Furthermore, more time and cost could be saved by applying SYBR Green real time PCR in clinical-research settings [Aldea et al., 2002; Park et al., 2009].

This paper describes a SYBR Green real time multiplex RT-PCR technique to detect

HCV and GBV-C simultaneously in plasma samples.

Materials and methods

Patients

Fifty six non cirrhotic HCV positive plasma samples (29 of genotype 3a and 27 of genotype 1a) were collected from patients referred to the Digestive Disease Research Center (DDRC), Shariati Hospital, Tehran, Iran before receiving treatment. None of these patients were co-infected with HIV or HBV. Informed consent was obtained prior to blood collection. All patients had positive HCV infection, confirmed by RT-PCR (Cobas Amplicor HCV Monitor Test) carried out in an independent clinical virology lab. The HCV genotype was determined by PCR-RFLP.

Viral RNA extraction from plasma samples

Whole blood samples were collected from HCV positive patients in EDTA-coated tubes. The tubes were then centrifuged at 800g for plasma separation. Plasma samples were stored in 1.5 ml microtubes at -70 ºC until use. For extraction and purification of viral nucleic acid, QIAamp Viral RNA Mini Spin kit (Qiagen, Hilden, Germany) was employed and purified RNA stored at -70 ºC until use. The procedure started with 140 µl of plasma sample and ended with 40 µl of eluted viral RNA.

RT-PCR

After RNA extraction, the samples were retrotranscribed. cDNA synthesis was performed in total volume of 20 µl using Expand RT kit and the steps were followed as manufacturer described (Roche Molecular Biochemicals, Mannheim, Germany). The master mix was prepared in a 0.5 ml microtube containing 4µl 5x RT buffer, 2µl DTT (10mM), 2µl dNTPs Mix (1mM), 1µl Random Hexamer (20Pmol/µl), 0.5µl RNase inhibitor (20 U), 1µl Expand RT (50U), 9.5µl RNA. Following the steps for cDNA synthesis, after mixing the mastermix (all reagents except Expand RT), microtubes were transferred to the ThermalCycler (Biometra, Gottingen, Germany) at 95 ºC for 5 min. In the next step, Expand RT was added to the mastermix and microtubes were transferred to the Thermal Cycler at 42 ºC for 60 min. At the end, cDNA was stored at -20 ºC until use.

Oligonucleotide primers

To prevent major mismatches of oligonucleotide primers, highly conserved 5´ untranslated region [5´ UTR] of HCV and GBV-C were selected as targets for specific primers. The selected primers were used for amplifying 175 bps and 262 bps amplicons within 5´ UTR regions of HCV and GBV-C genomes respectively (Table 1).

Table 1 Orientation of HCV and GBV-C primers within 5´UTR regions of HCV and GBV-C genomes.

Virus	Primer	Sequence	Amplicon
HCV	Sense	5'-GTGGTCTGCGGAACCGG-3'	175 bp
	Anti-sense	5'-GGGCACTCGCAAGCACCC-3'	
GBV-C	Sense	5'-GGTCGTAAATCCCGGTCACC-3'	262 bp
	Anti-sense	5'-CCCACTGGTCCTTGTCAACT-3'	

For selection and assessment of our primers' compatibility in a multiplex RT-PCR, the sequences of primers were analyzed by Mega 3.1, Gene Runner and Blast. The primer pairs were synthesized by TIB MOLBIOL (Berlin, Germany).

HCV/GBV-C positive control

One HCV positive sample with viral load of 10^5 IU/ml, confirmed by real time RT-PCR using a set of HCV reference standards from NIBSC (Hertfordshire, United Kingdom), and verified to be GBV-C positive by Nested RT-PCR, was selected as our HCV/GBV-C positive control. The sample was quantified and verified to have GBV-C viral load of 10^4 IU/ml carried out by an independent diagnostic laboratory. We utilized this sample as the double positive control (HCV/GBV-C positive control) in our research.

HCV and GBV-C detection by SYBR Green real time multiplex RT-PCR

HCV/GBV-C multiplex SYBR Green real time RT-PCR assay was developed using Fast Start DNA Master SYBR Green I kit (Roche, Germany) and the assay was performed in 20 µl glass capillary containing 2 µl Fast Start DNA Master SYBR Green I, 2.4 µl of 25 mM $MgCl_2$,0.2 µl of each HCV and GBV-C oligonucleotide primers, 12.8 µl H_2o and 2 µl of cDNA template.

