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Detection of the Predominant Hepatitis C Virus Genotypes using Linear Probe Assay for 5'NC Region in Iran

[1]Katayoon Samimi Rad, [2,3]Nafiseh Sadat Naghavi and [3]Mansoor Salehi
[1]Department of Virology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
[2]Department of Microbiology, Falavarjan Branch, Islamic Azad University, Isfahan, Iran
[3]Division of Genetics, Department of Biomedical Sciences, Medical School, Isfahan University of Medical Sciences, Isfahan, Iran

Abstract: The rates of infection, the major risk factors for the HCV infection and the distribution frequency of HCV genotypes evaluated among anti-HCV positive haemophiliacs and thalassemics in Iran. Nested RT-PCR used for detection of HCV infection among anti-HCV positive patients. The genotypes of viruses analyzed via Linear Probe Assay (LiPA) method. Sixty percent of the anti-HCV positive haemophiliacs and 50% of the anti-HCV positive thalassemics were HCV infected. Blood clotting factors injection rates in hemophiliacs and the first injection time in thalassemics determined as the main risk factors in HCV infection. Genotyping analysis showed type-1 in 62.5% of hemophiliacs and 87.5% of thalassemics, type 3a in 40.62% of hemophiliacs and 25% of thalassemics and type 6a in one of the haemophiliacs. A total of 9.37% of haemophiliacs and 37.5% of thalassemics were infected with mixed types. The first injection time and the rate of injection determined as the main risk factors in HCV infection. The predominant types of HCV were type 1 and 3a which is similar to the results from the other areas in Iran. Also we detected the type 6a which is not a common type in Iran and not seen in the previous studies.

Key words: Hepatitis C virus (HCV), rate of infection, genotyping, linear probe assay

INTRODUCTION

At least 170 million people are infected by Hepatitis C virus (HCV) all over the world. About 80% of these patients progress to chronic infection, 40% to the end stage cirrhosis and 50-76% to the hepatocellular carcinoma (Moradpour *et al*., 2001; WHO, 1999; Flamm, 2003).

Genotyping and subtyping of hepatitis C virus are from the most important analysis for determination of epidemiology, assessing of hepatitis C disease prognosis and the likelihood of response to therapy. HCV genotyping also helps to identify the source of HCV infection. Nucleotide sequence analysis is the gold standard method for identification of HCV types and subtypes. Although, because of the cost, time consuming and in convention for routine use, clinical laboratories are interested to use other methods for HCV genotyping, including LiPA, subtype-specific RT-PCR, DNA restriction fragment length polymorphism, heteroduplex mobility analysis, melting curve analysis and the serologic genotyping. One of the most widely used typing methods in clinical laboratories is the LiPA (Nolte *et al*., 2003; Verbeeck *et al*., 2008). This technique even has the ability to determine the genotype in the samples reported as unable to be genotyped by DNA sequencing and also useful for detection of mixed types (Verbeeck *et al*., 2008).

In the most studies comparing LiPA to other genotyping methods, over 97% of accuracies were shown between the methods by genotyping based on 5'NCR region of the HCV genome (Chen and Week, 2002; Lau *et al*., 1995; Ohba *et al*., 1995; Zekri *et al*., 2005; Stuyver *et al*., 1994). But at the subtype level the accuracies were lower (over 85.8%), probably because of the highly conservation of the 5'NC region in the viral genome and in several cases, only one or two nucleotide change results in distinguishing unique subtypes (Smith *et al*., 1995).

The aim of the present study was the evaluation of the rates and the risk factors affecting HCV infection. Also the distribution frequency of HCV genotypes was determined using LiPA method, among anti-HCV positive haemophiliacs and thalassemics in Iran.

Corresponding Author: Nafiseh Sadat Naghavi, Division of Genetics, Department of Biomedical Sciences, Medical School, Isfahan University of Medical Sciences, Isfahan, Iran and Department of Microbiology, Falavarjan Branch, Islamic Azad University, Isfahan, Iran Fax: 00983123120136

MATERIALS AND METHODS

Specimens and patients: A total of 108 blood samples from anti-HCV positive haemophiliacs (50 patients) and thalassemics (58 patients) collected between January 2010 and December 2010. The samples were studied for HCV infection by nested RT-PCR. Forty amplified cDNAs were detected for HCV genotypes via LiPA method. Information of the patients such as the age, sex and history of clotting factor and blood injection were achieved at the time of blood sampling. The data evaluated using SPSS version 12.

