

*Full Length Research Paper*

# Determination of hepatitis C virus genotypes among HCV positive patients in Shahrekord, Iran

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Hepatitis C is one of the most common causes of the liver failure and cancer and represents a major public health problem. Recent studies have focused on whether different hepatitis C virus (HCV) genotypes, are associated with different profiles of pathogenicity, infectivity and response to antiviral therapy. Genotyping system based on polymerase chain reaction (PCR) of the core region with genotype-specific PCR primers for the determination of HCV genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a, and 6a was developed. Different genotypes have been reported in different parts of the world. Genotype 1 is difficult to treat, while genotypes 2 and 3 are easy to treat. Therefore, identification of HCV genotype in patients is necessary to begin and follow up the treatment. In this study, viral genomic of 94 patients extracted from sera were detected by nested-real time (RT) PCR. PCR products were digested with proper enzymes and studied by restriction fragment length polymorphism (RFLP). The results of PCR-RFLP were as follows: 1a (54.26%), 1b (11.71%), 3a (27.66%), 2a (2.12%) and 4 (4.25%). This indicates that a high percentage of HCV infected patients.

**Key words:** Genotyping, Hepatitis C Virus, PCR, RFLP, Iran.

## INTRODUCTION

Hepatitis C virus (HCV) is a single stranded ribonucleic acid (RNA) virus; approximately 9.5 kb which belongs to the Flaviviridae family, HCV demonstrates a high degree of sequence variation throughout its genome (Choo et al., 1989). HCV is a causative agent for chronic, acute and fulminant hepatitis (Alavian et al., 2002). The association of HCV among patients with cirrhosis of liver and

hepatocellular carcinoma has been reported (Alavian et al., 2006). HCV infection is a global health problem and it is estimated that 200 million people of the world population are infected with HCV (Lee et al., 2008). Serological tests detecting antibody to HCV have shown that HCV is the major etiological agent for both transfusion acquired and sporadic non-A, non-B hepatitis (Alter et al., 1989; Kuo et al., 1989; Mosley et al., 1990). Chronic hepatitis occurs in more than 50% of HCV infected patients and can lead to cirrhosis and liver cancer. HCV causes 20% of acute hepatitis cases, 70% of all chronic hepatitis cases, 40% of all cases of cirrhosis of the liver, 60% of hepatocellular carcinomas, and 30% of liver transplants in Europe.

The study of genetic variability of HCV strains has led the consensus classification into six major genotypes. Some studies suggest that the clinical features of liver

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**Abbreviations;** HCV, Hepatitis C virus; RT PCR, reverse transcription polymerase chain reaction; 5'-UTR, 5' untranslated region; DNA, deoxyribonucleic acid; RIBA, recombinant immunoblot assay.

disease depend on HCV genotypes (Farshad et al., 2010). Classification of HCV is based on the diversity of the genome, and the criterion for HCV classification was proposed by Simmonds and colleagues (Simmonds et al., 1993).

The HCV genotypes have been determined primarily based on analysis of partial genome sequences. The most extensive database exists for the 5'-UTR, core, E1, and NS5B (Bukh et al., 1992a; Furione et al., 1999). Whereas the 5'-UTR is highly conserved and therefore preferred for diagnosis, the core, envelope, and NS5B regions are less conserved and therefore highly discriminative and may be preferred for subtyping (Prescott et al., 1997).

Sequence analysis of multiple strains of HCV has demonstrated that the nucleotide sequence can differ by as much as 30%. However, the levels of heterogeneity differ considerably among various regions of the virus. For example, sequence variation ranges from as little as 10% in the 5' untranslated region (5'-UTR) to as much as 50% or more within the E1 region. HCV isolates from around the world can be divided into distinct major groups or genotypes with about 66 to 69% nucleotide similarity, which can in turn be divided further into subtypes with about 77 to 80% nucleotide similarity (Chan et al., 1992; Bukh et al., 1993).

Since the first report of the HCV genome by the Chiron research group numerous complete or partial nucleotide sequences of HCV isolates have been reported worldwide. Comparison of these sequences revealed marked genetic heterogeneity of the HCV genome (Okamoto et al., 1990; 1992; Choo et al., 1991; Inchauspe et al., 1991).

