ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 4 (2): 139-143, 2005
ISSN 1682-296X

Determination of HCV Genotypes in Iran

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Abstract: In this study 55 serum samples of HCV suspected patients were subjected to RT-Nested-PCR. Thirty five samples were negative and 20 samples were positive and eligible for RFLP analysis. Four different enzymes including RsaI, NcoI, HaeIII and SmaI were used for genotyping differentiation of HCV, according to currently available genotype patterns. In this study, 11 cases (55%) were typed as type 1, 4 cases (20%) as type 3a and 5 cases (25%) were untypeable.

Key words: HCV, genotyping, Iran, PCR-RFLP

INTRODUCTION

Hepatitis C virus (HCV) is considered the major cause of post transfusion non A-non B hepatitis. The viral genome, a positive-sense single stranded RNA (ssRNA) of about 9400 nucleotides, is characterized by a high genetic heterogeneity like other RNA viruses[1]. There are three functional regions of the genome, the 5′ untranslated region (5′ UTR) and the coding region encoding the structural and nonstructural viral proteins and 3′UTR. HCV demonstrates a high degree of sequence variation throughout its genome. Levels of heterogeneity differ considerably among the various regions of the virus. 5′UTR region is the most highly conserved region among HCV strains and has made it the target of choice for most Reverse Transcriptase (RT)-PCR detection assays[2-4].

Sequence analysis on isolates from different geographic areas around the world has revealed the presence of six major types and several subtypes[5]. This HCV diversification is the result of the accumulation of mutations due to the lack of proof-reading activity of the RNA-dependent RNA polymerase and several selective pressures, among which epidemiological factors play a major role[6,7]. It has been postulated that differences in nucleotide sequence could result in differential activity of HCV proteins that could alter the rate of HCV replication, sensitivity to the antiviral activity of interferon or pathogenicity of the virus[7,8]. In addition to the determination of the presence or absence of HCV infection, has also been increased clinical interest in the genotyping of HCV. These genotyping results can be useful in viral transmission studies, as well as in epidemiological investigations, the progression of disease, interferon responsiveness and vaccine development[2-4,9-14].

In recent years, substantial evidence has indicated that, genotype 1 in particular, cannot be treated efficiently with IFN-α. While, genotypes 2 and 3 respond favorably, moreover genotype 1 infection may proceed more rapidly to severe forms of chronic hepatitis, cirrhosis and hepatocellular carcinoma, when compared with genotypes 2 and 3[4,7,9,10,15].

Several methods for HCV genotyping exist, such as Restriction Fragment Length Polymorphism (RFLP), DNA hybridization method called the line probe assay, serotyping and direct sequencing[2,4,9,15,16]. The aim of this study is the genotyping of hepatitis C virus in Iran.

MATERIALS AND METHODS

Sampling: Fifty five serum samples of HCV suspected patients analyzed in this study were obtained from different laboratories in Tehran.

RNA extraction: RNA was extracted from 50 μL of serum by RNX™ (plus) buffer (CinnaGen) and resuspended in 10 μL of diethyl pyro carbonate treated water.

cDNA synthesis: Nucleotide primer sequences specific for a 250 bp target located within the 5′UTR of the virus were used to generate amplification products and

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analysed by RFLP. Reverse Transcription (RT) was performed by incubating the template (equivalent to 50 µL of serum) in a 20 µL reaction mixture containing, 40 pmol of specific antisense external primer (HCV1R1 5`-GGT GCA CGG TCT ACG AGA CCT C-3`), 100 unit of Reverse Transcriptase Enzyme (RT), 1x RT buffer, 0.2 mM dNTP, for 1 h at 42°C. Reverse transcriptase was inactivated by heating at 95°C for 5´ and cDNA was kept cool until PCR amplification, which was performed immediately.

Nested-PCR amplification: The nest I PCR was performed as follows: 20 µL of the cDNA was added to a 10 µL PCR mixture containing, 1xPCR Buffer, 1.5 mM $MgCl_2$, 40 pmol each of specific sense and antisense external primers (HCV1F1 5`-CTGTGA GGAACTACTGTCTT-3` and HCV1R1 5`- GGT GCA CGGTCTACGAGACCT C-3`), 1.25 unit of Taq DNA Polymerase. These primers amplified a 296 bp fragment of HCV 5`UTR.

The nest I of PCR was done in thermocycler machine using the following cycling parameters: the first denaturation step at 94°C for 5´, 30 cycles with template denaturation at 94°C for 40", primer annealing at 54°C for 40", extension at 72°C for 40" and final extension step at 72°C for 5´to complete the amplification reaction.