The co-amplification was optimized and performed as follows: pre-incubation at 95 ºC for 10 min and followed by 45 cycles as 95 ºC for 10 s (Denaturation), 57 ºC for 4 s (Annealing) and 72 ºC for 8 s (Extension). Single fluorescence detection was performed in each cycle at 72 ºC to expose positive samples and to reduce primer dimers, despite the fact that such interference was inevitable especially in negative samples. Next step was determining melting curve temperature that was performed by a gradual increase in temperature (0.1 ºC/s) up to 95 ºC. At the end, a cooling step was performed as 40 ºC for 30 s. The LightCycler system (Roche Diagnostics, 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. Gel analysis was performed with Bio Doc analyzer (Biometra, Gottingen, Germany).

Results

SYBR Green real time multiplex RT-PCR optimization for HCV/GBV-C genomes detection

The optimal conditions for SYBR Green real time multiplex RT-PCR was acquired using DNA Master Mix SYBR Green I adjusted by adding 3 mM of magnesium chloride and 12.8 µl H_2O.In addition, concentrations of primers and of cDNA templates and the primers' annealing temperature were adapted to achieve maximum sensitivity.

We selected specific primer pairs 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 [Kong et al., 2009; Varga and James, 2006]. Distinctive melting temperature (Tm) 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 Tm = 88.3 ºC and a Tm = 91.7 ºC respectively (Fig 1).

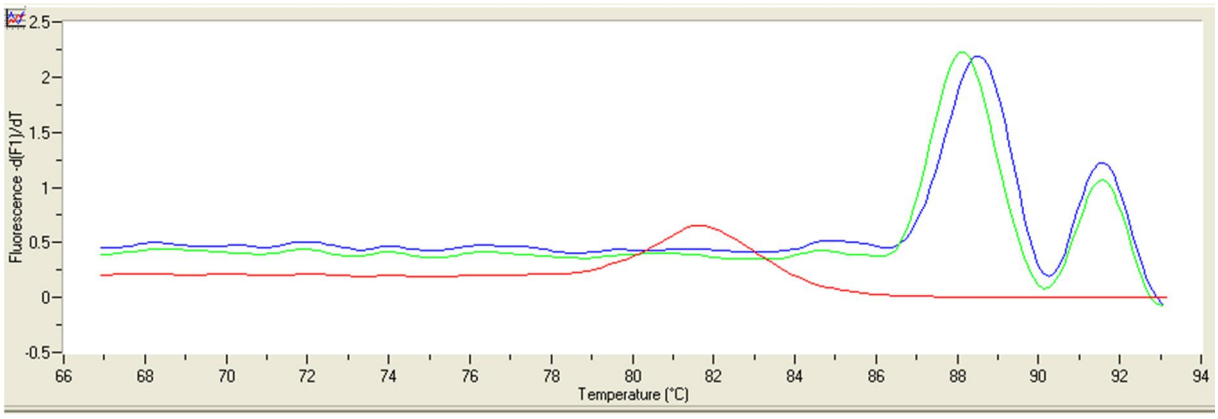

Figure 1 Melting curve analysis of two HCV/GBV-C co-infected samples and a negative sample. The HCV and GBV-C Tm were stated at 88.3 and 91.7 ºC, respectively.

SYBR Green real time multiplex RT-PCR assay specificity and sensitivity

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 to these controls, three HCV/GBV-C negative samples obtained from healthy individuals were examined that did not show any cross-reactivity with those of controls and samples.

To determine the detection limit of our assay, endpoint dilutions of cDNA retrotranscribed from our double positive control were prepared. The experiments were performed in duplicates from 10^5 IU/ml to 25 IU/ml and from 10^4 IU/ml to 25 IU/ml for HCV and GBV-C separately. The lowest dilution which could be detected by optimized assay considered as the detection sensitivity limit; itwas determined to be 100 IU/ml and 200 IU/ml for HCV and GBV-C respectively (Table 2 and Table 3). Finally, to test the assay for detecting HCV and GBV-C simultaneously, a set of dilutions were prepared from our double positive control and the results confirmed previous experiments performed with HCV or GBV-C as a single target.

Table 2 SYBR Green real time multiplex RT-PCR analytical sensitivity appraised by endpoint dilution analysis for HCV.

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

Table 3 SYBR Green real time multiplex RT-PCR analytical sensitivity appraised by endpoint dilution analysis for GBV-C.

GBV-C, IU/ml	Replicates [detected/replicates]
10000	10/10
1000	10/10
500	10/10
200	10/10
100	5/10
50	0/10
25	0/10

Performing SYBR Green real time multiplex RT-PCR assay on patients' plasma samples

In order to evaluate SYBR Green real time multiplex RT-PCR assay on patients' plasma samples, we collected 56 non cirrhotic HCV positive plasma samples (29 of genotype 3a and 27 of genotype 1a), a double HCV/GBV-C positive as positive control and 3 plasma samples from healthy individuals as negative controls. In addition to positive and negative controls, a non- template control (NTC) was also examined by our assay. The results demonstrated that 11HCV positive samples (5 of genotype 1a and 6 of genotype 3a) were GBV-C positive as well. On the other hand, all of 56 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. 2). The melting curve analysis revealed credible identification of HCV and GBV-C amplicons with distinct Tm with values of 88.3 ºC for HCV and 91.7 ºC for GBV-C. Anyhow, trivial melting temperature variations caused by different fragment internal sequences were occasionally observed. Agarose gel electrophoresis confirmed 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. 3).

