Genotyping of Hepatitis C Virus in Northwest of Iran

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Abstract: A study was conducted on patients with Hepatitis C virus (HCV) infection, to identify HCV genotypes in Northwest Iran. Sera from 50 patients with HCV infections were selected to study the HCV genotype in Northwest of Iran. We employed an improved and simplified method of genotyping based on PCR with genotype-specific primers deduced from the core gene. HCV RNA was detected in 28 cases by RT-PCR. Of 28 HCV isolates, 20 (71.4%) were typed as genotype 1a, 4 isolates (14.2%) 1b, 2 cases (7.1%) 2a and 2 HCV sera were not typed by this assay. Genotype 1 was detected in a significant majority of HCV infected patients in this area.

Key words: Hepatitis C virus, genotyping, polymerase chain reaction

INTRODUCTION

It is well known that HCV infection is the major etiological agent of post-transfusion hepatitis type Non-A, Non-B (Spada et al., 1998). HCV infection frequently progresses to chronic liver disease and HCV causes 20% of acute hepatitis cases, 70% of all chronic hepatitis cases, 40% of all cases of liver cirrhosis, 60% of hepatocellular carcinomas and 30% of liver transplants in Europe (Alter et al., 1999; Corbet et al., 2003). HCV infection is found in 0.5 to 8.0% of blood donor's worldwide (Mondelli and Silini, 1999). HCV continues to be a major disease burden on the world. In 1999, WHO estimated a worldwide prevalence of about 3% with the virus affecting 170 million people worldwide. (WHO, 1999). The limited available data indicate that the general population of Iran has a low prevalence of HCV infection ranging from 0.12 to 0.5% (Ghavanini and Sabri, 2000; Alavian et al., 2002, 2005). But the infection is emerging mostly because of various problems such as intravenous drug abusers and needle sharing among drug addicts. HCV infection is the most prevalent cause of chronic hepatitis and cirrhosis in hemophiliacs and thalassemic patients and also patients with renal failure in Iran (Alavian et al., 2002, 2005; Kabir et al., 2006). HCV is a single-stranded RNA virus containing linear genome with a length of about 9,600 nucleotides with positive polarity (Kazemi et al., 2004; Simmonds et al., 2001). The genome of HCV is extremely heterogeneous (Sandres-Saune et al., 2003). HCV isolates show four levels of genetic variation: types, subtypes, isolates and quasi species. Up to date, 6 major genotypes including more than 90 subtypes have been identified (Shobokshi et al., 2003). All HCV isolates are grouped to phylogenetically related clusters called subtypes. Subtypes can be classified into several major types that show sequence similarities of 65-75% of the total genome (Haushofer et al., 2003). These genotypes can differ up to 30% from each other in nucleotide sequence (Theodore and Jamal, 2006). The types have been numbered 1 to 6 and the subtypes are identified as a, b and c, in both cases in order of discovery (Roberson et al., 1998). HCV genotypes show a distinct geographical distribution. Genotypes 1a, 1b and 2a are the predominant genotypes in the United States and Western Europe. Genotype 4 is the predominant genotype of Middle East. Types 5 and 6 are largely confined to South Africa and South East Asia, respectively (Raghraman et al., 2003). Determining the HCV genotype is also useful epidemiologically. It enables new subtypes to be identified, the investigation of unconventional or new transmission routes and the pinpointing of nosocomial transmission (Hosseini-Moghadam et al., 2006). Depending on the HCV genotype, length of treatment can differ (Theodore and Jamal, 2006). HCV genotype 1, in particular 1b, is associated with more severe clinical manifestations, higher levels of viremia and less amenable to treatment such as Alpha interferon or Peg-interferon-alpha/ribavirin therapy. These findings indicate an important role of genotype identification for prediction of HCV infection outcome and the selection of patients for treatment (White et al., 2000; Chen and Week, 2002;
Therefore, significant efforts are being devoted to exploring the heterogeneity of HCV worldwide to obtain information on the prevalence of known genotypes. Although, the distribution of HCV genotypes in many countries is well documented, reliable data are still missing with respect to the frequency of the different HCV genotypes in different areas of Iran. We therefore conducted a study on patients with HCV infection, to identify HCV genotypes in Northwest Iran. Accurate knowledge of HCV genotypes in our community is essential for successful future research into vaccine development and control strategy. Such information is needed to correctly formulate health care policies, prioritize interventions and allocate resources. This is the first prospective study, in which samples of HCV are classified according to their genotypes and subtypes in Northwest Iran, based on PCR technique using specific primers.