Investigators of HCV genotyping have used sequence analysis of HCV NS5, core, E1, and 5'-UTRs. HCV genotyping by Okamoto et al. using type-specific primers was first introduced by using primers specific for the core region. (Okamoto et al., 1996) This method lacked acceptable sensitivity and specificity (McOmish et al., 1993). Several deoxyribonucleic acid (DNA) hybridization assays for HCV genotyping have been described.

A commercial kit for HCV genotyping has been introduced in Europe by Innogenetics (InnoLipa, Belgium) and is based on hybridization of 5'-UTR amplification products with genotype specific probes. Others have used restriction enzymes to determine viral genotype by restriction fragment length polymorphism (RFLP).

In this method, a PCR-amplified DNA fragment is digested into fragments with different lengths by enzymes (restriction endonucleases) that recognize cleavage sites specific for each genotype (Stuyver et al., 1993). Although all these methods are able to identify the major genotypic groups, only direct nucleotide sequencing is efficient in discriminating subtypes (Bukh et al., 1995).

In the current study, we have typed HCV strains with RFLP rapidly and reliably by digesting the amplified DNA from the primary specimens by selected restriction

enzymes and some by sequencing.

## MATERIALS AND METHODS

### Serological data

The sera were collected from 94 HCV infected patients, referred to Al Mahdi Laboratory (Shahrekord, Iran) during 2009-2010. All patients had elevated serum aminotransferases for at least 6 months, a positive test for anti-HCV antibodies (third generation ELISA [ORTHO HCV 3.0 ELISA Test system; Ortho Diagnostics, Raritan, New Jersey, USA], the confirmatory recombinant immunoblot assay (RIBA) test (Inno-LIA TM HCV Score) and HCV RNA in serum by reverse transcription nested PCR for the 5'-UTR of the HCV genome (Simmonds et al., 1993). The average age was varying from 18 to 64 year. while the mean age was 41.

### HCV RNA extraction and cDNA synthesis

For detection of HCV RNA in serum and for genotyping studies, RNA was extracted from 50 µl of serum by using STRP™ HCV detection kit (Cinna Gen Inc Company). For extraction of HCV RNA, add 50 µl serum to 450 µl cold RNXTM plus solution and vortex sample to dissolve clumps, then add 100 µl of chloroform and centrifuged at 12000 g and transfer the aqueous phase to a new tube then add equal volume of isopropanol, and stored at -20°C for at least 20 min, and centrifuged at 12000 g. Then discard aqueous phase and to the pellet 200 µl 70% ethanol and centrifuged at 12000 g, and discard aqueous phase and dry the pellet (RNA). At least dissolve RNA in 30 µl DEPC treated water and stored in -70°C. According to the kit protocol, cDNA was synthesis and by use specific primers RT-PCR was done.

### PCR genotyping primers

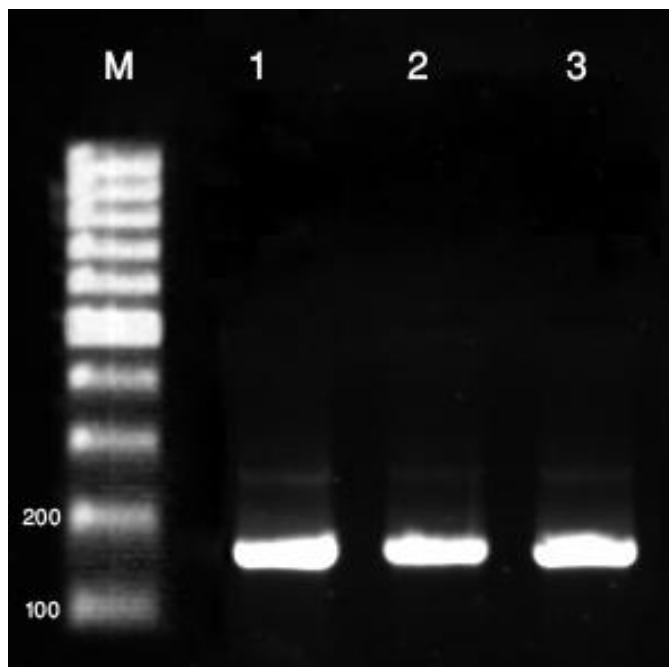
For specific and nested PCR, four oligonucleotide primers from 5'-UTR of HCV were designed using generunr (Hastings software) and synthesized at the Cinna Gene Company (Iran). In the first round of PCR, the primers corresponded to HCV-1 sense oriented nucleotides -268 to -251 F1 (AGCGTCTAGCCATGGCGT), numbered according to Bukh et al., (1992b) and antisense nucleotides -4 to -22 R1 (GCACGGTCTACGAGACCT). For the second round, the primer F2 (GTGGTCTGCGGAACCGG) corresponded to sense-oriented nucleotides -199 to -183 and R2 (GGGCACTCGCAAGCACCC) corresponded to antisense nucleotides -26 to -43.

