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ORIGINAL ARTICLE

Hepatitis C Virus Genotyping by Melting Curve Analysis in West Azerbaijan, Northwest of Iran

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Background and Aims: Hepatitis C virus (HCV) has contaminated almost 170 million people worldwide. The HCV genotype is a good predictor of response to antiviral therapies and prognosis. In this study, we tried by using real-time polymerase chain reaction (RT-PCR) and melting curve analysis, to identify the HCV genotype isolated from affected patients.

Methods: Using PCR and enzyme-linked immunosorbent assay (ELISA), serum sample of 310 suspicious patients with HCV were screened. Applying QIAamp Viral RNA Mini-kit, the viral RNA was collected from serum samples of 160 positive patients. Using one-step RT-PCR protocol, genotypes of the isolated HCV were specified according to the temperature melting (Tm) ranges.

Results: The frequency distribution of the 5 detected HCV genotypes (*i.e.*, 1a/b, 2a/c, 4, 2b, and 3a) in 160 cases, were as follows: 77 (48.12%) were genotype 3a; 35(21.87%) had genotype 2b; 19 (11.87%) were 2a/c; 16 (10.0%) were 4; and 13 (8.12%) had genotype 1a/b.

Conclusions: The most common HCV genotype in West Azerbaijan province in the Northwest of Iran is type 3a. We therefore concluded that it is possible to used PCR for routine HCV genotyping.

Keywords: Hepatitis C Virus, Genotype, Melting Curve Analysis, Real Time PCR

Introduction

The Centers for Disease Control and Prevention (CDC) estimates that in the US alone, approximately four million people and 170 million people worldwide are chronically infected with hepatitis C virus (HCV). In the US, 30,000 acute new infections occur each year and 10,000 people die annually of sequels of this infection [1-3]. Chronic HCV infection is the most prevalent disease leading to liver transplant in the US [4]. Taking into account the universal precautions and blood screening for HCV have significantly decreased the incidence of HCV infection in the US. Nonetheless, the annual mortality due to HCV-induced hepatic diseases is expected to triple in the next one or two decades [1].

HCV is a single-stranded RNA virus of the *Flaviviridae* family that causes both acute and chronic hepatitis [5]. Like the genomes of other single-stranded RNA viruses, the HCV genome is vulnerable to high rates of mutational change [6].

In early 1990s, the heterogeneity of the HCV genome was described. Subsequently, to identify various genotypes of the virus, laboratory tests for genotyping were developed, leading to various classifications in different parts of the world [7]. At least 11 genotypes and 70 subtypes were reported worldwide [1]. The entire viral genome differs by as much as 33% from genotype to genotype; within

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subtypes, the sequence has only 75%-86% similarity [2]. The most common HCV genotypes in the US are 1a, 1b, 2a/c, 2b, and 3a [3]. In North Africa, genotype 4; in the Middle East, genotype 5; and in the South Africa and Hong Kong, genotype 6 are the most common genotypes. Genotypes 7, 8, and 9 have been isolated only in Vietnamese patients, and genotypes 10 and 11 have been identified in patients from Indonesia [1, 3].

HCV genotyping provides clinically important information that can guide us in selection of the duration and type of antiviral therapy and to predict the likelihood of sustained HCV clearance after therapy [4]. Those infected with HCV genotype 1 may benefit from a longer course of therapy and patients with genotypes 2 and 3 HCV infection are more likely to respond to interferon-ribavirin combination therapy [7, 8].

The gold standard method for HCV genotyping is nucleotide sequencing followed by phylogenetic analysis. However, this method is time consuming and expensive [9]. Real time polymerase chain reaction (RT-PCR) has become increasingly employed in the diagnostic laboratories and has been utilized for HCV genotyping [1, 9, 10].

The available data on HCV genotypes in Iran are very heterogeneous and limited because of the number of cases studied or the method restriction fragment lengths polymorphism (RFLP-PCR) they applied for genotyping [11-13]. Therefore, in this study, we tried to identify the HCV genotypes by using melting curve analysis.