The nest II of PCR was performed in a volume of 30 µL using 1 µL of nest I reaction as template, PCR mixture containing 40 pmol each of specific sense and antisense primers internal primer (HCV2F2 5`-TTCACGCAGAAAGCGTCT AG-3` and HCV2R2 5`-GGGCACTCGCAAGCACCCTATC-3`), 1.5 mM $MgCl_2$, 1.25 unit Taq DNA Polymerase, 0.1 mM dNTP, 1xPCR buffer. Cycling parameters were as follows: First denaturation step at 94°C for 5´, followed by 30 cycles, denaturation step at 94°C for 40", primer annealing at 58°C for 40", extension at 72°C for 40" and final extension step at 72°C for 5´. These primers amplified a 253 bp fragment of HCV 5`UTR.

Electrophoresis: PCR products were analyzed by electrophoresis on a 2% agarose gel and DNA bands were visualized by UV transilluminator after ethidium bromide staining.

RFLP: To determine HCV genotypes, restriction fragment length polymorphism (RFLP) analysis of PCR products was performed by 4 restriction enzymes including HaeIII, NcoI, SmaI and RsaI. The nest II PCR products (250 bp) were digested with restriction enzymes as mentioned previously for 1 h at 37°C and digested patterns were visualized using electrophoresis on 10% poly acryl amide gel after ethidium bromide staining.

RESULTS

Fifty five serum samples of HCV suspected patients were selected to genotyping by RT-Nested-PCR. Figure 1 shows an 250 bp fragment as PCR product. Thirty five samples were negative and 20 samples were positive and eligible for RFLP analysis. Of the 20 HCV-RNA positive sera, which tested by RFLP, 11 cases (55%) were typed as type 1, 4 cases (20%) as type 3a (Fig. 2) and 5 cases (25%) were untypeable.

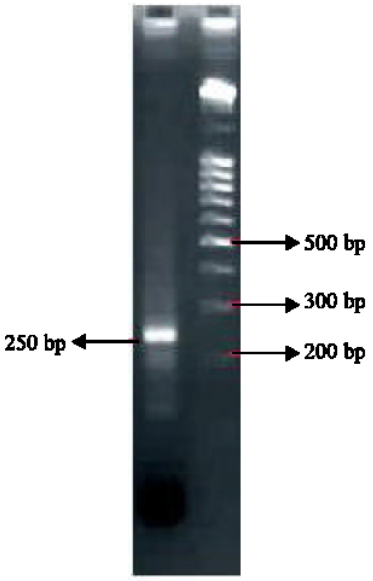

Fig. 1: PCR product amplified from the 5´UTR-region on 2% agarose gel

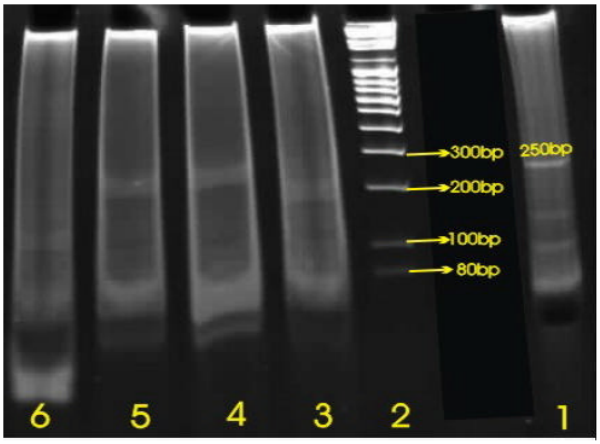

Fig. 2: RFLP analysed of PCR products amplified from the 5´UTR-region. The electrophoretic pattern of genotype I. PCR product were digested with: lane 3, SmaI; lane 4, NcoI; lane 5, HaeIII; lane 6, RsaI; lane 1, PCR product; lane 2, Molecular weight marker

DISCUSSION

Hepatitis caused by hepatitis C virus has become a major emerging infectious disease problem, with an estimated 170 million people infected worldwide[9]. In industrialized countries, HCV accounts for 20% of acute hepatitis cases, 70% of chronic hepatitis cases, 40% of end-stage cirrhosis cases and 60% of hepatocellular carcinoma cases. It has become one of the most common reasons for liver transplantation[9]. HCV exhibits a great degree of genetic heterogeneity and these differences influence the clinical outcome after infection, transmission patterns and clinical management of chronic HCV infection[4,9,17]. Several methods for HCV genotyping currently exist. The reference method is direct sequencing of products amplified. However, because this method is expensive and time-consuming and requiring special equipment for sequencing, it has been restricted to the research setting and considered impractical for large clinical studies. Instead RFLP is widely used in epidemiological studies with a high accuracy[2,3,9,16].