MATERIALS AND METHODS

Patients: Fifty patients with HCV infection referred from physicians to Molecular Biology Laboratory of Drug Applied Research Center, Tabriz, Iran, were selected for this study during 2003-2005. Patients were distributed within Northwest of Iran. All serum samples were aliquoted within 2 h and stored immediately at -70°C until use. Aliquots were not thawed more than once prior to analysis. The sources of infection mainly included: intravenous drug abusers, blood transfusion, renal dialysis, occupational exposure.

HCV ELISA test: The presence of HCV antibody in all serum samples was detected using second generation ELISA test (Abbot, HCV2.0, ELISA Kit) on the basis of HCV recombinant core region proteins. Human IgGs bound to the antigen were reacted with goat-anti-human IgG peroxidase conjugate as the secondary antibody and visualized by subsequent reactions with a chromogenic substrate. Positive samples generated a medium to dark blue color. All of the patients whose sera were positive for HCV genome entered for HCV genotyping study.

HCV genome detection: For the detection of HCV RNA in the serum of patients, a commercial kit (STRP HCV genome detection kit, Cinnagen) was used. This kit is designed for the detection of HCV RNA using single tube RT-PCR reaction followed by nested PCR. Briefly, RNA was extracted from serum samples by following procedure: 50 μL sera were added to 450 μL cold RNA-Plus solutions. The solution was vortexed to dissolve the clumps and incubated on ice. One hundred microliter of chloroform was added into the tubes, vortexed and centrifuged. Aqueous phase was transferred into a new tube and isopropanol (250-300 μL) was added. Then, the tubes were incubated at 20°C for at least 20 min and centrifuged. The aqueous phase was discarded and 200 μL ethanol 70% was added to the pellet and centrifuged. The aqueous phase was discarded again and RNA was dissolved in 30 μL DEPC water. cDNA synthesis was achieved by following RT-PCR procedure: 95°C for 1 min, 42°C for 20 min in the presence of the kit primers. Then, first round of PCR was performed by: 93°C for 40 sec, 60°C for 40 sec and 72°C for 40 sec (for 20 cycles). Three microliter of the first round PCR product was taken to the second PCR round according to the following program: 93°C for 40 sec, 60°C for 40 sec and 72°C for 40 sec (for 35 cycles). Three microliter of the first round PCR product was taken to the second PCR round according to the following program: 93°C for 40 sec, 60°C for 40 sec and 72°C for 40 sec (for 35 cycles). Three microliter of the first round PCR product was taken to the second PCR round according to the following program: 93°C for 40 sec, 60°C for 40 sec and 72°C for 40 sec (for 35 cycles).

Genotyping: HCV RNA positive sera were subjected to genotype detection by nested PCR according to modified Okamoto et al. (1996) method with further modification in PCR amplification. First, HCV RNA was extracted and reverse transcribed into cDNA by using an HCV specific primer and resultant cDNA was subjected to genotyping with type specific primers from the core region of HCV genome. Briefly, cDNA made by RT-PCR was subjected to PCR amplification (first round PCR) using universal primers resulting in generation of a 433 bp DNA band (Fig. 1). The amplified product was subjected to second round PCR with five different sets of sense and antisense primers specific for the five common HCV genotypes.

![Fig. 1: Agarose gel electrophoresis of first round PCR product, 100 bp DNA ladder (Lane 1), PCR product of first round with 433 bp size (Lane 2) ](image-url)
(Table 1). Genotypes 1a and 1b were determined using PCR technique in the presence of specific primers developed by Okamoto et al. (1996). In order to determine genotypes 2a and 2b, PCR was performed in the presence of specific primers for genotype 2a and genotype 2b with the following PCR program: 94°C for 3 min, followed by 25 cycles of 94°C for 30 sec, 53°C for 30 sec, 72°C for 30 sec and final extension was performed at 72°C for 8 min. Genotype 3a was tested using primers #397 and 473 with the following PCR program: 94°C for 3 min, followed by 25 cycles of 94°C for 30 sec, 57°C for 30 sec, 72°C for 30 sec and final extension was performed at 72°C for 10 min. For each amplification at the second round PCR, 1 µL of first round PCR product was used. The products of second round PCR were applied to 2% agarose gel electrophoresis or Acryl amide gel electrophoresis stained with ethidium bromide and observed under UV illumination.

