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GENOTYPIC PREVALENCE OF HEPATITIS C VIRUS AMONG HCV POSITIVE PATIENTS IN IRAN BY PCR-RFLP

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Abstract:

Hepatitis C virus (HCV) is the major cause of chronic liver disease such as acute and chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. This virus is responsible for more than 60% of chronic hepatitis. Based on its genetic variability, HCV is classified into at least six genotypes and a series of subtypes (1a, 1b, 2a, 2b, 3a, 3b, 4, 5a, and 6a), while Genotype one is difficult to treat but genotypes 2 and 3 are easy to treat. Different genotypes are seen in different parts of the world. HCV genotyping is important for prediction of success of chemotherapy and progression of liver diseases. So recent studies have focused on determination of HCV genotypes.

In this study, viral genomic of 86 patients (from different laboratories of Esfahan) 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 (52.3%), 1b (11.6%), 3a (29.1%), 2a (2.3%), 4 (4.7%). Our results showed that types 1a and 3a were the most prevalent HCV genotypes in our samples. The results of this study will guide clinicians in successful monitoring and treatment of HCV patients in our region.

Keywords: *Genotyping, Hepatitis C Virus, PCR, RFLP.*

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INTRODUCTION:

Hepatitis C virus (HCV) is a significant etiology of chronic hepatitis and the leading cause of hepatic transplantation in the world [1]. It is well established now that hepatitis C virus (HCV) is the leading cause of blood borne non-A, non-B hepatitis worldwide [2, 3].

HCV infection is a global health problem and it is estimated that 200 million people of the world population are infected with HCV [4]. HCV is a major cause of chronic liver disease, hepatocellular carcinoma, and the single most common indication for liver transplantation [5]. An estimate of 53,000 deaths per year caused due to HCV in world. Most HCV infected people remained unidentified until the development of late symptoms, while some remained carrier through their life and do not develop any complication [6].

This virus was discovered in 1989. It is an approximately 9.6 kb single stranded positive sense RNA virus. HCV belongs to the Flaviviridae family and Hepacivirus genus. The structure of the RNA is composed of three parts; structural (C- E1 – E2), nonstructural (NS1 –NS2 - NS3 –NS4 - NS5), and two untranslated regions (5´-UTR and 3´-UTR) at two ends of the viral genome [7, 8]. 5'-UTR is highly conserved and therefore preferred for diagnosis [9]. This region has some specific internal ribosomal entry sites (IRES) in its structure which help to distinguish between genotypes and subtypes [10, 11]. E1 and E2 regions display the most variable part of its genomic RNA [12]. The genetic variability is due to high mutation rate in the envelope gene coupled with the absence of a proofreading function in the virionencoded by RNA polymerase [13]. Based on genetic differences among HCV isolates, hepatitis C virus species is classified into 6 genotypes with 120 subtypes [8, 14, 15]. Genotype frequencies vary by geographic region. Some genotypes (1a, 1b, 2a, 2b, 3a) are widely distributed around the world [16], while others have a more restricted distribution. Genotype 4 is predominant in the Middle East (particularly Egypt), Zaire and Burundi [17] while genotype 5 has so far been mainly found in South Africa [18]. Genotypes 1 and 3 are commonly found in Iran [19, 20].

Genotyping is useful tool for investigating outbreaks and for understanding the epidemiology of the infection. Clinically, genotyping of HCV is important for predicting treatment responses and for determining the duration of antiviral therapy. Response to interferon (IFN)-based therapies in patients infected with HCV genotype 1 and 4 is much lower than in genotypes 2 and 3 [20, 21]; Thus, HCV genotyping is recommended before starting treatment.

HCV genotyping is performed by several molecular techniques, such as sequencing of cloned genome, hybridization, Restriction Fragment Length Polymorphism (RFLP) and genotype-specific primer PCR [9]. The gold standard method for HCV genotyping is sequencing but this technique is expensive and requires many equipments and facilities. Contrary, RFLP is a sensitive and cost-effective method. In RFLP, part of 5'-UTR is amplified by PCR and the amplicon is digested by restriction enzymes. The genotype of the HCV is determined based on the pattern of the fragments following digestion [22].

In the current study, we aimed to determine distribution of HCV genotypes and their prevalence in Esfahan by PCR-RFLP.

MATERIALS AND METHODS:

Serum Samples during 2013-2014, eighty six blood samples were obtained from HCV patients who were found to be HCV positive from different laboratory in Esfahan. HCV infection in patients had been confirmed by positive results in HCV-Ab. All patients had elevated serum aminotransferases for at least 6 months, a positive test for anti-HCV antibodies (thirdgeneration ELISA [Ortho Diagnostics, Raritan, N. J.]), and HCV RNA in serum by reverse transcription nested PCR for the 5'-UTR of the HCV genome [23, 24]. Patients including 54 males and 32 females, and the mean age was 39 (ranges was varying from 19 to 60 year). The collected bloods from the patients were stored at -70 °C until tested.

