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Determination of HCV Genotypes by Direct Sequencing Method in Kermanshah Province, Western Iran

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Hepatitis C virus is an important human pathogen that can cause acute and chronic hepatitis, liver cirrhosis, and possibility, hepatocellular carcinoma. Assays to determine the HCV genotypes have recently become relevant in the investigation of many aspects of HCV infection, including epidemiology, pathogenesis, treatment and control strategies. Six major hepatitis C virus genotypes have been characterized, which vary in their geographical distribution. Direct sequencing is gold standard method for HCV genotyping. In this study, the distribution of HCV genotypes by direct sequencing of Core region of HCV genome in an ABI-3130 DNA analyzer and their association with possible risk factors in a group of HCV infected patients from Kermanshah province of Iran was investigated. The genotypes of cases were revealed using direct sequencing of Core region of HCV genome. Risk factors were also recorded and a multivariate analysis was performed. Among 180 infected people, 138 (76.6%) with 3a genotype, 35 (19.4%) with 1a genotype, 3 (1.7%) with 1b genotype and 4 (2.2%) with 3a and 1b were determined. HCV was transmitted by different routes such as intravenous drug abuse (IVDA), tattooing, sexual, blood transfusion. IVDA is the main risk factor in this study and genotype 3a is the predominant genotype in the all groups. This study revealed that 3a is the most prevalent genotypes in Kermanshah province.

Keywords: HCV Genotyping , Kermanshah, Direct sequencing.

Infection to Hepatitis C virus (HCV) is one of the critical health problems worldwide. HCV infection is one of the main causes of chronic viral hepatitis, hepatocellular carcinoma and also cirrhosis[1]. HCV also is the leading reason for liver transplantation in the United States and it is controversial concepts in organ shortage[2]. HCV infection has reached epidemic proportions. Annually, more than one million infected new cases are reported in world[3]. Even though, the incidence of new HCV infection is declining, at least in industrialized countries, yet HCV infection with an estimated prevalence of 3% in the world population is known as heavily burdens public health[4].

The HCV genome is an enveloped positive-sense, single-stranded RNA genome, with approximately 10 kb long. It has marked similarities to those of members of the genera Pestivirus and Flavivirus[5]. In infected persons, HCV is a reservoir of related genetic variants, referred to as quasispecies[6]. The encoding genes for the envelope glycoproteins (E1 and E2), are the most heterogeneous, especially the 81 nucleotides encoding hypervariable region 1 (HVR1) of E2. HVR1 mutation occurs during the natural course

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of HCV infection in untreated immunocompetent persons[7]. The mutation rate has been estimated to be 0.1 to 0.2 nucleotide substitutions per genome site per year, and several amino acid changes occur over a period of 1 year or more[8].

Up to now, many complete or partial sequences of HCV genome have been reported[5, 9-12], that disclosed marked genetic heterogeneity of the HCV genome[13]. HCV strains isolated in different part of the world were classiûed into 6 genotypes (genotypes 1-6) and numerous subtypes (e.g., subtypes 1a and 1b). Based on the identiûcation of these genomic differences, HCV has been classiûed into different strains. Maybe genetic heterogeneity of HCV has an important role for some of the differences in disease outcome and that genotypes are the strongest predictor of the virological response to treatment with interferon (IFN)[2, 14]. Genotypes 1, 2, and 3 are widespread, whereas others are limited to certain geographical areas[2, 15]. The prevalence of hepatitis C virus (HCV) in an area is not constant, and depends on the changes in route of infection, which may change over time.

Kermanshah province, located in the west of Iran, has 1.95 million population which 69.7% reside in urban. While literacy of Kermanshah is 81.7%, this province has more drug abusers compared to neghlibore provinces (http://www.amar.org.ir/Default.aspx?tabid=133). Furthermore, Kermanshah shares border with Iraq in the west. The aim of this study was to determine HCV genotype prevalence in Kermanshah Province, Iran. The distribution of HCV genotypes and their association with possible transmission routes (risk factors) in a group of HCV infected patients from Kermanshah province where located in western of Iran was investigated, as the data exclusively related to this area is limited.