RESULTS

A total number of 50 sera were tested for HCV detection and genotyping in Tabriz University of Medical Sciences. Of 50 patients, 48 were male and 2 female and the mean age of the patients was 27 (range 10-45 years). The elicited risk factors for patients with HCV included intravenous drug abusers, blood transfusion, renal dialysis and occupational exposure. All serum samples used in this study were tested for anti-HCV antibody using ELISA kit. ELISA results showed that all tested samples were seropositive for anti-HCV antibody. Then, the presence of HCV RNA in the serum of the patients was tested using HCV genome detection kit. A positive serum sample for HCV RNA was addressed by the presence of a DNA band with 216 bp size (Fig. 2). Among 50 serum specimens, 28 (56%) specimens were HCV-RNA positive which entered for HCV genotyping analysis. Genotype classification was carried out using modified Okamoto et al. (1996) protocol. The second generation protocol of Okamoto et al. (1996) by PCR technique could distinguish the five common genotypes of HCV RNA (including 1a, 1b, III/2a, IV/2b, V/3a) and discriminate them from the other genetic groups. The simplified and modified Okamoto procedure correctly determined the genotypes of 28 HCV isolates for three common genotypes. Of 28 samples determined, 20 patients (71.4%) carried type 1a genotype which was confirmed by PCR amplification of a DNA band with 128 bp size. Four cases (14.2%) were classified as genotype 1b according to the production of a 125 bp DNA band and another 2 samples (7.1%) were determined as genotype 2a with regard to PCR production of a 69 bp DNA band. The electrophoresis patterns from the three reaction of second round PCR are shown in Fig. 3. We didn’t find any 2b and

<table>
<thead>
<tr>
<th>No</th>
<th>Sequence (5'-3')</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>#475</td>
<td>5'-GGAGGAAGCCTCTAGAGACCTGAC-3'</td>
<td>Universal</td>
</tr>
<tr>
<td>#188</td>
<td>5'-ATGTACCCATGAGGTTGCGA-3'</td>
<td>Universal</td>
</tr>
<tr>
<td>#699</td>
<td>5'-GTTGCAAGCTCCAAGCTTAC-3'</td>
<td>1a</td>
</tr>
<tr>
<td>#462</td>
<td>5'-GAGCAATGCACGAGCCACAGC-3'</td>
<td>1a</td>
</tr>
<tr>
<td>#389</td>
<td>5'-GAGCAATGCAGGGAGCAAGA-3'</td>
<td>1b</td>
</tr>
<tr>
<td>#402</td>
<td>5'-GAAGCAATGCCTCTACCCCCAGCA-3'</td>
<td>1b</td>
</tr>
<tr>
<td>#472</td>
<td>5'-GGCAGAGTCTGCTTCAACACTA-3'</td>
<td>2a</td>
</tr>
<tr>
<td>#468</td>
<td>5'-GCTTACCCAGGCCTGCTTAC-3'</td>
<td>2a</td>
</tr>
<tr>
<td>#491</td>
<td>5'-GACTTCAGCACTCCCTCCCTGAA-3'</td>
<td>2b</td>
</tr>
<tr>
<td>#394</td>
<td>5'-GAGCAATGCACGAGCCACAGC-3'</td>
<td>2b</td>
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<tr>
<td>#397</td>
<td>5'-GAGAAATTGCAACTTCTCAG-3'</td>
<td>3a</td>
</tr>
<tr>
<td>#473</td>
<td>5'-GGAGGAAGCCTCTCGTCGCGA-3'</td>
<td>3a</td>
</tr>
</tbody>
</table>
3a genotypes in our samples and the predominant type was genotype 1a (71.4%). It should be noted that 24 isolates (85.7%) were determined as type 1 and 2 isolates (7.1%) as type 2. The HCV genotype of 2 patients (7.1%) was not determined.

