Iranian Journal of Virology 2015;9(2): 1-7

Original Article

Comparison of Hepatitis C Genotypes in Plasma and Peripheral Blood Mononuclear Cells (PMBCs) in Hepatitis C-Infected Patients

Shahidokht M[1], Moghimi M[1], Ahmadi-Vasmehjani A[2*], Doosti M[2]

1. Department of Pathology, Yazd University of Medical Science, Yazd, Iran.
2. Infectious Research Center, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.

Abstract

Background and Aims: The Hepatitis C Virus (HCV) is essentially hepatotropic but virus compartment has also been found in other important extra hepatic sites. Detection of HCV RNA in extra hepatic reservoirs such as peripheral blood mononuclear cells (PBMCs) is important for determining of disease progression and effective treatment. The present study aimed to determine of different HCV genotypes in plasma and PBMCs specimens from patients of Yazd province of Iran.

Materials and Methods: Blood samples of 50 patients with established HCV referred to Shahid Sadoughi Hospital were collected. These patients had positive anti-HCV and positive plasma HCV RNA. After RNA extraction from plasma and a pellet of approximately 3-5× 10^6 PBMCs, CDNA synthesis was done and stuck in -70°C. Then HCV genotyping by using of restriction fragment length polymorphisms (RFLPs) method was carried out. Finally, Data analysis was done by SPSS software.

Results: The mean age of subjects infected with HCV 38/2±14/6 (ages 21-66) who were the majority of those between ages lower 30 years. 3a and 1a genotype distribution in plasma was significantly higher in those aged over 40 than other age that this difference in genotype distribution at the age of 40 is significant (P value> 0.04) but in PBMCs was not significant (p=0.25). 1a and 3a genotype distribution in plasma and PBMCs in between 40-30 ages showed a lower frequency than other ages. In none of the samples from patients with genotype 2 and 1b genotypes but only 3a and 1a such as mixed infections was detected. Typically, mixing virus infection in 8 patients (16%) in plasma and in 16 samples (32%) was identified in PBMCs.

Conclusions: It suggested that plasma subtyping as the target genotype for considering of antiviral therapy is essential, but may be result to the goal of therapy. HCV genotyping in PBMCs samples along with plasma specimen might be beneficial. Therefore, determine of HCV genotype in PBMCs in before therapy because detection of occult infection is useful

Keywords: Hepatitis C virus (HCV), HCV genotypes, PBMCs, Polymerase Chain Reaction

Introduction

Hepatitis C is classified to at least 7 major genotypes that each genotype has several genomic subtype and generally about 67 subtype due to changes in the genome (1). The geographical distribution of HCV genotypes is varied in different regions of world although some strains in some region are endemic (2, 3). Hepatitis C virus is necessarily hepatotropic but replication in other areas such as including in peripheral mononuclear blood cells (PBMCs) of infected people has also been found (4). These cells are including in lymphocytes, Macrophages, and Dendritic cells which are a major component of the immune cells (5). It is shown that virus genotypes in these cells in a patient's infected-HCV may be distinct with circulating strains in the plasma, so suggested that the proliferation in PBMCs is considered as an independent area. The detection of virus genotypes in this area it is possible important the type of transmission, disease progression and treatment regime (6, 7). Hepatitis C virus in PBMCs and liver is proliferating and for this reason many different subtypes of the virus during replication is created so in these two area can be distinct subtype of virus genotypes and two genotypes are varies from this aspect of sequence them together. It is probably due to the different genotypes of the virus in serum and PBMCs can have an impact on the progress of disease recurrence in during and after therapy (6). Therefore, it is essential to determine the HCV genotype in PBMCs before beginning the therapy. The pathological significance and the relationship between HCV RNA serum levels and PBMCs in during and end of therapy are not well known in Iran and low study has been conducted on this subject (8). This is due to the scanty and conflicting data available based on sustained virological responses (SVRs), obtained when HCV RNA is undetectable in PBMCs. Herein, there are no published data about the presence and prevalence of various HCV genotypes in plasma and PBMCs in Yazd (central of Iran) city, therefore the present study was conducted to investigate this issue in detail.