Materials and Methods

From 2006 to 2008, in a cross-sectional study, we studied 310 patients referred to a medical laboratory in the West Azerbaijan province, northwestern Iran, for HCV antibodies and RNA. From each patient 5 mL whole blood was obtained. Serum was separated using bench type centrifugation method and frozen at -40 °C till examined.

Of the 310 examined patients whose blood samples were tested with both enzyme immunoassay (EIA) and PCR, 128 (41.3%) were found negative. Among the 182 remaining patients who had positive EIA, 22 were found negative in PCR. Therefore, serum of the 160 patients that were positive by both PCR and EIA method were kept for genotyping and amplification according to the method described previously [14].

For purification of viral RNA from the collected serum samples, 140 µL of sera were used according to the manufacture instruction (QIAamp Viral RNA Mini-kit, QIAGEN, USA). To enhance binding of viral nucleic acids to the QIAamp Mini membrane, especially if there were very few target molecules in the sample, carrier RNA was also added to the reaction, as recommended. The addition of large amounts of carrier RNA reduces the chance of viral RNA degradation in the rare event that RNase molecules escape denaturation by the chaotropic salts and detergent in buffer. If carrier RNA is not added to buffer, this may lead to reduced viral RNA recovery. Amplified PCR product (225 bp) run on 2% agarose gel. The PCR program used is summarized in table 1.

Table 1. PCR thermal cycling program used for amplification of HCV-RNA.

Segment name	Temperature (°C)	Incubation period	No of cycles
Reverse transcription	50	30 min	1
Taq activation	95	15 min	1
Denaturation	94	30 sec	45
Annealing	57	15 sec	
Elongation	72	30 sec	
Post-elongation	72	10 min	1

Hepatitis C genotype determination by melting curve analysis with a single set of fluorescence resonance energy transfer probes was performed using two primers with complementary sequences located in the 5'-UTR that are conserved among known HCV genotypes [1]. Primers and probes (FITC and Red640) were purchased from TIB MOLBIOL Company (Berlin, Germany) and used in the process as recommended by the manufacturer. Probe preparation procedure is summarized in table 2.

Table 2. Preparation of probes for use in the genotype reaction process. In each step, 8 reactions were prepared.

H_2O:	12.6 µL × 8	100.8 µL
10 X Buffer:	2 µL × 8	16 µL
$MgCl_2$:	2 µL × 8	16 µL
RED640:	0.2 µL × 8	1.6 µL
FITC:	0.2 µL × 8	1.6 µL
PCR Product: 3 µL		
Total: 136 µL		
Divide: 17 µL		

3 µL of our quantative PCR product with a concentration of 20,000 IU/mL HCV or higher was mixed with 17 µL of the prepared probes and left in

the Rotor-GeneTM 3000 PCR machine and programmed for melting analysis for HCV genotyping with the following program. A hold temperature of 95°C for two minutes; a ramp of 45-75 degree; then raising by one degree in each step; wait for 60 sec in the first step and then in each step for six sec. The produced melting curve was interpreted based on the melting point results (Fig. 1). HCV genotypes were specified and determined according to the melting temperature (Tm) ranges [1].

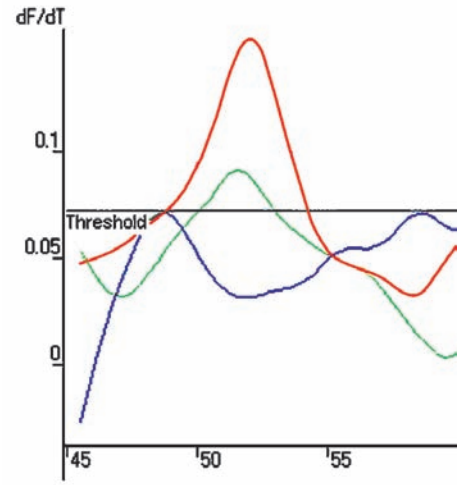

Figure 1. A representative genotyping experiment using real-time Rotor-GeneTM 3000 PCR machine. Melting curve analysis was generated using Rotor-Gene Real-Time Analysis Software 6.0. Patient sample was genotyped using 2 mM $MgCl_2$. Water blank was included to control for reagent contamination.

