Iranian J Publ Health, Vol. 35, No. 4, 2006, pp.54-61

Determination of HCV Genotypes, in Iran by PCR-RFLP

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(Received 10 Dec 2005; accepted 24 May 2006)

Abstract

Hepatitis C is a major cause of liver related morbidity and mortality worldwide and represents a major public health problem. Depending on genomic organization, the virus is divided into six genotypes and a number of subtypes. Different genotypes are seen in different parts of the world. Genotype one 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 materials of 214 patients' sera were detected by nested-RT PCR. Based on genomic differences among different genotypes, the PCR products were digested with proper enzymes and studied by RFLP. Except for one, sequencing of 14 samples, representative of all genotypes, confirmed the results of PCR-RFLP. The results of PCR-RFLP were as follows: 1a (52.88%), 1b (14.01%), 3a (27.57%), 2a (2.1%), 4 (3.44%). This indicates that a high percentage of HCV infected patients in Iran are infected with 1a or 3a genotypes. These findings reveal that the pattern of HCV genotypes in Iran differs from those of other middle-eastern countries.

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

Introduction

Hepatitis C virus (HCV) is an important cause of chronic liver disease. 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 (1). Recently, HCV infection has drawn great attention due to similar risk factors and co-infectivity with HIV infection. WHO estimation of HCV prevalence, suggests that up to 3% of the worlds population (170 million) have been infected with HCV (2). In Iran, it seems that the prevalence of general population is less than 1 percent, which is much lower than that of most of neighboring countries in this region (2). However, the infection is emerging mostly due to problems of intravenous drug abuse and needle sharing in the country (2). On the other hand, increase in number of centers providing hemodialysis and transfusion facilities for hemoglobinopathies generated new sources and susceptible populations in Iran (3). HCV, the causative agent of most cases of non A, non B hepatitis, exists as a heterogeneous group of viruses sharing at least 65% homology among different strains. This virus has a positive sense, single stranded RNA genome of approximately 9.5 kb. Consistent with related members of the Flaviviridae family, HCV demonstrates a high degree of sequence variation throughout its genome. Sequence analysis of multiple strains of HCV has demonstrated that the nucleotide sequence can differ by as much as 30% (4). 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 (5, 6). There are three functional regions of the genome, the 5'-UTR, the

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coding region encoding the structural and nonstructural viral proteins, and the 3'-UTR (7). The polyprotein is processed into structural and nonstructural proteins. The core and the two envelope proteins (E1 and E2) are parts of the virion (7-9). 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 (10). Phylogenetic analysis may aid in the separation of sequences into distinct types (11). Six major genotypes (HCV-1 to HCV-6) have been described so far, each containing multiple subtypes (e.g., 1a, 1b, etc). The isolates formerly published as genotypes 7 to 11 are now considered subtypes within genotypes 3 (subtype 10) and 6 (subtypes 7, 8, 9, and 11) (12).

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 (13- 17). 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 (17).

The standard and most definitive method for HCV genotyping is sequencing of a specific PCR-amplified portion of the HCV genome obtained from the patient, followed by phylogenetic analysis. Investigators of HCV genotyping have used sequence analysis of HCV NS5, core, E1, and 5'-UTRs. However; direct sequencing of amplified DNA does not usually identify mixed infections with two different HCV genotypes (17). Other methods that have been reported depend mainly on the amplification of HCV RNA from clinical specimens, followed by either reamplification with type-specific primers or hybridization with type-specific probes (18-21) or by digestion of PCR products with restriction endonucleases that recognize genotype-specific cleavage site (22). HCV genotyping by using type-specific primers was first introduced by Okamoto et al. using primers specific for the core region (23). This method lacked acceptable sensitivity and specificity (24). Several 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 (25). 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 (26). Although all these methods are able to identify the major genotypic groups, only direct nucleotide sequencing is efficient in discriminating subtypes (27, 28).

