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A Novel and Simple Method for HCV Genotyping

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ABSTRACT

Hepatitis C virus (HCV) is a major global health problem and one of the most common causes of chronic liver diseases. The current study was carried out to investigate the ability of High Resolution Melting (HRM) method in HCV genotyping. 40 HCV-Positive sera with unknown and 12 sera with known HCV genotypes were collected from different clinical laboratories in Tehran as well as Rasht in north of Iran. The RT-PCR was performed on RNA extracted from each sample. The first PCR amplification product was used as template for second run in a Nested-PCR followed by HRM method. Unknown genotypes were determined with comparing of their normalized and difference graphs with those obtained by known genotypes as standard. All results were confirmed by direct sequencing. In a comparison between results obtained by HRM method and those of direct sequencing, only 2 out of 40 samples with unclear HCV genotypes had been incorrectly genotyped by HRM method. The results obtained by this study show that HRM can be introduced as a simple, fast, and reliable method for HCV genotyping in clinical laboratories.

1. Introduction

Hepatitis C Virus (HCV) infection is a major health burden with about 170-200 millions infected people all around the world (Hariss et al., 2001). It is estimated that 5-20% of patients with HCV infection will develop to cirrhosis and 1-4% to hepatocellular carcinoma, annually (Yen et al., 2003). HCV isolates are classified into six genotypes and more than 70 subtypes based on their sequences (Okamoto et al. 1992; Ross *et al*. 2000; Davarpanah et al., 2009). It has been shown that different genotypes of HCV represent different level of response to antiviral drugs. For instance, genotypes 1 and 4 show a lower level of response to interferon in compare to genotype 2 and 3 (Alavian et al., 2002; Omrani et al., 2009). Therefore, genotyping of HCV could be considered as an important procedure in antiviral therapy. HCV genotyping is also important regarding determination of the severity and progression rate of liver infection (Davis et al., 1997).

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Several methods have been developed for HCV genotyping including Restriction Fragment Length Polymorphism, Type-Specific PCR, direct DNA sequencing, primer-specific and Mispair extension, Line Probe Assays, Heteroduplex Mobility Analysis by Temperature Gradient Capillary Electrophoresis, and Denaturing High-Performance Liquid Chromatography (Halfon et al., 2001). High Resolution Melting (HRM) analysis is a highly sensitive method to determine SNP genotypes (Smith et al., 2009). It is now becoming the method of choice for scanning large numbers of samples for genetic variants, and is widely used in clinical studies. In this method, the nucleotide polymorphism in PCR amplicons are distinguishable based on the melting curves created by detecting fluorescence produced by a saturating dye (Reed et al., 2007).

2. Material and methods

2.1. Sample Collection

Sera from 40 HCV-positive patients with unknown and 12 patients with known HCV genotypes were collected from Keyvan Virology laboratory in Tehran and Ashtiani Pathology laboratory in Rasht, a city in north of Iran, during years 2009-2010. The genotype determination performed by COBAS commercial kit (Roche). 200 µl of serum was used for viral RNA extraction using a High Pure Viral RNA kit (Roche, Germany) according to manufacturer instructions.

2.2. RT-PCR

RT-PCR was performed in a volume of 50 µl containing 10 µl RT Buffer (5X), 4 mM of dNTPs mixture, 4 mM of MgCl2, 20 units of RNasin (Qiagen, USA), 20 pmole of forward and reverse primers (F: 5'-GCAGAAAGCGTCTAGCCATGGCGT-3' and R: 5'-CTCGCAAGCACCCTATCAGGCAGT-3'), 200 units of RT enzyme (Fermentas, Rusia) and 15µl of extracted RNA. The thermal cycler condition included 42 ^{o}C for 60 minutes, followed by 20 cycles of 95 ^{o}C for 40 seconds, 60 ^{o}C for 40 seconds, and 72 ^{o}C for 40 seconds. Amplified product of this reaction was a 244 bp fragment located at 5'-UTR region of HCV genome.

2.3. Nested PCR and HRM

To amplify a 150 bp fragment, a nested-PCR was performed by Rotor-Gene 6000^{TM} system at a volume of 25 µl containing 4 µl of RT-PCR product, 2.5 µl of 10x Buffer, 1 mM of MgCl2, 10 pmole of internal primer (F: 5'-TGCGGACCGGTGAGTACAC-3' and R: 5'-CGACCCAACACTACTCGGCTAG-3') 2 mM of dNTPs mixture, 10 µmole of SYTO9 solution (invitrogen, USA), and 2.5 units of Hot start Taq DNA polymerase enzyme (Fermentas, Rusia). The thermal cycler condition involved primary denaturation at 95 ^{o}C for 2 minutes, followed by 40 cycles of 95 ^{o}C for 20 seconds, 60 ^{o}C for 20 seconds, and 72 ^{o}C for 20 seconds. The nested-PCR was followed by HRM analysis based on a 0.1 $^{\circ}$C/s thermal shift from 75-90 ^{o}C.