Results

Performing this study allowed us to determine for the first time, the HCV genotypes of affected patient with hepatitis C in the west Azerbaijan province located in the northwest of Iran. HCV genotypes of the 160 serum samples were as follows: in 77 (48.12%) it was genotype 3a; in 35 (21.9%) it was 2b; in 19 (11.9%) the genotype was 2a/c; in 16 (10 %) it was 4; and in 13 (8.1%) the genotype was 1a/b.

Discussion

Since the severity of the HCV infection, its progression and response to therapy may vary according to the virus genotype, we have witnessed increasing interest for determining HCV genotype worldwide [15]. Treatment for hepatitis C consists of the administration of pegylated or non-pegylated interferon-α (IFN-α) in combination with ribavirin. The duration of treatment for each patient is determined according to the type of virus [3-5].

Various studies have shown that there are substantial regional differences in the distribution of HCV genotypes [16]. Therefore, it is very important to determine the predominant and less prevalent subtypes of this virus in different geographic regions. Therefore, knowledge on the distribution of various genotypes in our country is critical for its prognostic implications on chronic hepatitis C infection. In the present study, the frequency of genotypes of HCV among patients infected with virus for the first time in the West Azerbaijan province was reported.

Based on the findings of this study, the frequency of the observed HCV genotypes in descending order was 3a (48.1%), 2b (21.9%), 2a/c (11.9%), 4 (10.0%), 1a/b (8.1%). Like Pakistan, the subtype 3a is the most frequent HCV genotype (49.05%) observed, though other genotypes had totally different distributions; subtypes 2b and 4 had frequencies of 0.8% and 1.49%, respectively, in Pakistan [15].

In the reported genotypes among Saudi and Egyptian population, this difference is more obvious. In both nationalities no case with 3a genotype was reported. Instead, genotype 4 with frequencies of 45.9% in Saudi and 91.6% in Egyptian people, respectively, was responsible for HCV infection in these countries [16].

In another study from India, genotype 3a was responsible for 13.8%, 3b for 5.5% and 4 for 2.7% of infections. In 47.2% of patients, both 3a and 3b genotypes were isolated [17].

In a report from Canada, the most common HCV genotype was 1, including 1a and 1b (61.1%). The frequency of other genotypes were 3 (15.3%); 2b (7.6%); 2a/c (8.3%); 4(1.9 %); and 1a/b (0.6%) [18].

A report from our country by Kabir *et al.* in 2006, mentioned that the most frequent HCV subtype was 1a (37.8%), followed by 3a (28.9%) and 1b (16.7%) [11]. At this stage, it is hard to state whether the observed difference between our result and them is due to geographical or ethnical difference of the studied patients or due to the different methods used for HCV genotyping. Kabir *et al.* used third-generation commercially available enzyme-linked immunosorbent assay (ELISA) kits for the diagnosis of chronic hepatitis C while we used both EIA and PCR.

In another report from Iran by Hejazi *et al.* subtype 1a had the highest frequency of 71.4%; it

followed by 1b (14.2%) and 2a (7.1%) [12]. The major limitation of this study was firstly, the low sample size (28 patients), and secondly, their indirect method for genotyping-amplification refractory mutation system (ARMS-PCR).

Study conducted by Kazemi *et al.* on 20 patients using RFLP-PCR, showed that 55% of their patients were of type 1, 20% had type 3a and 25% were non-typable [13].

Like the previous one, this report had also limitations; firstly, they studied only 20 patients, and secondly, they used RFLP which is an indirect method by using only two enzymes.

To reach a consensus results about HCV types and subtypes in Iranian population, more studies in various regions of Iran are needed; our study can be assumed as a part of this project in the future.

In conclusion, performing this study allowed us to determine the frequency of the HCV genotype in west Azerbaijan province. This paper should help gastroenterologists in choosing the right protocol and course of therapy in their patients.

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