RNA extraction and cDNA synthesizes: Total RNA was extracted from the sera using RNX^{TM}plus, chloroform and isopropanol (Sambrook and Russell, 2001). The cDNA synthesized using random hexamer primer and reverse transcription (RevertAid^{TM} first strand cDNA synthesis kit, Fermentas).

RT-PCR: Nested RT-PCR performed using a pair of flanking (1, 2) and a pair of inside (3, 4) primer. The sequence of primers is shown in Table 1. A 234 nucleotide sequence within the position of -299 to 6 region in the 5'NCR in the HCV genome was amplified.

Genotyping: Genotyping performed via linear probe assay (Stuyver *et al*., 1994) in 4 steps as following:

- Denaturing of DNA using sodium hydroxide 1.7% at room temperature for 5 min
- Hybridization of the single stranded DNA with the specific oligonucleotide probes on the nitrocellulose strips using SSC buffer (VERSAT LiPA, Bayer Inc.) with detergents at 50°C in the closed lid shaking water bath (80 rpm) for 60 min
- Washing the strips using wash solutions containing SSC buffer and detergent followed by washing with phosphate buffer with 0.5% 2-chloroacetamide, NaCl and detergent
- Addition of 1.6% 5-bromo-4-chloro 3-indolyl-phosphate as substrate; 4-nitro blue tetrazoliom in 83% dimethyl formamide; and the conjugate solution containing straptavidin labeled with alkaline phosphatase. A color developing was resulted by enzyme activity on the substrate in hybridized lines on the strips

Table 1: Characteristics of the primers used in nested PCR

Primer No.	Primer sequence	Primer concentration (pmol μL^{-1})
1	5'- CCC TGT GAG GAA CTCTG TCT TCA CGC-3′	19
2	5'- GCT CAT GT GCA CGG TCT ACG AGA CCT-3	22
3	5'- CAC TCG CAA GCA CCC TAT CAG GCA GT-3'	18
4	5'- TCT AGC CAT GGC GTT AGT A G AGT GT-3′	56

RESULTS

Amplification by PCR: The amplified region was in the nucleotide position of -299 to 6 of the HCV genome. Agarose gel electrophoresis of the PCR products is shown in Fig. 1.

Rates of infections: The product of the second step of PCR was detected as a 234 bp amplified fragment in the agarose gel electrophoresis. Total 30 patients of the 50 anti-HCV positive haemophiliacs (60%) and 31 of 53 anti-HCV positive thalassemics (50%) were detected for HCV infection by nested RT-PCR.

Serological background: All patients were serologically (ELISA) positive for anti-HCV antibodies. Within the Patients with RIBA analysis background, 95.79% were RIBA positive. The results of serological test in the patients are shown in Table 2.

Risk factors: The rates of blood clotting factors injection in haemophiliacs (OR = 3) and the first blood injecting time in thalassemic patients (OR = 5.5) were the main risk factors for HCV infection (Table 3). A 2.6 folds decrease in HCV infection observed in the patients treated by interferon plus ribavirin for 1 year compared to the patients without treatment.

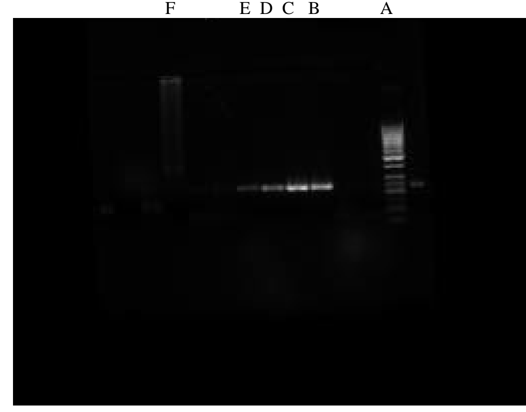

Fig. 1: The results of agarose gel electrophoresis of the PCR products. A: Fifty bp DNA size marker. B, C, D: amplified fragment with the size of 234 bp (the product of the second step of PCR). E: positive control. F: negative control (from amplification of PCR mix using water instead of target DNA)

Table 2: Serological background of the 103 patients which analyzed by PCR for HCV infection

Types of the patients	No. of patients	ELISA positive patients (%)	RIBA positive patients (%)	RIBA negative patients (%)	Patients without RIBA analysis background (%)
PCR positive haemophiliacs	30	100	34.6	0.0	63.6
PCR negative haemophiliacs	20	100	00.0	2.0	98.0
PCR positive thalassemic patients	32	100	26.1	0.0	73.9
PCR positive thalassemic patients	22	100	16.7	1.5	98.5