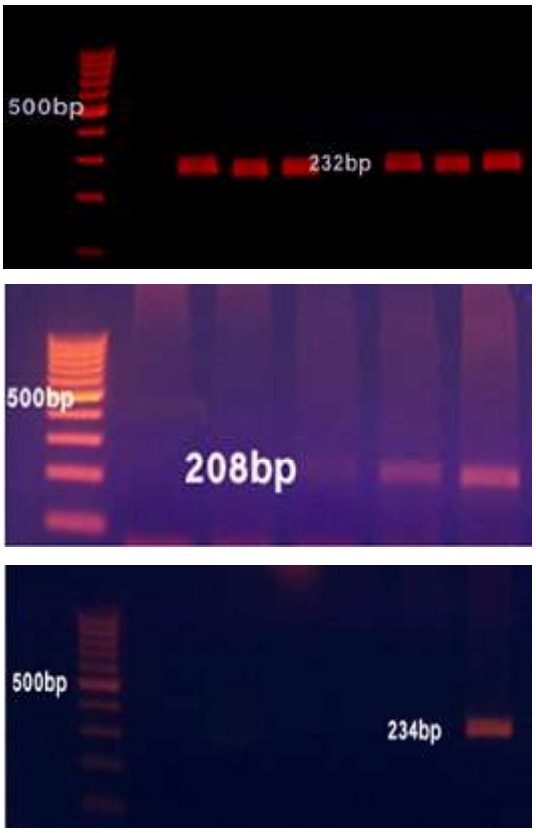

Fig. 1. 2.5% agarose gel electrophoresis of the PCR products of the HCV DNA from different genotypes. DNA marker 500 bp (lane M); A: Genotype 3a; B: Genotype 1a; C: Genotype 1b

Patients and methods

Patients and ethics

In a cross-sectional study, a total of 300 people suspect to HCV infection by specialist

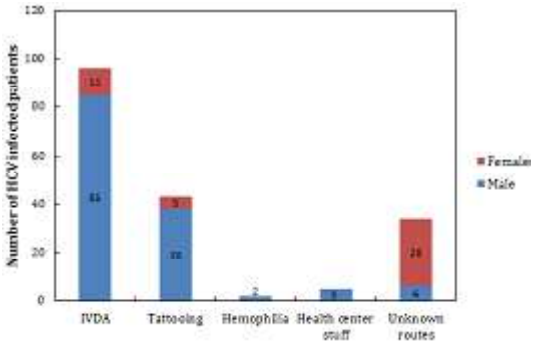

Fig. 2. Routes of infection in HCV infected patients

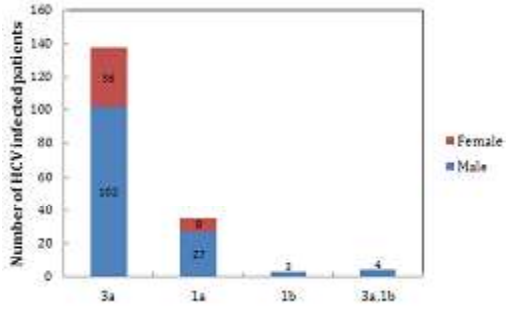

Fig. 3. HCV genotype distribution in Kermanshah province

Table 1. PCR, sequencing, and genotyping Oligonucleotide primers (modified from Ohno et al., 1997 (13)).