HCV RNA extraction For detection of HCV RNA in serum and for genotyping studies, RNA was extracted from fifty µl of serum by acid guanidinium-isothiocyanate- phenol- chloroform method [25], then precipitated with isopropanol, and rinsed with 70% cold ethanol. The RNA pellet was resuspended in 25 µl of diethyl pyrocarbonate (DEPC) treated water.

cDNA synthesis Five microliter of the extracted RNA was used as the template for synthesis of cDNA by QIAGEN Sensiscript RT kit. In a microtube, 2 µl 10x RT buffer, 2 µl 5mM dNTPs, 2 µl 10mM random primer, 1 µl 10U/µl RNase inhibitor were added. The final volume of RT reaction was adjusted to 20 µl by DEPC water. The reaction tube was incubated at 37 ºC for 1 hour and the product was kept at -20 ºC.

PCR genotyping primers For specific and nested PCR, four oligonucleotide primers form 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, numbered according to Bukh et al., [26] and antisense nucleotides -4 to -22 R1. For the second round, the primer F2 corresponded to sense-oriented nucleotides -199 to -183 and R2 corresponded to antisense nucleotides -26 to -43. The sequences of primers were follow:
F1: 5'- AGCGTCTAGCCATGGCGT -3'
R1: 5'- GCACGGTCTACGAGACCT-3'
F2: 5'- GTGGTCTGCGGAACCGG -3'
R2: 5'- GGGCACTCGCAAGCACCC -3'

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 [27].

Genotyping by RFLP To do RLFP of HCV, 25 µl of each nested-PCR products were divided into three tubes containing appropriate buffers. Restriction enzymes, *Apa* I, *Hinf* I, *EcoR* II and *Bsh*1236 (Fermentas, Co.) used as the following combinations: 1. *Apa* I / *Hinf* I; 2. *EcoR* II/*Hinf* I; 3. *Bsh1236* I. [28]. The tubes were incubated with 1 U of the enzyme mixture for 3 h at 37°C. The digested products were separated by electrophoresis on polyacrylamide 13% gel at 100 V for 3 h and then the DNA fragments were visualized by ethidium bromide staining.

RESULTS:

Primarily, serum samples were screened for anti-HCV antibody. Sera samples from 86 patients were positive for anti-HCV antibody and all of them also were showed positive by nested-PCR. Figure 1 shows the 174 bp nested RT-PCR amplification of HCV RNA extracted from blood samples. Table one 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 [26]. Figure 2 shows the pattern of the digested products of 1a and 3a HCV genotype by RFLP on polyacrylamide gel electrophoresis.
In this present study, 5 subtypes were detected. The RFLP results were as follows: 1a (52.3%), 1b (11.6%), 3a (29.1%), 2a (2.3%), 4 (4.7%). This indicates that a high percentage of HCV infected patients in Iran are infected with 1a or 3a genotypes (Table 2). Analysis of population previously infected with HCV showed that 9.3% of patients were less than 25 years of age; also 30.2% were above 50 years old and demonstrated that 3a was the most frequent genotype in this patients.