Methods

Study Population. In this cross-sectional study, 50 established anti-HCV positive individuals, referred to the Shahid Sadoughi Hospital (affiliated with the Yazd University of Medical Sciences, Yazd, Iran) from November 2014 to March 2015 were enrolled. Written consent forms, compiled by the Ethics Committee of Yazd University of Medical Sciences were signed by the patients. Inclusion criteria were positive anti-HCV accompanied by the presence of plasma HCV RNA. Patients who were positive for anti-HCV antibodies but negative for HCV RNA were excluded from the study. Anti-HCV detection was performed by a third generation enzyme immunoassay, based on the manufacturers' guidance (DIA.PRO, Diagnostic Bioprobes Srl, Italy). Hepatitis C plasma RNA was detected by PCR method. Plasma samples were kept frozen and stored at -70°C until use. Also, a questionnaire, consisting of the demographic characteristics and risk factors ,as the possible modes of transmission (intra-venous drug abuse, multi-transfusion and blood transfusion was completed for each patient.

HCV RNA Extraction and cDNA Synthesis. RNA was extracted from 200 µL of plasma, using the AccuPrep Viral RNA Extraction Kit (Bioneer, South Korea). About 5 mL of peripheral blood was kept in EDTA-containing tubes and then the PBMCs were isolated, using Ficoll-Hypaque density gradient centrifugation (Ficoll-Hypaque; Pharmacia, Uppsala, Sweden). The PBMCs were then washed three times with phosphate-buffered saline (pH 7.3±0.1) and counted and then RNA was extracted from a pellet of approximately 5 × 106 PBMCs, then total RNA was prepared from the blood cells after lysis with ammonium chloride and TRizol reagent (Invitrogen, Paisley, UK). The final volume, in the reaction for synthesis of cDNA, was 20 µL, including 5

* **Corresponding author:** Abbas Ahmadi Vasmehjani. Infectious and Tropical Disease Research Center, Shahid Sadoughi University of Medical Sciences, Yazd, Iran
Email: ahvasmehjani@gmail.com

µL RNA, 1 µL random hexamer (Fermentas GmbH, Germany), 6.5 µL of diethylpyrocarbonate (DEPC) treated water, 4 µL reverse transcriptase reaction buffer 5x, 2 µL dNTP, 10 mM stock, (Fermentase GmbH, Germany), 0.5 µL RNase Inhibitor (Fermentas GmbH, Germany) and 200 IU of reverse transcriptase (Fermentas GmbH, Germany), which was then incubated at 65°C for 5 minutes, 42°C for 60 minutes.

HCV Genotyping. HCV genotype was determined using specific-primers of 5'-UTR region of HCV genome have already been published (9). Each 25 µl reaction mixture contained of 5 µl of template, 2.5 µl of 10X reaction buffer (Applied Biosystems, Foster City, CA), and 1.5 mM MgCl2 (AB), 0.5 mM of each dNTPs (AB), HCV-specific primers, and AmpliTaq DNA polymerase (AB). The amount of specific-HCV primers in each reaction based on the type of primer was different. 2 µL of this cDNA was amplified for 40 cycles with the following parameters: a preliminary 20 cycles of amplification at 94 0C for 1 min (denaturing), 45 0C for 1 min (annealing), and 72 0C for 1 min (extension), followed by 20 additional cycles of 94 0C for 1 min, 60 0C for 1 min, and 72 0C for 1 min. For the second-round PCR, 5 µL of first-round PCR product was amplified for 30 cycles; each cycle consisted of 94 0C for 1 min, 62 0C for 45 s, and 72 0C for 1 min. Finally, the analysis of results was performed on the basis of the presence or absence of specific bands of amplified DNA in the 2% agarose gel (Fig.1).

Statistical Analysis. Analysis was performed using the Statistical Package for Social Sciences (SPSS) software version 17.0, using descriptive values such as mean, standard deviation and Fisher's exact probability test. P value<0.05 was considered statistically significant.

Results

The average age of the 50 HCV infected-people were 14.6±38.2 (ages 21-66) was that most people were between ages 50-41 years. The majority of participants were males (82.5%) and the rest of females (17.5%). The frequency distribution of genotypes in plasma

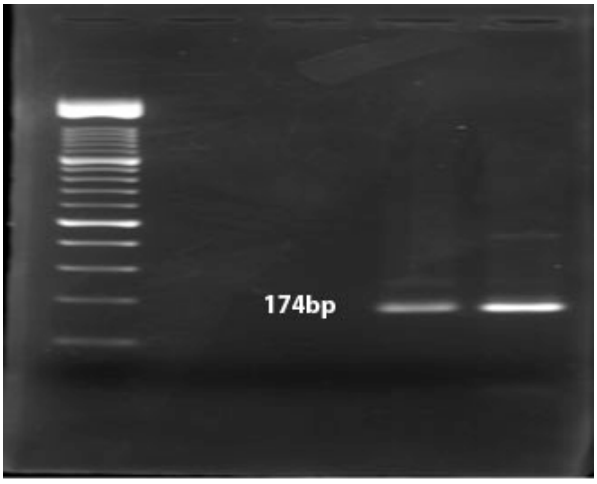

Fig. 1. Electrophoresis patterns of PCR products from different HCV genotypes. The left to right pattern, molecular size markers (GeneRuler™100 bp Plus DNA Ladder, ready-to-use; Fermentas UAB, Vilnius, Lithuania) are indicated on lane 1, lane 1 and 2 was negative sample, lane 3 and 4 was positive control.

