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Development of 1a and 3a HCV genotyping based on 5'-UTR region by hybridization probe Real-time-PCR

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ABSTRACT

HCV with millions of patients is one of the leading causes of liver cell cancer and liver cirrhosis. Based on the nucleotide differences, HCV has several types that play an important role in response to treatment. this study aims to develop a fast and accurate way to identify the most common genotypes of this virus in Iran based on the 5'-UTR region using probe hybridization method. In this study, 45 positive serum samples with a Titer of more than 1000U/mL, all of which were infected with genotypes 1a and 3a, were prepared from golestan hospital. After designing and synthesizing the primers and probes, the system hybridization of the probe and the extraction of viral RNA with High Pure RNA Viral Extraction kit (Roche, Germany) the reaction optimization was performed. The results of the resulting genotyping were then compared with the initial results of the samples. The results obtained on the device showed that genotypes 1a and 3a have different melting point peaks with a difference of about 2 degrees. Also, the results of the initial genotyping, which was based on the sequence determination of the 5'-UTR region, are consistent with the results obtained in this study. Although the hybridization method of the probe developed in this study is not perfect and is not able to identify 7 types of HCV, but despite the high frequency of 1a and 3a Genotypes in Iran (about 90%) and the speed of reaction and its single-stage can be Use it for initial screening.

1. Introduction

Hepatitis C virus is a single-stranded genomic virus that can cause both acute and chronic infection with a range of symptoms from severe to mild (Choo et al., 1991, Brown et al., 1992). In infected people, this virus can cause liver cirrhosis and hepatocellular carcinoma (Hasan et al., 1990; Poynard et al., 1997; Lee et al., 1999). More than 5% (about 500,000 people) of the Iranian population are infected to HCV and according to the higher prevalence of this infection in neighboring countries (so Iraq and Afghanistan), the risk of HCV infection in Iran is high (Mahmud et al., 2018). Early detection

and timely treatment can infect fewer fractions of the liver cells to HCV, which can reduce the likelihood of subsequent consequences of a viral infection (Choo et al., 1991).

So as that get proper treatment and choosing the drug type and dose and treatment period, it is important know the viral load and genotype. About 85% of hepatitis C infections in Iran are 1 and 3a genotypes (Lee et al., 1999). Several molecular methods have been developed to identify the HCV genotype (Nakao et al., 1999; Mcomish et al., 1993) based on the 5'-UTR and NS5B (Bukh et al., 1992; Norder et al., 1993)

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region including the PCR Sequencing (Lai et al., 1998), PCR-RFLP (Okamoto et al., 1992), Reverse Line Blot (Hawkins et al., 1993), ARMS-PCR (Beld et al., 2002), Probe Array (Liu et al., 1993) and so etc.

This study aims was to develop a rapid method for the identification of 1 and 3a HCV genotypes based on 5'-UTR region by the probe hybridization Real-Time PCR technique.

2. Materials and Methods

2.1. Collection of plasma samples

A total of 45 samples of hepatitis C plasma were collected from January to September 2018 from Golestan hospital in Tehran with the permission of the Medical Ethics Committee with the code IR.IAU.TON.REC.1399.005. All samples have a viral load of more than 1000 IU / ml and genotypes 1a and 3a were genotyped in this center using 5'-UTR PCR-Sequencing method.

2.2. Design of primers and Hybridization Probes

Primers and probes for Real-time PCR in this study were designed based on the 5'-UTR sequence of the hepatitis C genome (GeneBank, NC_004102, NC_009823, GQ275355, KJ470615, JF735126, FJ024281, AF290978, JX463616, AB426117, AB435162, KJ439767,

KJ439780, NC_009825, JX227964, NC_009826, NC_009827) and using Primer Express Software (Applied Biosystems) and synthesized by Metabion Inc (Germany). It should be noted that all primers and probes used in the research were designed by us. The primer sequences and probes are shown in Table 1. Also, the nucleotide differences of the Detector probe binding site in the different genotypes are shown in Table 2.