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

Samples During 2003-2004, 214 sera from HCV-infected Iranian patients with chronic hepatitis, referred to Keivan Virology Laboratory (Tehran, Iran) were studied. Etiological factors other than chronic HCV infection were ruled out in all cases. All patients had elevated serum aminotransferases for at least 6 months, a positive test for anti-HCV antibodies (third-generation ELISA [Ortho Diagnostics, Raritan, N. J.]), and HCV RNA in serum by reverse transcription nested PCR for the 5'-UTR of the HCV genome (29, 30). The mean age of patients was 41 (range, 13 to 70) yr. One hundred forty one patients were male and 73 were female. One hundred seventy nine patients had one, two, or even three of the following criteria: 102 had a history of blood transfusion, 54 had a history of intravenous drug use (IVDU), 65 had long duration of hemodialysis and thalassemia, 43 had been tattooed, 138 had a history of ma-

jor surgery, and 76 had minor surgery. The possible source of infection was not determined in 35 patients. Results of histological investigations revealed mild chronic hepatitis in 59 cases, moderate chronic hepatitis in 49, and severe chronic hepatitis in 35 patients. There was no histological information for 71 patients. Pretherapy blood samples were obtained under appropriate conditions to improve preservation of RNA and immediately stored at -70 °C until tested (31).

HCV RNA extraction and cDNA synthesis

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 (32), 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 was synthesized from 7 µl of RNA with 200 U of AMV reverse transcriptase (Promega, USA) AMV reverse transcriptase 5X reaction buffer, 10 mM of the dNTPs in the presence of 40 U random primers and 20 U of RNase inhibitor (Promega, USA). The guidelines of Kwok and Higuchi (33) were strictly observed to prevent carryover contamination, and appropriate negative controls for RNA extraction, cDNA synthesis, and PCRs were routinely included in each PCR round. The amplification procedure involved reverse transcription at 42°C for 30 min and RNase inhibitor inactivation at 94°C for 3 min.

Primers For RT-PCR and nested PCR, four oligonucleotide primers for the 5'-UTR of HCV were designed using generunr (Hastings software) and synthesized at the SeqLab Company (Germany). In the first round of PCR, the primers corresponded to HCV-1 sense oriented nucleotides -268 to -251 F (AGCGTCTAGCCATGGCGT), numbered according to Bukh et al. (10), and antisense nucleotides -4 to -22 R (GCACGGTCTACGAGACCT). For the second round, the primer FN (GTGGTCTGCGGAACCGG) corresponded to sense-oriented nucleotides -199 to -183 and RN (GGGCACTCGCAAGCACCC) corresponded to antisense nucleotides-26 to -43.

PCR In the first round of PCR, reagent I composed of fifty mM KCl, 1.5 Mm MgCl 2, 10 mM Tris-HCl (pH 9.0), 1X Taq DNA polymerase buffer (Fermentas, Ukraine), 200 µM each of the four dNTPs, 0.4 µM each of the first round primers and 1 U Taq DNA polymerase (Fermentas, Ukraine), were used. The amplification procedure included denaturation at 94°C for 3 min, followed by 35 cycles of amplification for 45 s each at 94 °C, 58 °C, and 72 °C and finally 5 min at 72 °C for final extension.

For the second round, PCR reagent II, composed of 50 mM KCl, 1.5 mM MgCl 2, 10 mM Tris-HCl (pH 9.0), 1X Taq DNA polymerase buffer (Fermentas) 200 µM each of the four dNTPs, 0.4 µM each of the second round primers and 1 U Taq DNA polymerase, were used. The amplification procedure was as follows: predenaturation at 94 °C for 3 min, 25 cycles for 1 min steps at 94 °C, 65 °C, and 72 °C, respectively, and final extension at 72°C for 4 min.

Fifteen µl out of the 25 µl of PCR and nested-PCR products were analyzed by horizontal gel electrophoresis on 2% agarose in TBE 1X buffer at 100 V/50 min. A single, 264 bp band was sometimes visible after the first round, but a 174 bp was preserve in all cases after the second PCR.

Genotyping by RFLP Total volume of each nested-PCR product (25 µl) was divided into three tubes containing appropriate buffers. Restriction enzymes, *Apa I*, *Hinf I*, *EcoR II* and *Bsh1236* (Fermentas, Ukraine) used as the following combinations: 1.*Apa I* / *Hinf I*; 2. *EcoR II* / *Hinf I*; 3. *Bsh1236 I*. *Bsh1236 I* was found to be informative by sequence analysis of different strains, while other enzymes had been reported previously by McOmish et al. (34). Tubes were incubated with 1 µl of the enzyme mixture for 3 h at 37 °C. The digests were heated for 5 min before analysis by vertical 12% polyacryl amide gel electrophoresis. After ethidium bromide staining, the DNA fragments were identified under ultraviolet light. Molecular weight 100 bp plus marker (Fermentas, Ukraine) and undigested PCR products was included in each

analysis. The genotypes were deduced from the fragmentation patterns of the amplified DNA.