### PCR

The first round was carried out for 30 cycles which consisted of initial denaturation at 94°C for 5 min, denaturation at 94°C for 35 s, annealing at 58°C for 40 s, extension at 72°C for 45 s and, the final extension at 72°C for 5 min. The second round was followed for 25 cycles which consisted of initial denaturation at 94°C for 5 min, denaturation at 94°C for 35 s, annealing at 64°C for 40 s, extension at 72°C for 45 s, and the final extension at 72°C for 5 min. The 174-bp second PCR product was submitted to electrophoresis by using a 1.5% agarose gel in 0.5X TBE buffer, and was visualized by ethidium bromide staining under ultraviolet light (Han et al., 1991).

### Genotyping by RFLP

Total volumes of each nested-PCR product (25 µl) were divided into



**Figure 1.** Ethidium bromide stained gel of PCR products amplified with HCV primers. DNA 100 bp markers (lane M), samples positive (lanes 1, 2 and 3).

**Table 1.** Demonstrates cutting sites of *Hinf* I, *Apa* I, *EcoR* II and *Bsh1236* I restriction enzymes for different strains of HCV as published by Bukh et al. (1992).

Genotype	Segment (bp)		
	Tube A	Tube B	Tube C
1a	97	97	129
1b	97	97	99
2a	97	174	174
2b	174	174	174
3a	129	145	99
3b	97	145	99
4	97	145	129
5	97	174	99
6	97	97	174

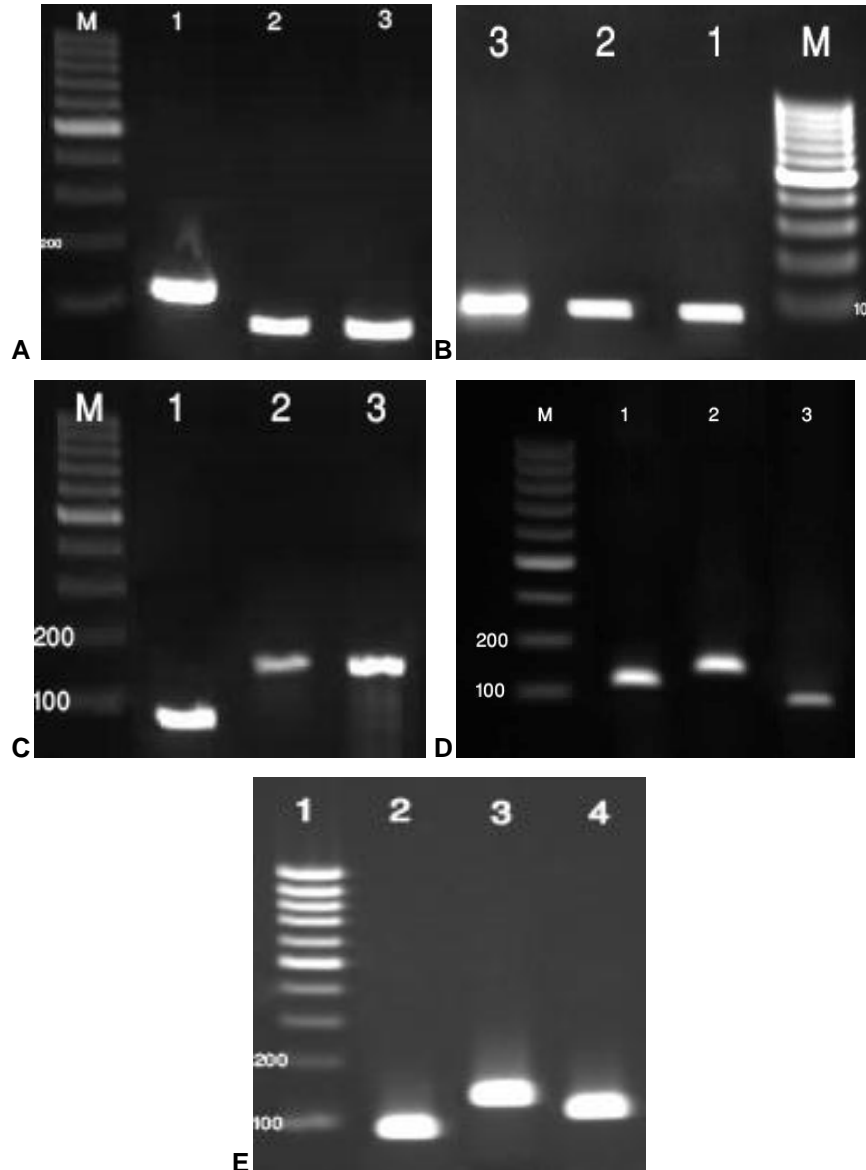
three tubes containing appropriate buffers. Restriction enzymes, *Apa* I, *Hinf* I, *EcoR* II and *Bsh1236* (Fermentas, Co.) used as the following combinations: 1. *Apa* I / *Hinf* I; 2. *EcoR* II/*Hinf* I; 3. *Bsh1236* I. The other enzymes were similar to McOmish et al (1994) method.