2.3. Hybridization Probe Real-time PCR Assay

Total RNA of the positive HCV plasma was extracted using the High Pure RNA Viral Extraction kit (Roche, Germany) according to the manufacturer's instructions. The extracted RNA after heat treatment at 65 °C immediately put into ice for 5 minutes and then added to the Real-Time PCR reaction mixture. The reaction mixture was included 2X Hot Master Mix (Amplicon, Denmark) 1X, M-MuLV Reverse transcriptase 200 U (Promega, USA), RNasin 20U (Promega, USA), HG1 Primer 20 pmol, HG2 Primer 40 pmol, Detector Probe 10 pmol, Anchor Probe 10 pmol and 10 µl extracted RNA. The reaction was performed in an Onestep RT-Real-Time PCR application according to the thermal conditions listed in Table 3 in the Rotor-Gene Q device (Giagen, USA).

Table1. Primers and Probes sequence of Hybridization RT-PCR Technique.

Primer Name	Sequence	Position region 5-UTR
HG1	GGCGACACTCCACCATAGATC	17-38
HG2	GGTGACCGGTCTACGAGACCT	321-341
P1(Detector Probe)	5- CTCAATACCCAGAAATTTG-FAM	211-229
P2 (Anchor probe)	5-Cy5-CGTGCCCGCAGA-Phosphate	232-246

Table2. Nucleotides different Detector Probe in 1 and 3a HCV genotypes in binding site.

HCV Genotype	Detector Probe Binding Site Sequence	Position in 5-UTR
1	5- CTCAATGCCTGGAGATTTG-3	211-229
3	5- CTCAATACCCAGAAATTTG-3	

Table3. Temperature program and reaction cycles Onestep RT-Real-Time PCR.

Steps	Temperature (°C)	Time	Cycles
Incubation	42°C	30min	-
Initial denaturation	95°C	15min	-
Cycles	95°C	20sec	40
	55°C	30sec	40
	72°C	30sec	40
denaturation	95°C	2min	-
hybridization step	35°C	2min	-
Melting	40 °C to 85 °C	5sec for each step	1

3. Results

Onestep RT Real-Time PCR based on the Hybridization probe for HCV genotyping was successfully performed on the RG-6000 device (Corbett Research Inc) (figure 1). to evaluate the accuracy and effectiveness of this method, we used 45 positive HCV plasma samples that were divided into two groups of 1a and 3a genotypes. Our results showed a 100% concordance between the probe hybridization method and those determined by the PCR Sequencing method. The values of T_m obtained for the two

genotypes 1a and 3a are shown in (Table 4), and the difference of T_m between these two genotypes is 7°C. This difference of T_m shows that these two genotypes can be measured by measuring their T_m from also recognized. we also found that when the virus load in clinical specimens is less than 500 IU/ml, (Three clinical samples with a viral load of 140, 210, and 380 IU/ml respectively were examined. The results are not mentioned) Hybridization probe efficiency is reduced and genotyping is not possible. These results indicate that only samples with a viral load greater than 500 IU / ml can be genotyped by this method.

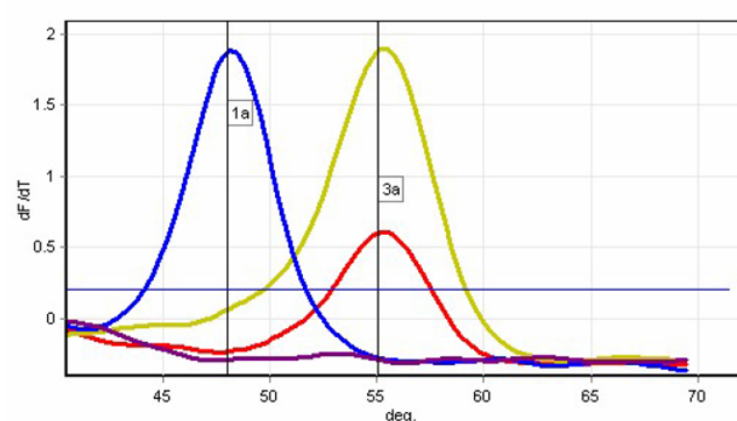


Figure 1: 1a and 3a HCV genotyping by Hybridization Probe and Melting Curve Analysis. 1 genotype lose fluorescent signal at a lower temperature ($T_m=48^\circ\text{C}$), while 3a genotype released fluorescent signal at ($T_m= 55^\circ\text{C}$); -d, negative derivative fluorescent; dt, derivative concerning time.