HRM analysis was performed by Normalized and difference graphs generated by Rotor-Gene 6000 v1.7 software. In difference graphs, any line with a gradient more than four units (+4U) in compare to baseline was considered as a different genotype. To verify the results obtained by HRM technique, all samples were sent to Macrogen Company (South Korea) to be genotyped by DNA sequencing.

3. Results

Figure 1 is showing normalized graphs for 3a, 1a, and 1b genotypes. The difference graphs based on 3a, 1a, and 1b are also shown in Figures 2, 3, and 4, respectively. The comparison between results obtained for sera with known and unknown HCV genotypes examined by HRM showed that of 40 samples with unknown genotypes, 14 could be considered as belonging to 3a, 19 to 1a, and 7 to 1b genotypes. However, based on direct DNA sequencing of PCR amplified fragments, 15 samples were belonging to 3a, 17 to 1a, and 8 to 1b genotypes. One 3a and one 1b genotypes had incorrectly been genotyped as 1a by HRM.

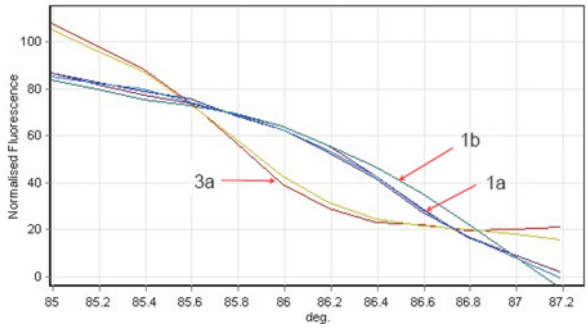

Figure 1. Normalized graph for 3a, 1a, and 1b genotypes. Red and yellow lines show 3a genotype, blue and violet lines show 1a genotype, and Green lines show 1b genotype.

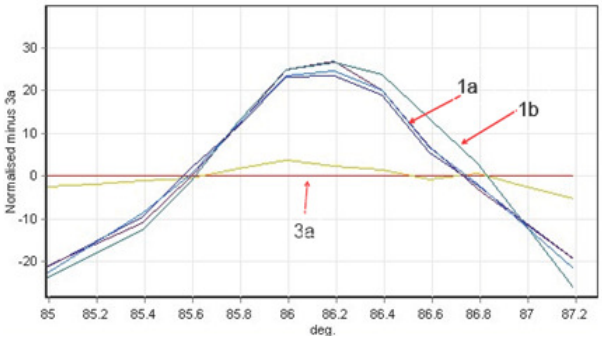

Figure 2. Difference graphs generated based on 3a genotype as baseline. Green and blue/violet lines are detected as 1b and 1a genotypes, respectively, since they are showing a gradient more than 4 units (+4U) over baseline.

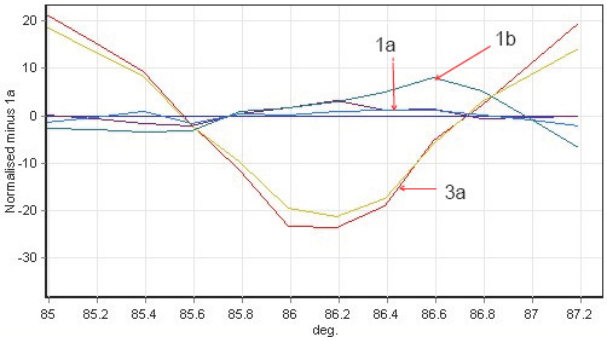

Figure 3. Difference graph generated based on 1a genotype as baseline. Green and yellow/red lines are showing a gradient more than 4 units (+4U) over baseline, and are detected as 1b and 3a genotypes, respectively.

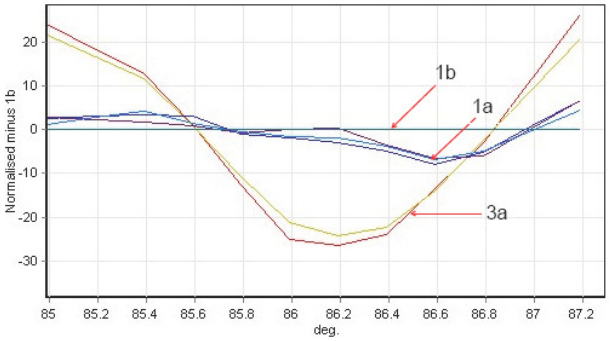

Figure 4. Difference graph generated based on 1a genotype as baseline. Blue/violet and yellow/red lines are detected as 1a, 3a genotypes, respectively, as they show a gradient more than 4 units (+4U) over baseline.