PCR round	Sequence (5'-3')	Primer
1st round PCR	GGGAGGTCTCGTAGACCGTGCACCATG	Sc2
1st round PCR	GAG(AC)GG(GT)AT(AG)TACCCCATGAG(AG)TCGGC	Ac2
2nd -round PCR for sequencing	AGACCGTGCACCATGAGCAC	S7
2nd -round PCR for sequencing	TACGCCGGGGGTCA(TG)T(GA)GGGCCCCA	A5
		Mix 1
2nd -round PCR for genotyping	AGACCGTGCACCATGAGCAC	S7
2nd -round PCR for genotyping	AACACTAACCGTCGCCCACAA	S2a
2nd -round PCR for genotyping	CCTGCCCTCGGGTTGGCTA(AG)	G1b
2nd -round PCR for genotyping	CACGTGGCTGGGATCGCTCC	G2a
2nd -round PCR for genotyping	GGCCCCAATTAGGACGAGAC	G2b
2nd -round PCR for genotyping	CGCTCGGAAGTCTTACGTAC	G3b
		Mix2
2nd -round PCR for genotyping	AGACCGTGCACCATGAGCAC	S7
2nd -round PCR for genotyping	GGATAGGCTGACGTCTACCT	G1a
2nd -round PCR for genotyping	GCCCAGGACCGGCCTTCGCT	G3a
2nd -round PCR for genotyping	CCCGGGAACTTAACGTCCAT	G4
2nd -round PCR for genotyping	GAACCTCGGGGGGAGAGCAA	G5a
2nd -round PCR for genotyping	GGTCATTGGGGCCCCAATGT	G6a

Table 2. Hepatitis C virus genotype subtypes prevalence within transmission route groups

Presentation of patients	HCV-genotype				Sig.
	3a (N = 138)	1a (N = 35)	1b (N = 3)	3a,1b (N = 4)	
Male/Female (%male)	102/36 (73.91)	27/8 (77.14)	3/0 (100)	4/0 (100)	
Transmission routes					
IVDA	77	16	0	3	0.05
Tattooing	29	10	3	1	ns
Hemophilia	0	2	0	0	ns
Health center stuff	4	1	0	0	ns
Unknown routes	28	6	3	0	ns

physicians referred to the Central Medical Laboratory of Kermanshah from 2010 to 2013. Informed consent was signed by all participants. Data were stored in the database with no reference to the subjects' names. The study protocol and the consent forms were reviewed and approved by the Ethic and Research Committee of Kermanshah University of Medical Sciences. EDTA-anticoagulated specimens in less than 2 hours centrifuged and plasma separated.

Sampling

Plasma samples were aliquoted and frozen in -70°C until RNA extraction. Total RNA was extracted by commercial QIAamp Viral RNA Mini kit (QIAGEN).

Determination of viral load by quantitative Real-time PCR

The viral load was estimated in the peripheral blood specimens using a commercial artus HCV RG RT-PCR kit (QIAGEN) for the detection of HCV RNA using Real-time PCR on ABI Prism 7500 instrument (Applied Biosystems, Foster City, CA). The amplification reaction was performed according to the manufacturer's instructions.

HCV genotyping

HCV isolates of viral load positive samples were genotyped using universally accepted method of Ohno *et al*[13]. Brieûy, based on this method for HCV genotypes manifestation, two

rounds of nested PCR was done by one step RT PCR kit (QIAGEN). Core specific primers, Sc2 and Ac2, were applied for the first- turn PCR and two mixtures of primers were used for the second- turn of Multiplex PCR. Primes of mixture A were specific for the detection of 1b, 2a, 2b, and 3b HCV genotypes (234 bp, 139 bp, 337 bp and 176 bp, respectively), while primes of mixture B were used for the detection of 1a, 3a, 4, 5a, and 6a HCV genotypes (208bp, 232bp, 99bp and 336bp, respectively). Meanwhile, the PCR programs of these processes described by Ohno *et al* (1997)[13] and primer sequences are shown in Table 1. The genotype speciûc band was visualized on a 2.5% agarose gel by ethidium bromide and UV light (Figure 1).