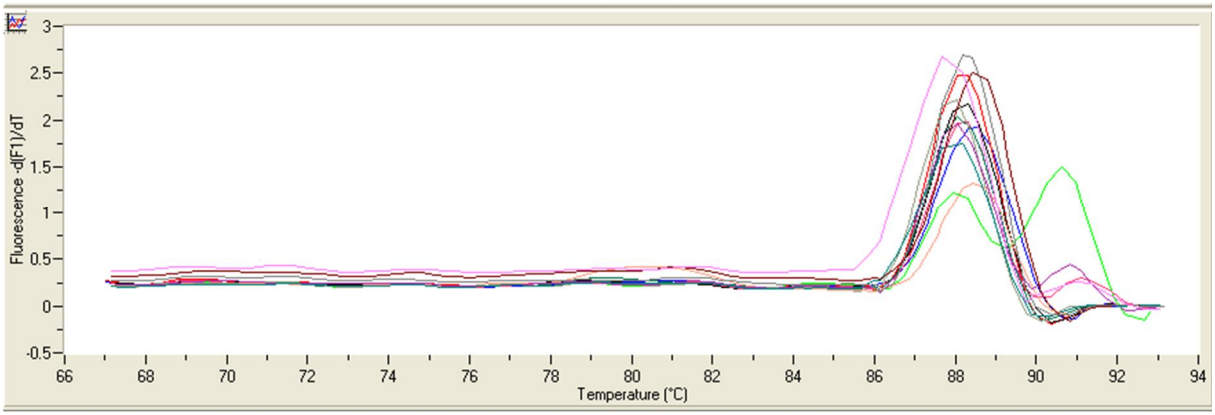

Figure 2 A typical melting curve analysis of three HCV/GBV-C positive plasma samples and a HCV/GBV-C positive control beside some HCV positive but GBV-C negative plasma samples.

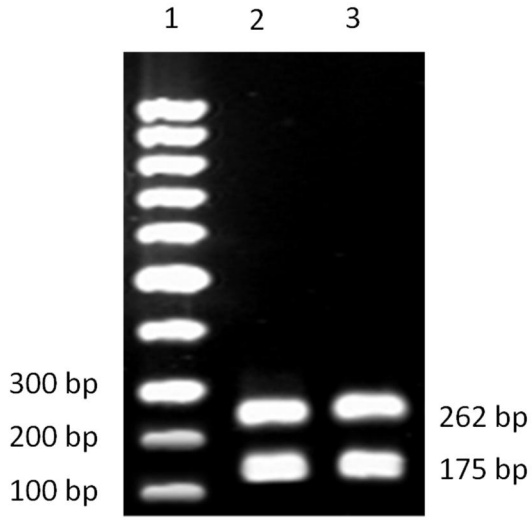

Figure 3 Agarose gel electrophoresis analysis of HCV/GBV-C co-amplified samples after SYBR Green real time multiplex RT-PCR. Lane 1 represents molecular markers. Lane 2and 3 demonstrate a co-amplification of two HCV/GBV-C positive samples. The HCV specific amplicon is at 175 pbs, whereas the GBV-C specific amplicon is at 262 bps.

Discussion

Present report depicts the progress of development a SYBR Green real time multiplex RT-PCR for the simultaneous detection of HCV and GBV-C genomes in plasma samples. The multiplex RT-PCR was performed using random hexamer in order to retrotranscribe HCV and GBV-C RNA genomes into cDNA. 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 (c) 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 Tm of HCV and GBV-C melting curves which were 88.3 ºC and 91.7 ºC, 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

therefore elevating the speed, obviation of post amplification electrophoresis, high sensitivity, 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 is more expensive and paraphrasing the results could be much complicated [Liu and Zhang, 2008; Martinez et al., 2008]. Albeit, SYBR Green real time multiplex 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 100 IU/ml for HCV and 200 IU/ml for GBV-C.

After development and optimization of SYBR Green real time multiplex RT-PCR assay, we applied this technique on 56 non cirrhotic HCV positive patients' samples [29 of genotype 3a and 27 of genotype 1a] which 20.6% of genotype 3a, 18.7% of genotype 1a and 19.6% of overall samples had HCV/GBV-C co-infection. We applied this technique on samples 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.

In conclusion, we described a SYBR Green real time multiplex 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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