Table 3: The statistical analysis of risk factors for HCV infections

Risk factors in haemophiliacs	OR (90%CI)	p-value	Risk factors in thalassemic patients	OR (90%CI)	p-value
Patient age (year):	1.43(0.26,7.7)		Patient age (year):	1.47(021,10.2)	
Up to 15		1	Up to 10		1
Over than 15		0.69	Over than 10		1
The first injection:	1.2(0.17,8.4)		The first injections:	2.3(0.30,18.7)	
After 1996		1	After 1996		1
Before 1996		1	Before 1996		0.58
Patient's sex:	1.8(0.1,32.0)		Patient's sex:	1.48(0.35,6.3)	
Male		1	Male		1
Female		1	Female		0.7
Blood clotting factor injection history (unit)[1]	3.0(0.56,16.1)		Blood injection history (unit)[2]:	1.75(0.25,12.0)	
Up to 250000		1	Up to 300		1
Over than 250000		0.6	Over than 300		0.23

[1]1 unit is equal to 1 unit of concentrated clotting factor, [2]1 unit is equal to 250 mL blood

Table 4: The distribution frequency of genotypes in the patients

Haemophiliacs	Distribution frequency	Thalassemics	Distribution frequency
Genotype 1 (% of total)	20 (62.50)	Genotype 1 (% of total)	7 (87.50)
Subtype 1a (%)	3 (15.00)	Subtype 1a (%)	2 (28.57)
Subtype not detected (%)	11 (55.00)	Subtype not detected (%)	4 (57.14)
Genotype 3 (% of total)	13 (40.62)	Genotype 3 (% of total)	2 (25.00)
Subtype 3a (%)	6 (46.15)	Subtype 3a (%)	2 (100.00)
Subtype 3b (%)	0 (0.00)	Subtype 3b (%)	0 (0.00)
Subtype not detected (%)	7 (53.84)	Subtype not detected (%)	0 (0.00)
Genotype 6 (% of total)	1 (3.12)	Mixed genotype (% of total)	3 (37.50)
Subtype 6a (%)	1 (100.00)	1-3a (%)	2 (66.66)
Subtype 6b (%)	0 (0.00)	1a-1b (%)	1 (33.4)
Subtype not detected (%)			
Mixed genotypes (% of total)	3 (9.37)	Genotype not detected (% of total)	1 (12.50)
1b-3a (%)	1 (33.33)		
1-3a-6a (%)	1 (33.33)		
1a-1b (%)	1 (33.33)		
Genotype not detected (% of total)	1 (3.12)		
Total number of patients	32	Total No. of patients	8

Genotyping: The distribution of different HCV genotypes in haemophiliacs and thalassemics shown in Table 4.

The genotypes 1 (subtypes la, b) and 3 (subtype 3a) detected as the major genotypes. There was a case with the genotypes 6 in one of the hemophiliacs.

DISCUSSION

In the present study, the relative prevalence of HCV infection were detected among haemophiliacs and thalasssemics in Iran. The rates which were obtained were lower than western countries (Adamowicz-Salach *et al*., 1999; Franchini *et al*., 2001). This difference is likely because of the variability in the susceptibility and confidence of PCR procedures, the rates of curing from infection, patient ages, treatment strategies or the rate of contaminated clotting factors injection in different countries between 1970 and 1985.

The Lipa has been reported as a rapid, sensitive and accurate method which can be used as a routine tool for the detection of HCV genotypes and subtypes (Verbeeck *et al*., 2008). The predominant genotypes detected in this study were 1 and 3a which are similar to the results of our investigation based on sequencing and PCR methods in different areas in Iran (Samimi-Rad *et al*., 2004; Kazemi *et al*., 2005; Hejazi *et al*., 2007). Detection of mixed types and the type 6a is from notably importance, because the type 6a was not detected in previous studies. The origin of this type may be from the foreign concentrated clotting factors at the time in which the factors were not decontaminated with heating processes. This type was detected mixed with the types 1 and 3a by LiPA method for HCV genotyping.

CONCLUSION

In conclusion, in the most different methods for HCV genotyping, only one predominant type of HCV had been detectable (Simmonds *et al.*, 1993; Van Doorn *et al.*, 1996). We reported the mixed types and a non routine type (6a) of HCV in haemophiliacs and thalassemics in Iran using LiPA method. Because of the differences in pathogenicity and the response to treatment in different HCV genotypes, detection of mixed types by using methods such as LiPA is important for epidemiologic studies and selection of a successful protocol for treatment of the infection.

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