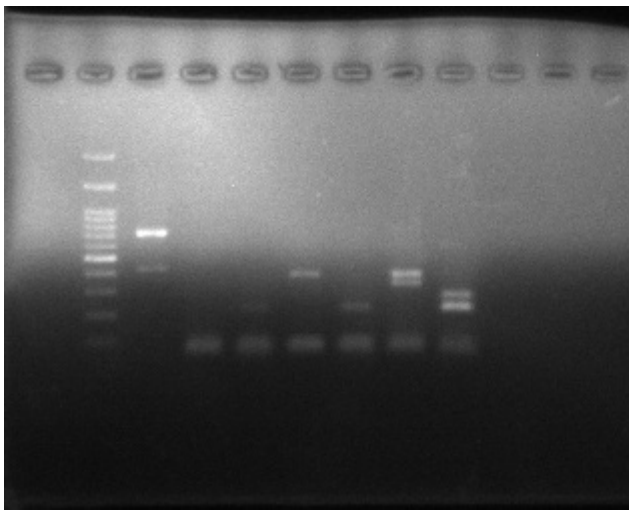

Fig. 2. Typical electrophoresis patterns of PCR products from different HCV genotypes. Patterns of four serum samples left to right are shown for containing HCV genotypes 1a (lane 5), 1b (lane 2), 3a (lanes 4 and 6), Mix genotype 2/3a (lane 8) and 1a/1b (line 7). The patterns of molecular size markers (GeneRuler™100 bp Plus DNA Ladder, ready-to-use; Fermentas UAB, Vilnius, Lithuania) are indicated on lane 1. The lengths of PCR products were 338 bp for genotype 1a, 398 bp for 1b, 286 bp for 2 and 227 bp for 3a (10).

Table 1: Distribution of Different HCV Genotypes in Plasma and PBMCs.

HCV Genotypes	Plasma	PBMCs
1a	% 20 (10)	% 18 (9)
3a	% 64 (32)	% 50 (25)
Mix (1a/3a)	% 16 (8)	% 32 (16)
Total	50	50

Table 2: The Difference of HCV Genotypes in Plasma and PBMCs.

NO	*Age /Gender*	*Plasma*	*PBMCs*
1	M/42	1a/3a	1a/3a
2	M/43	3a	1a/3a
3	M/48	3a	1a/3a
4	M/48	1a/3a	1a/3a
5	M/47	1a/3a	1a/3a
6	M/48	3a	1a/3a
7	M/44	3a	1a/3a
8	M/32	1a/3a	1a/3a
9	M/50	1a	1a/3a
10	M/32	3a	1a/3a
11	M/36	1a/3a	1a/3a
12	M/48	3a	1a/3a
13	M/31	3a	1a/3a
14	M/29	1a/3a	1a/3a
15	M/47	3a	1a/3a
16	M/34	1a/3a	1a/3a

Table 3: Distribution of plasma HCV genotypes based on different age groups.

Age	No	1a	3a	Mix
<30	11	3	5	3
30-40	10	3	5	2
>40	29	4	22	3
Total	50	10	32	8

Table 4: Distribution of plasma HCV genotypes based on different age groups.

Age	No	1a	3a	Mix
<30	11	3	4	4
30-40	10	2	3	5
>40	29	4	18	7
Total	50	9	25	16

and PBMCs you can see in Table 1. Genotype distribution in plasma and PBMCs are different in some cases Table 2. In none of the samples from patients with genotype 2 and 1b were detected but genotype 3a and 1a were detected along with mixed infections. Mixed infection with genotype 3a and 1a in 8 patients (16%) in plasma and in 16 samples (32%) in PBMCs was detected.

PCR and HCV genotyping: Genotyping of 50 sera from patients who were either recently infected by HCV or with history of previous HCV infection and positive PCR results was performed. The first, all of samples due to HCV detection was check using Nested-PCR that the size product was 174 bp (Figure 1). Then The HCV genotypes were determined in all 50 positive PCR patients based on HCV

genotyping kit (InterLabService, Sadovnicheskaya str. Moscow, Russia) (Figure 2).