Sequencing method cannot determine the mixed infection; one of the best advantages of RFLP is to distinguish mixed infection in patients[4,15]. However RFLP cannot distinguish all virus subtypes or some novel genotypes[6,18,19]. We modified Park's RFLP method[12] which performed for HCV genotyping in Korea, but they used NicI and BstuI instead of SmaI and NcoI. In this study 55 serum samples of HCV suspected patients were subjected to RT-Nested-PCR. Thirty five samples were negative and 15 samples were weakly positive, possibly due to low viral titer of HCV-RNA in serum[3,6] and 20 samples were positive that subjected to RFLP.

Eleven cases (55%) were typed as type 1, 4 cases (20%) as type 3a and 5 cases (25%) were untypeable. This method identified 6 major types of virus but unable to distinguish between subtypes 1a and 1b, 2a and 2b, because of similar RFLP patterns. In one of the unnoticeable sample RFLP with SmaI, RsaI, HaeIII was similar to type 1 but RFLP with NcoI gave discordant results. In another, RFLP with HaeIII, SmaI and NcoI was similar to type 1 but RFLP with RsaI show different results. RFLP pattern with SmaI, RsaI and HaeIII in another sample was similar to type 2 but RFLP pattern with NcoI was not similar. Two another untypeable cases had completely different RFLP pattern (Table 1). Determination of these untypeable samples is possible probably by using another restriction enzymes, or another method such as direct sequencing. Perhaps these samples belong to novel HCV strain.

According to these results appears that type 1 and then type 3a is more prevalent than other HCV types. It's necessary to be considered that the population in this study was small. To determine the prevalence of HCV genotype there is a great need for epidemiological studies. These results are similar to the results obtained from Iran[14], Estonia[17,20], Uzbekistan[21] and England[22]. Zali *et al.*[14] performed HCV genotyping by type-specific primers deduced from the HCV core gene. Fourteen cases were grouped into five genotypes as follows: type 1a in 7 cases (47%), type 1b in 3 patients, type 3a in 4 patients (27%), one patient (6%) was found to have type 4. These results showed that type 1 and then type 3a are more prevalent in Iran[14]. HCV genotyping according to PCR-RFLP was used by many of research groups in word and appears that it is more efficient than the other methods (except direct sequencing which has high accuracy). The concordance of genotyping results base on RFLP and other methods was acceptable[6,16,17,23,24]. Also untypeable samples observed in other studies[6,7,15,16] and impossibility of assigning a virus type (was with RFLP) is obviously better than a wrong assignment[6].

Table 1: RFLP pattern due to HaeIII, NcoI, RsaI and SmaI digestion of 253 bp, 5'UTR region

	Virus types								
Enzymes	1a	1b	2a	2b	3a	3b	4	5	6
HaeIII	35	35	35	35	35	35	63	63	35
	215	215	55	55	215	55	187	187	55
			160	160		160			162
NcoI	21	21	21	21	21	21	none	none	19
	229	229	229	229	229	229	none	none	231
RsaI	26	26	26	26	26	26	15	54	26
	101	101	43	43	32	32	54	73	41
	123	123	58	58	69	69	58	123	58
			123	123	123	123	123		125
SmaI	66	66	66	66		none	38	38	64
	184	184	184	184	184	none	212	212	186

Davidson *et al.*[25] demonstrated that a combination of six restriction enzymes could identify virus subtypes 1a, 1b, 2a, 2b, 3a, 3b and virus genotypes. The accuracy of PCR-RFLP typing for HCV genotypes 1 to 6 was 97 and 95% for differentiation between subtype 1a and 1b. They used enzymes HaeIII, RsaI, HinfI, MvaI, BstuI, ScrfI, but PCR-RFLP system couldn't distinguish all virus subtypes or some novel genotypes discovered in Thailand and Vietnam[25]. There is interesting point in many researches that discrepant results according to the analyzed different HCV genomic region have been reported[3,6,15,16,24]. In this regard, at least three potential explanations should be considered:

a) The coexistence of HCV genotypes for which primers used during RT-PCR. Displayed different sensitivity and/or specificity.
b) *In vitro* template shuffling during RT-PCR amplification.

c) Hypothetical possibility of recombination between different HCV variants showing nucleotide sequences belonging to different genotypes throughout viral genomes[16].

Even when different restriction enzymes used in the same region, observed different genotyping results in some studies so the restriction enzymes to determine the genotype and subtype distribution must be selected carefully[7,16,18]. To reach more accurate genotyping results is better direct sequencing is performed and RFLP pattern in 5′UTR and core region are combined to determinate genotypes 1-6 and different subtypes[6,7].

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