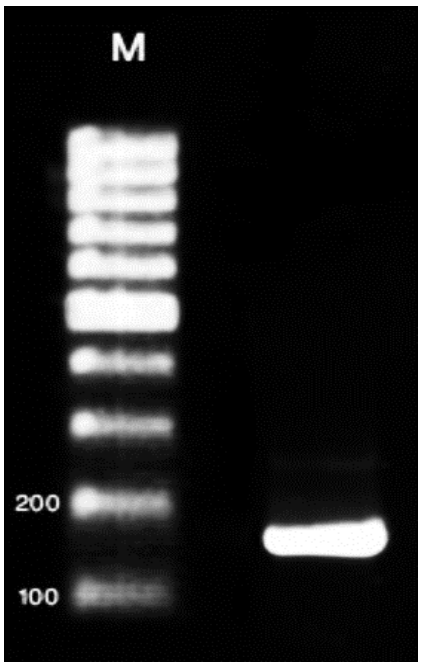
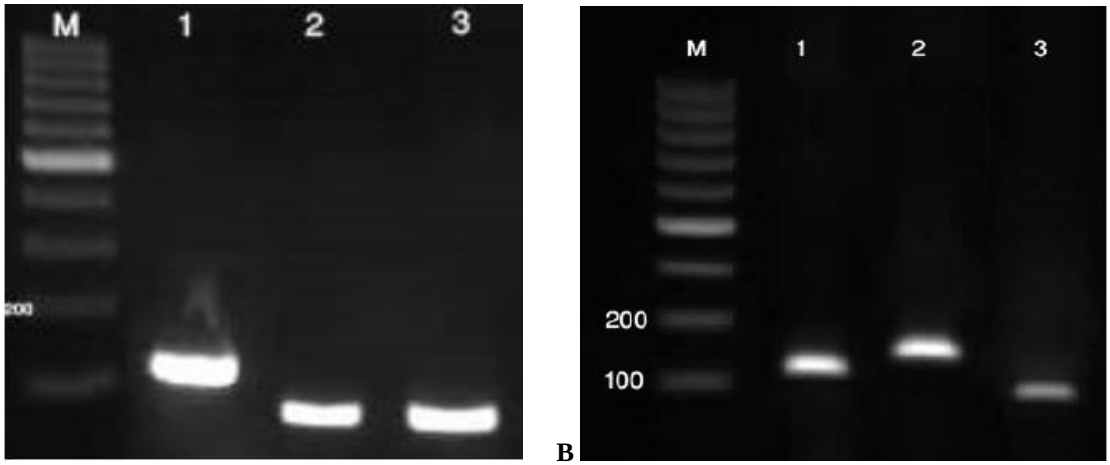


Fig 1: Ethidium bromide stained gel of PCR products amplified with HCV primers. DNA 100 bp markers (lane M), samples positive



A B

Fig 2. 12% polyacrylamide gel electrophoresis of RLFP patterns of genotypes of 1a and 3a. Marker; DNA 100 bp (lane M), A: Genotype 1a (129, 97 and 97 bp) B: Genotype 3a (129, 145 and 99 bp)

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. [26]

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

Table 2: Hepatitis C virus genotypes in 86 patients with RFLP method

Genotype	Number	%
1a	45	52.3
1b	10	11.6
2a	2	2.3
3a	25	29.1
4	4	4.7
Total	86	100

DISCUSSION:

HCV contain a positive polarity, single-stranded RNA genome with *5'* and 3' UTR. The core (C), envelope 1 (El) and envelope 2 (E2) proteins are encoded at the 5' terminus and the non-structural proteins (NS) are encoded at the 3' terminus of the single open-reading kame of the genome [29].

HCV infection is still one of the major health problems in the world. HCV nucleotide sequences, which are different from each other up to 30 % of the time, are divided into six genotypes and more than 80 subtypes [4].

Different genotypes of HCV demonstrate different geographical distribution, besides, the severity of HCV hepatitis and the outcome of chemotherapy against HCV genotypes is not similar. HCV type 1 infections are common worldwide. Genotypes 1, 2 and 3 are more common genotypes and are observed mostly at Europe, North America, China, Japan and Australia. There are more significant differences in the distribution of subtypes. Type 1a often was found in Northern Europe and North America, and Iran; type 1b is the most common genotype in Japan, South and Eastern Europe. Genotype 1 was found in 71 % of the infected population in USA and type 2 is rarer than type 1 in the world. HCV subtypes 2a and 2b are relatively common in North America, Europe and Japan and subtype 2c is most common in northern Italy [9, 30, 31].

The majority of patients are infected with HCV genotype 3 in Thailand, Malaysia, India and Pakistan [32]. HCV genotype 4 appears to be prevalent in North Africa and the Middle East. This genotype is reported to be dominant in Yemen, Kuwait, Saudi Arabia, Iraq, Zaire, Gabon and Gambia. Genotypes 5 and 6 seem to be confined to South Africa and Southeast Asia, respectively [9, 33, 34]. In our study HCV genotypes were found, 1a (52.3%), 1b (11.6%), 3a (29.1%), 2a (2.3%), 4 (4.7%).

HCV genotyping has been particularly important for studying the relationship between type/subtype and clinical status, pathogenesis, and disease outcome. This is useful for vaccine research and development, specifically because different genotypes often respond differently to antiviral treatment [9, 35, 36]. In addition to treatment purposes, detection of HCV genotypes in different regions can be used for the purpose of molecular epidemiology [37].

HCV genotyping can be performed with several molecular techniques. According to Furione *et al* the sensitivity of RFLP for genotyping of HCV is 96.2 % [22]; therefore, we decided to use RLFP for genotyping of HCV in chronic hepatic patients.

As a result of this study genotype 1a (52.3%) was the most common HCV genotype in our region. In studies conducted in our country also the predominant type is often 1a. The results of this study will guide clinicians in successful monitoring and treatment of HCV patients in our region.

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