Direct sequencing of 5'-UTR PCR fragments were cut out from the agarose gel and purified using a mini column system (MN Germany). Cycle sequencing was performed using the ABI PRISM The BigDye Terminator v3.1 Cycle Sequencing Kit (USA) following the manufacturer's instructions. Inner PCR primers (FN, RN) were used as sequencing primers to obtain the entire nucleotide sequences of the two strands. Sequencing reactions were prepared according to the manufacturer's instructions before automatic electrophoresis of the sequencing products by an ABI PRISM 3700 (USA) Genetic Analyzer.

Results

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. (EMBL/Gen Bank/DDBJ, accession No.11443). This table shows fragments yielded upon restriction enzyme digestion of 5'-UTR region. The cutting sites of restriction enzymes are shown (A) *Apa I*/*Hinf I*; (B) *EcoR II* /*Hinf I*; (C) *Bsh1236 I.*

Fig. 1 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 214 sera from patients who were either recently infected by HCV or with history of previous HCV infection and positive PCR results was performed. Study of results of sequencing of 14 PCR positive sera demonstrated that except in 1 case, results of RFLP genotyping and sequencing were comparable. The RFLP result indicated 1b subtype but the sequencing showed 3 h. Table 2 shows the results of sequencing in comparison to those of RFLP. The results of PCR-RFLP were as follows: 1a (52.88%), 1b (14.01%), 3a (27.57%), 2a (2.1%), 4 (3.44%). This indicates that a high percentage of HCV infected patients in Iran are infected with 1a or 3a genotypes (Table 3).

Analysis of population previously infected with HCV showed that only 26 % were above 50 yr of age (Table 4). The most frequent genotype in patients above 50 was 3a, while 1a genotype was more prevalent in patients under 50 yr old. Hemodialysis patients and cases by known history of transfusion were known to be infected by only subtypes 1a and 3a. Also the patients under 30 yr old were infected by only subtypes 1a and 3a. A higher percentage of mixed infection (1a/ 3a) was observed in IVDU and Thalassemic patients in comparison with other groups.

The 12 HCV sequences (5'-UTR) from the Iranian patients have been submitted to GenBank; the 5'-UTR sequences were given these accession numbers: AY523463 to AY523466, AY515300, and AY545671 to AY545678.

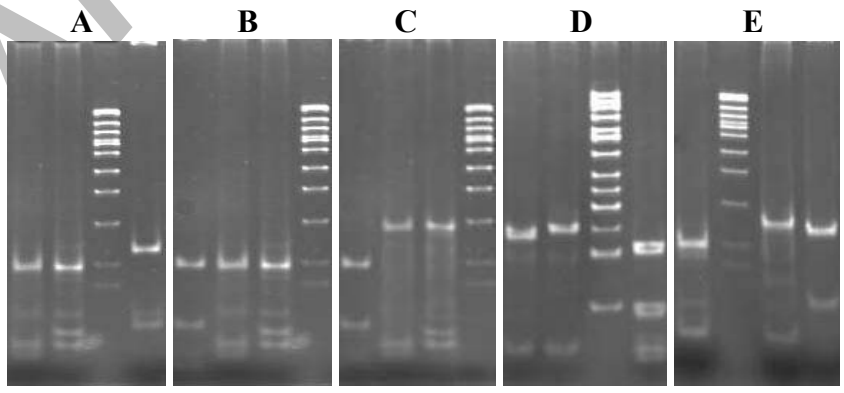

Fig. 1: 12% polyacrylamide gel electrophoresis of the digestion products of the amplified DNA from different genotypes. Marker; 50bp (Fermentas, Ukraine) A; **1a (97bp, 97bp, 129bp)**, B; **1b (97bp, 97bp, 99bp)**, C; **2a (97bp, 174bp, 174bp)**, D; **3a (129bp, 145bp, 99bp)**, E; **4(97bp, 145bp, 129bp)**