HCV genotype distribution based on different age groups:

***Plasma*.** 1a and 3a genotype distribution was in over 40 years higher than in other ages that this difference with other ages was significant (P Value> 0.04). Distribution of the genotypes 1a and 3a as well as between the ages of 40-30 years showed the lowest frequency than other ages. The distribution of different genotypes of HCV in plasma of different ages was shown in Table 3.

***PBMCs*.** 1a and 3a genotype distribution was in over 40 years higher than in other ages but this difference with other ages was not significant (P=0.25). Distribution of the genotypes 1a and 3a as well as between the ages of 40-30 years showed the lowest frequency than other ages. HCV genotypes distribution in PBMCs of different ages can be seen in Table 4.

Discussion

In the recent study, we were evaluated the genotype of the virus in plasma and PBMCs samples in 50 patients infected to HCV. In our study, it was shown that the prevalence of genotype 1a and 3a is higher in patients over age 30 that were different from that obtained in other studies in Iran but was similar to other study (11) which can be due to small sample size in the study (12, 13).

The distribution of HCV genotype in plasma and PBMCs are different in some cases but HCV genotypes were detected to all plasma and PBMCs that were reported in previous studies (13, 14). Mix genotype was detected in eight plasma samples and 16 samples of PBMCs. Mixed infection is a person when has two or more distinct genotypes of HCV virus is at the same time. Mix viral infection has clinical importance, for example, may lead to exacerbation, treatment failure or relapse after completion of the disease. This means that the PBMCs may contain various genotypes that cannot be detected in plasma. Previous studies have concluded that the PBMCs as a place extrahepatic replication that is confirmed(14). Several studies in HCV-infected patients have shown that the genome of the virus in other locations as the site of viral replication in the liver, such as PBMCs after completion of treatment was detected (15-17).

In fact, this achievement stressed that infection with one genotype is no longer a barrier to infect with another genotype. So, multiple contacts with HCV, especially with high risk people such as hemophilia, thalassemia, may lead to re-infection and the stability of mixed infection in some patients(18). Also super-infection with a new type may be to suppress a HCV virus and this type was as dominant type of treatment therefore this event may alter treatment outcome.

In our study, the prevalence of mixed infections in 8 patients (16%) in plasma and in 16 samples (32%) in PBMCs was estimated in individuals infected with HCV. Previous studies was indicated there has been evidence of persisting HCV RNA in PBMCs despite sustained clearance of HCV RNA from serum (19). Mixed infection with two different genotypes and in others with a different genotype than in serum was detected in PBMCs. In other studies, the rate of 6.1% to 31% in hemophiliacs has been detected with high transfusion (20). Studies, mixed infections was concluded that 15% of patients with different genotypes, also in another study investigated 2.5% of mixed infections in plasma was detected. Our study showed that a significant proportion of people infected with HCV genotypes are different in the PBMCs over plasma.

Our study had some limitations, including the lack of liver biopsy noted that was hazard because liver biopsy is the main place HCV replication. Since liver biopsy can be performed for all patients is not recommended due to the different genotypes of the virus have been detected in PBMCs over plasma PBMCs that can be used as a reservoir for assess of infection.

Conclusion

Our study suggests that we should be assessment mix infection in patients with high transfusion using examining PBMCs. Because according to the results we can say that the lack of response to treatment or recurrent disease is caused by a lack of adequate knowledge of the genotype of the virus. So, evaluate genotypes of HCV virus in PBMCs samples as a complementary tool can be help to for treatment planning.

Acknowledgement

This study was supported by Shahid Sadoughi University of medical sciences, Yazd, Iran, grant and we are grateful to all member of our laboratory for their cooperation.