4. Discussion

According to the World Health Organization (WHO) report, the worldwide prevalence of HCV infection is about 3%. Nevertheless, its prevalence in Iran is estimated to be less than 1%, which is much lower than that of most neighboring countries in this region (Alavian et al., 2002).

Considerable nucleotide changes are incorporated throughout HCV genome. In early 1990s, the heterogeneity of HCV genome was used to identify various genotypes of the virus. Subsequently, HCV was classified in different parts of the world as laboratory tests for genotyping were developed (Ross et al., 2000; Omrani et al., 2009). The level of heterogeneity differs considerably among different regions of the HCV genome. The sequence variation ranges from as little as 10% in the 5' Untranslated region (5'-UTR) to as much as 50% or even more within the E1 region (Chan et al., 1992; Bukh et al., 1993; Ahmadi Pour et al., 2006). Since 5'-UTR region of HCV genome is highly conserved, it is more used for diagnosis of HCV infection, while NS5B and core, envelope regions are useful for subtyping due to their highly variable nature (Prescott et al., 1997). HCV is divided into some major groups, or genotypes, with about 66-69% nucleotide similarity, while each genotype can be further divided into some subtypes with about 77-80% nucleotide similarity (Bukh et al., 1992; Ahmadi Pour et al., 2006). By now, at least 6 main genotypes of HCV, each consisting of several subtypes, have been described.

HCV genotypes and subtypes are different in terms of geographical distribution and *antigenicity* (Okamoto *et al*. 1992; Davarpanah et al., 2009). Genotypes 1, 2 and 3 have a universal distribution (Cha et al., 1992; Bukh et al., 1993; Davarpanah et al., 2009) while genotypes 4, 5, and 6 are distributed in separated areas of the world (Takada et al., 1992; Cha et al., 1992; Davarpanah et al., 2009). In addition to different geographical distribution and distinct nucleotide sequences, some evidences have been found regarding the biological differences between HCV genotypes. It has been shown that patients infected by HCV subtype 1b poorly respond to Alfa interferon therapy (Kanai et al., 1992; EASL 1999a; Davarpanah et al., 2009). Also, patients infected by genotypes 1 and 4, with viral load more than 800,000 IU/ml, need to be treated for a longer period of time in compare to those infected by other genotypes (McOmish *et al*. 1993; Zylberberg et al., 2000; Davarpanah et al., 2009). As a result, HCV genotyping and viral load determination in different hosts seem to be necessary for prediction of level of response to the drug and duration of treatment (Davis et al., 1997; McHutchison, et al., 1998; Poynard et al., 1998; Zylberberg et al., 2000; Anonymous, 2002). HCV genotyping is also essential for epidemiological estimates (Pawlotsky et al., 1995; Bourliere et al., 2002; Martial et al., 2004) and defining the source of HCV infection (Le Pogam et al., 1998; Norder et al., 1998; Izopet et al., 1999; Ackerman et al., 2000; Halfon et al., 2001).

Although direct DNA sequencing is the golden standard for HCV genotyping, however, it is a difficult to do, rather expensive, and time consuming method. In contrast, High Resolution Melting (HRM) is a simple, rapid, and low-cost Method (Ross et al., 2000; Hariss et al., 2001; Alavian et al., 2002; Moghaddam et al., 2006). As an advantage of HRM, PCR amplification and melting curves analysis are both performed within the same tube, without any need for post-PCR processing. Nevertheless, there are some drawbacks with this method. To genotype any unknown sample, it is required to include all typical known genotypes as standards in every reaction. Furthermore, the sensitivity of HRM analysis can be affected by lenghth of amplicon. Melting profiles created by shorter amplicons have less complexity than longer ones (Applied Biosystems 2009). So far, HRM method has been utilized for detection of nucleotide polymorphisms in both diploid and haploid organisms, such as Beta-globin, CFTR, factor V, and prothrombin genes in human beings, and mycobacterial typing using *hsp65*, bacterial speciation using *16s rRNA* gene, and identifying *gyrA* mutations in microorganisms (Reed et al., 2007). Here, for the first time, HRM is going to

be introduced as a method of choice for HCV genotyping as well. The results of current study show that the accuracy of HRM technique in HCV genotyping is 95%. The inaccuracy of the method in some cases seems to be more related to low viral loads in samples. It is likely that a higher level of accuracy would be achievable by using a higher amount of template RNA and optimizing some other parameters in the method.

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