The tubes were incubated with 1 U of the enzyme mixture for 3 h at 37°C. The digestion temperature was 37°C. If the samples could not be analyzed immediately after digestion, they were stored at -20°C before the analysis vertical 12% polyacrylamide gel electrophoresis and the digested products were heated for 5 min. After ethidium bromide staining, the DNA fragments were identified under ultraviolet light. Molecular weight 100 bp plus marker (Fermentas, Co.) and undigested PCR products was included in each analysis. The genotypes were deduced from the

fragmentation patterns of the amplified DNA.

## RESULTS

The 5'- UTR of 94 HCV positive serum samples were amplified and digested by appropriate restriction enzymes for genotype determination. The RFLP results revealed: 1a (54.26%), 1b (11.71%), 3a (27.66%), 2a (2.12%), 4 (4.25%). Figure 1 shows the 174 bp nested RT-PCR amplification of HCV RNA extracted from blood samples. Table 1 show fragments yielded upon restriction



**Figure 2.** 12% polyacrylamide gel electrophoresis of the digestion products of the amplified DNA from different genotypes. Marker; DNA 100 bp (lane M), A : Genotype 1a (129, 97 and 97 bp). B: Genotype 1b (99, 97 and 97 bp), C: Genotype 2a (97, 174 and 174 bp), D: Genotype 3a (129, 145 and 99 bp) E: Genotype 4 (97, 145 and 129 bp).

enzyme digestion of 5'-UTR region. The cutting sites of restriction enzymes are shown (Tube A) *Apa I/Hinf I*; (Tube B) *EcoR II/Hinf I*; (Tube C) *Bsh1236 I*.

Figure 2 demonstrates the analytical polyacrylamide gel electrophoresis of HCV types 1a, 1b, 2a, 3a and 4 after digestion of the amplified DNA with the selected restriction enzymes.

Genotyping of 94 sera from patients who were either recently infected by HCV or with history of previous HCV infection and positive PCR results were performed. The results of PCR-RFLP were as follows: 1a (54.26%), 1b (11.71%), 3a (27.66%), 2a (2.12%) and 4 (4.25%). This

indicates that a high percentage of HCV infected patients in Iran are infected with 1a or 3a genotypes (Table 2).

Out of 94 patients, 63 patients were male and 32 were female. Analysis of population previously infected with HCV showed that 26 patients (27 %) were less than 30 years of age, 43 patients (45.74%) between 30-50 and 25 patients (26.5%) were above 50 yr of age. The most frequent genotype in patients above 50 was 3a, while 1a genotype was more prevalent in patients under 50 years old. Hemodialysis patients and cases by known history of transfusion were known to be infected by only subtypes 1a and 3a. Also the patients fewer than 30 year old were

**Table 2.** Hepatitis C virus genotypes in 94 Iranian patients with RFLP method.

Genotype	Number	%
1a	51	54/26
1b	11	11/71
2a	2	2/12
3a	26	27/66
4	4	4/25
Total	94	100

infected by only subtypes 1a and 3a.