Direct Sequencing

To test the validity of PCR based genotyping, the nucleotide sequences of HCV core gene from 25 of the 180 specimens were sequenced by ABI 3130 genetic Analyzer instrument (Applied Biosystems, Foster City, Calif.).

Statistical analyses

Data are presented as percentage (%) or number of patients. Chi-Square and Fisher's Exact tests were carried out by SPSS statistical package version 14.0 for windows. *P* values less than 0.05 were considered significant.

RESULTS

From 300 cases referred to Reference Clinical Laboratory 180 case shown viral load positive by Real time PCR assay. Among these 180 HCV positive cases, 44 (24.4%) were female and 136 (75.6%) were male with an age range between 18 to 76 years old. There is a notable statistic; from 180 infected patients, 146 cases (81.1%) were connected with suspicious blood; 96 cases were addicted (IVDA), 43 cases had history of tattoo, 5 cases were health center staff, 2 cases had hemophilia and 34 cases (18.9%) have unknown risk factors (Figure 2) that maybe is related to high risk sexual behavior that didn't declare because of cultural reasons. One important thing in the mentioned data is that 7 cases means 3.9% of total patients, 5 cases of Health staffs and 2 hemophilia cases, are infected due to fault in Health systems because frequency of HCV infection in normal population is 0.5-1% (16). This is an unfortunate report that could be a catastrophe for a Health system. Interestingly, 82.36% of unknown routes group that may referred to high risk sexual behavior cases comprises of women. These cases are not IVDA but almost have addicted spouse.

As shown in Figure 3, RT-PCR genotyping results for 180 people infected by HCV showed that 138 people (76.6%) had 3a genotype, 35 people (19.4%) had 1a genotype, 3 (1.7%) had 1b genotype and finally 4 (2.2%) had 3a and 1b genotype. Genotypes 2a, 2b and 3b were not detected in any samples. Additionally, the results of direct sequencing for 25 cases, randomly selected, strongly confirmed RT-PCR results.

Classification of cases based on risk factors revealed that the predominant HCV genotype is 3a among drug abusers, tattooing and maybe high risk sexual behavior (unknown risk factors) groups (Table 2). These groups comprise of <45 years old cases which consist with previous study that demonstrate usually IVDA cases have 3a genotype[17].

DISCUSSION

Different studies indicated that HCV is the most important etiological factor for transfusion-acquired and sporadic non-A, non-B hepatitis[13, 18]. The variability in HCV genomes has been proven by comparative analysis of HCV isolates from different geographical regions. The variability in HCV genotypes leads to different serological reactivity and differences in treatment response[2, 15, 19-22]. Based on sequence variation in both the coding and non-coding regions, several classiûcation systems have been proposed. Ohno *et al* classified HCV to 1a, 1b, 1d, 2a, 2b, 3a, 3b, 4, 5a, and 6a isolates, based on the core region PCR with genotype-speciûc primers, that is widely acceptable[13]. Genotypes 1, 2, and 3 of HCV become manifest to have a worldwide distribution; however, their relative prevalence differs from one geographic region to another. 1b subtype, in Japan is responsible for about 73% of HCV infection[23]. In the United States and Europe, 1a and 1b subtypes are the predominant genotypes[24-27]. HCV genotype 3a is more common among the abusers of intravenous drug in the United States and Europe[17]. In Middle East genotype 4 of HCV is common[28, 29]. HCV genotype distribution in Tehran, located in

the center of Iran, indicated that 3a genotype was the most frequent type (46.6%), and then type 1 (43.2) is more common[30]. Another study in Northeastern of Iran shows 3a (40%) and 1a (39.2%) types are most prevalent[31]. A survey in Southern Iran shows 3a (26.2%) and 1 (11.1%) genotypes are common[32]. The present study in Western Iran, Kermanshah Province, revealed that 3a (76.7%) and 1a (20%) genotypes are prevalent.

In conclusion, these results indicate that type 3a of HCV is most prevalent in Kermanshah Province, Iran, which is different from other reports around the world.

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