References

1. Smith DB, Bukh J, Kuiken C, Muerhoff AS, Rice CM, Stapleton JT, et al. Expanded classification of hepatitis C virus into 7 genotypes and 67 subtypes: updated criteria and genotype assignment web resource. Hepatology. 2014;59(1):318-27.
2. Messina JP, Humphreys I, Flaxman A, Brown A, Cooke GS, Pybus OG, et al. Global distribution and prevalence of hepatitis C virus genotypes. Hepatology. 2015;61(1):77-87.
3. Alavian S-M, Adibi P, Zali M-R. Hepatitis C virus in Iran: Epidemiology of an emerging infection .Arch Iranian Med. 2005;8(2):84-90.
4. Castillo I, Rodriguez-Inigo E, Bartolome J, De Lucas S, Ortiz-Movilla N, Lopez-Alcorocho J, et al. Hepatitis C virus replicates in peripheral blood mononuclear cells of patients with occult hepatitis C virus infection. Gut. 2005;54(5):682-5.
5. Houghton M, Weiner A, Han J, Kuo G, Choo QL. Molecular biology of the hepatitis C viruses: implications for diagnosis, development and control of viral disease. Hepatology. 1991;14(2):381-8.
6. Inglot M, Pawlowski T, Szymczak A, Malyszczak K, Zalewska M, Radkowski M. Replication of hepatitis C virus in peripheral blood mononuclear cells in patients with chronic hepatitis C treated with pegylated interferon alpha and ribavirin Replikacja wirusa zapalenia wątroby typu C (HCV) w jednojądrowych komórkach krwi obwodowej (PBMC) u pacjentów z przewlekłym zapaleniem wątroby typu C, leczonych pegylowanym interferonem alfa i rybawiryną. Journal cover. 2016;70.
7. Chang C-C, Lin C-C, Hsieh W-L, Lai H-W, Tsai C-H, Cheng Y-W. MicroRNA expression profiling in PBMCs: a potential diagnostic biomarker of chronic hepatitis C. Disease markers. 2014;2014.
8. Zarepour F, Sharifi Z. Study on the Presence of HCV RNA in PBMC Compared with Plasma of Patients with Hepatitis C after Treatment. 2. [Original article]. 2014;8(2):7-11.
9. Pohjanpelto P, Lappalainen M, Widell A, Asikainen K, Paunio M. Hepatitis C genotypes in Finland determined by RFLP. Clinical and diagnostic virology. 1996;7(1):7-16.
10. Nasab SDM, Baharlou R, Piroozmand A, Toghyani H, Shadmand E, Fazel H, et al. Distribution of IL-28B genotypes in patients with hepatitis C and healthy individuals in Jahrom city. Gastroenterology and hepatology from bed to bench. 2015;8(4):278.
11 . Jahanbakhsh Sefidi F KH, Monavari SH, Alavian SM, Fakhim S, Bokharaei-Salim F. Distribution of Hepatitis C Virus Genotypes in Iranian Chronic Infected Patients. Hepatitis Monthly. 2013;13(1):e7991.
12. Sefidi FJ, Keyvani H, Monavari SH, Alavian SM, Fakhim S, Bokharaei-Salim F. Distribution of hepatitis C virus genotypes in Iranian chronic infected patients. Hepatitis Monthly. 2013;13(1).
13. Hafez AA, Baharlou R, Nasab SDM, Vasmehjani AA, Shayestehpour M, Joharinia N, et al. Molecular epidemiology of different hepatitis C genotypes in serum and peripheral blood mononuclear cells in jahrom city of iran. Hepatitis Monthly. 2014;14(5).
14. Castillo I R-IE, Bartolomé J, et al. Hepatitis C virus replicates in peripheral blood mononuclear cells of patients with occult hepatitis C virus infection. Gut. 2005;54(5):682-685.
15.Januszkiewicz-Lewandowska D WJ, Pernak M, Nowicka K, Zawada M, Rembowska J, et al. Presence of hepatitis C virus (HCV)-RNA in peripheral blood mononuclear cells in HCV serum , Dis.2007;60(1 32–29:(npdiartJJI.
16. Hanno AFF MK, Alshayeb AF, Deghedy A. HCV RNA in peripheral blood mononuclear cells (PBMCs) as a predictor of the response to antiviral therapy in chronic hepatitis C. Alexandria J Med.2013;50(1).
17. Laskus T, Operskalski EA, Radkowski M, Wilkinson J, Mack WJ, Al-Harthi L, et al. Negative-strand hepatitis C virus (HCV) RNA in

peripheral blood mononuclear cells from anti-HCV–positive/HIV-infected women. Journal of Infectious Diseases. 2007;195(1):124-33.
18. Bastani M-N, Bokharaei-Salim F, Keyvani H, Esghaei M, Monavari SH, Ebrahimi M, et al. Prevalence of occult hepatitis C virus infection in Iranian patients with beta thalassemia major. Archives of virology. 2016:1-8.
19. Mohamad HI, El-Bab H, Kamal AM. HCV RNA in serum and peripheral blood mononuclear cells after successful interferon therapy. Hepato-Gastroenterology. 2010;58(107-108):932-6.
20. Telfer P, Devereux H, Savage K, Scott F, Dhillon A, Dusheiko G, et al. Chronic hepatitis C virus infection in haemophilic patients: clinical significance of viral genotype. Thrombosis and haemostasis. 1995;74(5):1259-64.