## DISCUSSION

The study of viral diversity provides a better understanding of the origin and dynamics of viral infections. Genetics variants of HCV are known to be widely spread around the world. Genotypes 1, 2 and 3 are found on all countries, but in some geographical areas, such as Africa and Southeast Asia, viral isolates are highly divergent and particular genotypes or subtypes are predominant (Moller et al., 1995). These data suggest the existence of a long term endemic infection in these areas and some researchers have hypothesized that HCV have originated in such places (Simmonds et al., 1993).

Epidemiological studies in different regions of the world show the virus is distributed worldwide with prevalence varying between different countries from 0.2 up to 40%. It is clearly revealed that the incidence of HCV is higher among less developed nations. In Iran, HCV prevalence in general population is less than 1%.

In our study HCV genotypes were found, 1a (54.26%), 1b (11.73%), 3a (27.66%), 2a (2.12%), 4 (4.25%). HCV is highly variable, leading to the classification of at least six genotypes, each with several subtypes. This heterogeneity is, at least partly, responsible for lack of availability of an effective vaccine (Samimi-Rad et al., 2004). Investigators of HCV genotyping have used sequence analysis of HCV NS5, Core, E1 and 5'-UTRs. However, direct sequencing is not practical on a large scale. RFLP has been used widely for this aim, especially for screening of large number of specimens.

The use of 5'-UTR assay designed for the detection of HCV in clinical specimens provides a sensitive, standardized amplification protocol specifically designed for large-volume testing and rapid turnaround time and is also used widely for HCV genotyping by different investigators. In this report, we have focused on chronically infected group of patients, to determine the most prevalent genotypes in Iran (Ahmadi et al., 2006).

RFLP of HCV PCR positive sera and sequencing of 174 bp fragment of 5'-UTR region was used to achieve this purpose. In our study, HCV genotype 1a was the

most frequent (%54/26), followed by genotype 3a (%27/66) and genotype 1b (%11/71). This is compatible with the findings of Zali et al. (2000), Samimi-Rad et al. (2004) and Ahmadi et al. (2006) in Iranian patient.

Ahmadi et al. (2006) reported 1a (%52.88), 3a (%27.57) and 1b (14%) genotypes HCV among Iranian patients by RFLP method. Samimi-Rad et al. (2004) found 1a (47%), 3a (36%) and 1b (8%) genotypes HCV among Iranian patients, and revealed that genotype 1a is frequent in the South of Iran (70%), while 3a is more prevalent in the North-West of Iran (83%).

In the present study, our data showed the same result as those demonstrated by Zali et al. (2000), Samimi-Rad et al. (2004) and Ahmadi et al. (2006). Although the frequency of genotype 1a was slightly higher in these study. Patients infected by blood products more frequently had genotype 1a (57%), while younger drug users had genotype 3a (54%) more frequently (Farshad et al., 2010).

Keyvani et al. (2007), reported HCV genotypes in Iran. In their study, genotype 1a with 39.7% had the highest frequency. Genotype 3a (27.5%) and 1b (12.1%) were the other frequent genotypes (Farshad et al., 2010). The prevalence of HCV among blood donors is less than 1% in Northern European countries (Choo et al., 1989). Higher rates have been reported in South East Asian countries, including India (1.5%), Malaysia (2.3%), and the Philippines (2.3%) (Farshad et al., 2010).

The incidence in Japan is 1.2% (Kato et al., 1990). Alarming rates of 14.5% are reported in Egypt (Farshad et al., 2010). HCV genotype 4 is common in countries such as Yemen, Kuwait, Iraq, and Saudi Arabia (Samimi-Rad et al., 2004).

However, in Turkey genotype 1b, in Pakistan genotypes 3a and 3b, in Uzbekistan 1a, 1b, 2a, 2k and 3a, in Lebanon 1g are reported to be the dominant genotypes (Pavio and Lai, 2003).

Genotype 4 is the main genotype circulating in most Arabic countries. In Bahrein 4a and in Saudi Arabia HCV genotype 4 were detected in 50% of patients and genotype 1b was found in nearly 40% of patients (Elahi et al., 2003).

Genotype information is important when HCV treatment is being considered, since some genotypes respond more favorably to the medications. Genotype also determines the length of therapy, for example, treatment for genotypes 2 and 3 requires only 24 weeks while genotypes 1 and 4 require 48 weeks (44).

In addition to treatment purposes, detection of HCV genotypes in different regions can be used for the purpose of molecular epidemiology (Pavio and Lai, 2003).

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