Table4. T_m values in genotypes 1a and 3a

No.	Colour	Name	Genotype	Peak 1
1	Red	unkown sample	3a	55.3 (3a)
2	Yellow	3a genotype	3a	55.3 (3a)
3	Blue	1a genotype	1a	48.2 (1a)
4	Purple	ntc		

4. Discussion

Determining of HCV genotype is clinically important in the treatment plan for this infection. The high variability and RNA HCV genome cause that HCV genotyping is a difficult process. So far, several commercial and homemade methods have been developed for this purpose that each one has disadvantages in terms of repeatability, time, and price. On the other hand, many researchers have worked on determining the genotype of this virus. For example, in 2010, Murad Abdolvahab and his colleagues examined the genotypes of the hepatitis C virus in 95 anti-HCV positive samples using RT-PCR method. Finally, they were able to determine the genotype in 77 samples successfully and 17 samples were not genotyped by the studied method (Moradi et al., 2010). In another study conducted by Katayoun Vahdat and his colleague in 2007 to determine the genotype of hepatitis C virus using nested-RT PCR method and PCR-RFLP was performed on 69 patients, of which 60 were HCV positive and their predominant genotypes were 1a (7.36%) and 3a (3.38%) and the remaining 85.36% could not determine the genotype (Vahdat et al., 2013). In another study conducted by Farah Bukharaei Salim et al. In 2011, they examined the presence of different genotypes of hepatitis C virus in the serum of 152 patients with chronic hepatitis C infection using 5'-UTR PCR-Sequencing method and the results. They showed that a significant proportion of patients infected with the hepatitis C virus had different genotypes. Infected with the virus, which may not be detectable in patient's plasma (Bokharai et al., 2012). Muhammad Ammar Athar and colleagues in 2015 performed real-time PCR for rapid detection of HCV genotypes 1a, 1b, 2a, 3a, 3b, and 6a. The results of their work show that using a system designed based on the 5'-UTR region, they can detect 6 types of HCV virus well (Athar et al., 2015).

Various studies have shown that probes and primers that are appropriately designed for the 5'-UTR conserved region of the HCV virus are highly specific and are able to identify HCV genotypes but from different regions of the virus genome (Elkady et al., 2010). HCVs such as E1, NS4, NS5, and core are also used to determine the genotype of the virus (Laperche et al., 2005),

but among these regions, the 5'-UTR region is the most widely used (Ansari et al., 2012). On the other hand, the 5'-UTR protected region lacks sequence variability, and this feature sometimes causes errors in genotype determination and determines inappropriate genotype (Lole et al., 2003). In our study, primers and probes designed to detect genotypes 1a and 3a were designed based on the 5'-UTR protected area. In the initial bioinformatics studies, the selected site, 211-229, had appropriate nucleotide differences (in 4 nucleotides) between these two genotypes. This resulted in a difference of more than 2°C at the melting point of the 1 and 3a genotype probes. Although the hybridization probe method developed in this study have disadvantages such as the inability of 1 genotype subtyping (1b and 1c), incompatibility with all Real-time devices and inefficiency in low virus load (< 500 IU/ml), but despite the speed, accuracy and ease of HCV genotyping it can be used as a new method in molecular biology laboratories. despite the high frequency of genotypes 1a and 3a in Iran (about 90%), this method can be used for primary screening. If the studied samples were not one of the genotypes 1a and 3a, used other methods to determine the genotype.

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