**DISCUSSION**

Studies suggest that infections caused by different HCV subtypes may have different clinical outcome and that some subtypes are associated more frequently with advanced liver disease and hepatocellular carcinoma (Pawlotsky et al., 1995; McHutchison et al., 1998). The duration of treatment should be based on the HCV genotype and the pretreatment viral load. However, since tests for the quantification of HCV RNA are still not standardized and the viral load naturally fluctuates over time, the viral load currently is not routinely used for determining the treatment regimen (Lauer and Walker, 2001). A number of HCV genotyping systems were also developed which include: restriction fragment length polymorphism (Ahmadi Pour et al., 2006), PCR with specific primers (Okamoto et al., 1996; Holland et al., 1996; Hu et al., 2000), reverse line probe assay (Le Pogam et al., 1998), heteroduplex mobility analysis (White et al., 2000), pyrosequencing (Elahi et al., 2003), direct sequencing (Furion et al., 1999), serological based detection methods (Schoeter et al., 1999), CLIP sequencing (Ross et al., 2000). Among PCR based assays for HCV genotyping, the core primers have been most widely used in epidemiological and clinical investigations (Sanimi-Rad et al., 2004; Elahi et al., 2003). Okamoto et al. (1996) and Holland et al. (1996) reported their methods as a useful protocol for clear distinction between subtypes 1a and 1b. For HCV genotyping, an assay adapted from the original techniques of Okamoto (1993, 1996) and Holland (1996), which uses type specific primers located in the core region for amplification of genotype-specific sequences, was employed in this study. In present analysis, we didn’t find any mixed infection genotype, however, mixed infections detected by a type specific PCR should always be interpreted with caution, especially if the assignment of genotype involves different therapeutic choices (Spada et al., 1998). In present study, efficacy was very satisfactory because of a very low rate of untypeable samples (two samples). It may be related to low-level viremia or the presence of variations in the core region that prevent annealing of the primers or new genotype. In this regard, it should be noted that present results are different from those previously reported from Italy and USA (Furione et al., 1999; Lau et al., 1995) suggesting that the application of a core based PCR assay for genotyping produces a high rate of undetermined results. In the currently widely used classification system, variants of HCV collected from different parts of the world are divided into six main genotypes and a series of subtypes (Zan et al., 1996; McHutchison et al., 1998). Within genotypes, further clustering of HCV variants into subtypes is remarkably uniform. The distribution of HCV genotypes varies in different geographical areas. However, population migration and travel may considerably modify the current world HCV genotype map. The most prevalent subtype, 1a found in this study is in accordance with other regions of Iran (Sanimi-Rad et al., 2004; Elahi et al., 2003; Zali et al., 2000; Kazemi et al., 2005), which is also the prevalent genotype in England, Scotland, Australia and Denmark (Corbet et al., 2003; Mellor et al., 1995; McCaw et al., 1997; Harris et al., 1999). Zali et al. (2000) performed HCV genotyping by type specific primers. Fifteen cases were grouped into four genotypes as follow: type 1a in 7 (47%) patients, type 1b in 5 patients, type 3a in 4 patients, one patient was found to have type 4. Kazemi et al. (2005) reported 11 cases (55%) as type 1, 4 cases (20%) as type 3a and 5 cases (25%) were untypeable. Kabir et al. (2006) showed the most frequent HCV subtypes in Iran were 1a, 3a and 1b. Therefore the subtype's distribution within Iran did not differ distinctly according to geographical origin. HCV genotype 1 is the most prevalent worldwide (White et al., 2000). The results of present study also showed that the most common genotype among HCV infected patients in Northwest of Iran is type 1 (85.7%), in contrast, genotypes 3 and 4 were rare or absent. In addition, we need to develop more effective therapies for persons with infection, particularly for those with genotype 1, the most difficult to treat (Alter et al., 1999; McHutchison et al., 1998) as well as approaches to the treatment of current or former injection drug abusers. Genotype 1a and 3a are more prevalent in intravenous drug abusers in Europe and USA (McOmish et al., 1994; Alter et al., 1999; Silva et al., 2000). In the present study 60% patients with intravenous drug abusers had genotype 1a. On the other hand, the majority of HCV infection in young people was detected as 1a. In multiple PCR reaction conditions, the number and size of fragments must be precisely evaluated. In order to avoid unnecessary complications in the procedure secondary to the addition of primers for all new types, as well as taking into account that the range of virus types and subtypes circulating in a defined geographical area is generally limited (McOmish et al., 1994; White et al., 2000; Pawlotsky et al., 1995), only primers for new and relevant types should be incorporated in the assay. There are no epidemiological data concerning the circulation of
CONCLUSIONS

This is the first time that HCV genotype determination was conducted in Northwest Iran. The results of present study showed that the most common genotype among HCV infected patients in Northwest Iran is type 1. Considering that patients infected with HCV genotype 1 would need a different therapy regimen from that for patients with HCV genotype 2 and 3, the HCV genotyping test is useful for our region. Two untypeable samples may be novel types or other subtypes of HCV, further study by another method such as direct sequencing is needed to clarify the matter.

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