Table 1: RFLP of the 174 bp DNA fragment from 5'-UTR of different HCV genotypes. (1) *Apa* I/*Hin*f I; (2) *Eco*R II/ *Hin*f I; (3) *Bsh*1236 I. The sequences were from Bukh et al. (1992, 1994), except for 3a, obtained from EMBL

Genotypes	*Segments*		
	Tube A	*Tube B*	*Tube C*
1a	97bp	97bp	129bp
1b	97bp	97bp	99bp
2a	97bp	174bp	174bp
2b	174bp	174bp	174bp
3a	129bp	145bp	99bp
3b	97bp	145bp	99bp
4	97bp	145bp	129bp
5	97bp	174bp	99bp
6	97bp	97bp	174bp

Table 2: Comparison of RFLP typing and Sequencing

	Sequencing					
RFLP	1a	1b	2a	3a	4	**3h**
1a	3	-	-	-	-	-
1b	-	3	-	-	-	-
2a	-	-	1	-	-	-
3a	-	-	-	4	-	-
4	-	-	-	-	2	-
1b	**-**	**-**	**-**	**-**	**-**	**1**

Table 3: Hepatitis C Virus genotypes in 214 Iranian patients with RFLP method

Genotype	**No.**	**(%)**
1a	117	(52.88)
1b	28	(14.01)
2a	1	(2.1)
3a	63	(27.57)
4	5	(3.44)
Total	214	(100)

Table 4: HCV genotype distribution among Iranian subjects as determined by 5'-UTR RFLP analysis

		Level (n)	*HCV Genotype: No. (%)*					
			1a	*1b*	*2a*	*3a*	*4*	*1a/3a*
Age(year)	***<30***	60	67	-	-	33	-	-
	30-50	98	50	16	1	25	3	5
	>50	56	33	11	-	45	-	11
Gender	***Male***	141	49	11	1	32	3	4
	Female	73	63	11	-	21	1	4
Transfusion		102	61	-	-	39	-	-
Hemodialysis		32	51.4	-	-	48.6	-	-
IVDU		54	59.7	8	1	20.3	3	8
Thalassemic		33	61	11	-	21	1	6
Tattoo		43	61	13	-	26	-	-
Minor surgery		76	51	17	-	24	-	8
Major surgery		138	55	13	-	32	-	-

Discussion

Hepatitis C Virus (HCV) is one of the main causes of acute and chronic hepatitis. The virus is distributed worldwide with prevalence varying between different countries from 0.2 up to 40% (35). 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 (36).

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 purpose, especially for screening of large number of specimens (37).

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. RFLP of HCV PCR positive sera and sequencing of 174 bp fragment of 5'-UTR region was used to achieve this purpose. By RFLP, we have classified all of HCV isolates from Iranian patients to 5 genotypes. We could not determine genotype of one sample by RFLP, and through sequencing it was determined to be subtype 3b. We have recently shown that subtypes 1b and 3b could be very similar in their 5'-UTR region (38). It seems that sequencing of another region of HCV genome (Core, E1, and NS5B) followed by phylogenetic analysis may clarify the differences between these two subtypes.

In the first report in Iranian patients, the prevalence of specific genotypes in 15 samples was studied in Tehran and the results were as follows: Type I/1a in seven cases, Type II/1b in three cases-and Type V/3a in four patients (39). One sample disclosed Type 4. A recently published article in Iranian patients with anti-HCV Ab positive from Tehran and five cities from different locations of Iran, showed that genotype 1a was predominant (47%), and 3a, 1b, as well as 4 were 36%, 8%, and 7%, respectively (40).

There are limitation in determining the precise geographic distribution of HCV genotypes and subtypes in many areas of the world. Only a few countries have detailed information about the molecular epidemiology of HCV. In Western Europe, the most common type is 1a, whereas in southern and Eastern Europe type 1b is more common (26, 41).

There is also difference between southeastern Europe and Turkey (mainly type 1b) and the Middle East. The major genotype in the Republic of Yemen, Kuwait, Iraq, and Saudi Arabia is type 4 (42).

It was concluded that the HCV genotypes pattern in Iran differs from other middle-eastern countries and is more similar to Western European region.

Acknowledgments

We acknowledge Mr. M Abachi, Mr. M Karimi, Mr. A Adeli and Mr. S Farajnia for their excellent technical assistance and advice. This work was supported by grants from: Keyvan Virology Lab. and Pasteur Institute of Iran, Tehran, Iran.

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