ARTICLE

COMPARISON OF TWO GENOTYPING METHODS OF HCV- RT-PCR AND DIRECT SEQUENCING

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ABSTRACT

Hepatitis C virus (HCV) is an important human pathogen that can cause acute and chronic hepatitis, liver cirrhosis, and, hepatocellular carcinoma. HCV has been classified into 6 major genotypes and many subtypes. Several methods have been developed for HCV genotyping and direct sequencing is gold standard method. HCV was transmitted by different routes such as intravenous drug abuse (IVDA), tattooing, sexual, blood transfusion and other risk factors. In this study collected 180 samples of patients infected with HCV were referred to Reference laboratory in Kermanshah University of Medical Sciences, for HCV genotype testing and samples were genotyped by RT-PCR and direct sequencing of Core gene. Among them , 138 samples (76.6%) was 3a genotype, 35 (19.4%) was 1a genotype, 3 (1.7%) was 1b genotype and 4 (2.2%) was 3a and 1b. This study revealed that 3a is the most prevalent genotypes in Kermanshah province and IVDA is the main risk factor in this province and also Results of genotyping by RT-PCR was confirmed by direct sequencing .

KEY WORDS
HCV Genotyping, Direct sequencing, IRAN, Kermanshah

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INTRODUCTION

Chronic hepatitis, hepatocellular carcinoma and cirrhosis are most important liver diseases in the world [1]. Approximations 130–170 million persons, or 2–3% of the world's population, are infected with HCV. Prevalence estimates are 400 000 chronically infected subjects in Australia and Oceania, 14 million in the Americas, 16 million in the Middle East, 17.5 million in Europe, 28 million in Africa, and 83 million in Asia [2]. The researches show that the main factors responsible for the epidemic spread of HCV were the routes for transmission: blood transfusions or blood product administration, the medical use of unsterilized needles for injections and for large scale vaccination, intravenous drug use associated with sharing of injection equipment, heterosexual or homosexual activity with multiple sexual partners and tattooing[3]. Heavy alcohol consumption, particularly in females, age and HCV/HIV co-infection may be associated with more rapid progression of HCV liver disease, especially fibrosis. The HCV genome is a positive-sense, single-stranded RNA genome approximately 10 kb long. It has marked similarities to those of members of the genera and Flavivirus. Hepatitis C is containing one long open reading frame [ORF] that encodes for a non-functional polyprotein of approximately 3010 amino acids in length .This non-functional polyprotein is cleaved by cellular and viral proteases to yield at least ten different functional protein products. Structural proteins are the major are coded in the 5' quarter of the ORF and arranged as C ,E1 ,E2 and P7, while the nonstructural proteins are encoded in the 3' three-quarters of the ORF in the order NS2–NS3–NS4A/B–NS5A/B [4].

HCV has a high viral heterogeneity and different HCV isolates from around the world show substantial nucleotide sequence variability throughout the viral genome. Genotypes differ from each other by approximately 30% in amino acid sequence[5]. HCV genotypes have a striking geographical and epidemiological distribution and genotype identification is clinically important to tailor dosage and duration of the treatment [6]. Interestingly, not only do the HCV genotypes seem to differ in nucleotide sequence and geographical distribution, but there is also evidence of biological differences between the HCV genotypes[7]. Therefore, genotyping is an important tool for prognosis and follow-up of infected patients. Viral genotype determination before treatment is currently advised as a routine assay. Mode of transmission may also affect distribution of HCV genotypes [8]. Hepatitis C genotype is the most important virological factor predicting response to antiviral therapy[9]. Several regions of the HCV genome have been analyzed with the purpose of genotypic classification. The 5' NCR, Core, E1 and NS5B regions have been frequently amplified and studied for the purpose of genotypic classification. The NS5B region more often used for differentiation of subtypes and confirmation of genotyping results in research settings [10,11].

Several methods for genotyping HCV have been developed, including direct DNA sequencing [12], type specific PCR [13], restriction fragment length polymorphism, line probe assays, primer-specific and mispair extension analysis [14,15], heteroduplex mobility analysis by temperature gradient capillary electrophoresis [16], denaturing high preference liquid chromatography and gold standard method is direct DNA sequencing [17]. The aim of this study was to determine HCV genotype prevalence and comparison of two methods of HCV genotyping; RT-PCR and direct sequencing of Core gene in Kermanshah Province, the west of Iran. The distribution of HCV genotypes and their association with

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possible transmission routes [risk factors] in a group of HCV infected patients from Kermanshah province was investigated, as the data exclusively related to this area is limited.

Kermanshah province, located in the middle of the western part 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 other provinces of Iran [http://www.ar.org.ir/Default.aspx?tabid=133].

MATERIALS AND METHODS

In a cross-sectional study, a total of 180 people infected to HCV infection 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 was centrifuged and plasma separated and the RNA was extracted by commercial QIAamp Viral RNA Mini kit [QIAGEN]. For purification of viral RNA from the collected serum samples, 140 µL of sera were used according to the manufacture instruction [QIAamp Viral RNA Mini-kit, QIAGEN, USA] To enhance binding of viral nucleic acids to the QIAamp Mini membrane, especially if there were very few target molecules in the sample, carrier RNA was also added to the reaction, as recommended. The addition of large amounts of carrier RNA reduces the chance of viral RNA degradation in the rare event that RNase molecules escape denaturation by the chaotropic salts and detergent in buffer. If carrier RNA is not added to buffer, this may lead to reduced viral RNA recovery. The HCV RNA in sera was measured by the commercially standardized quantitative reverse-transcription polymerase chain reaction [RT-PCR] artus HCV RG RT-PCR kit and ABI 7500 Real Time PCR instrument.The results are given in international units per milliliter [IU/ml] The following protocol in the instrument:

45°C for 10 min, 1 cycle;
95°C for 15 min, 1 cycle;
95°C for 15 sec and 60°C for 60sec 40 cycles.

Multiplex RT- PCR method with One Step-RT PCR Qiagen kit and specific primers for Core region of HCV genome were performed to determine viral genotype analysis. The primers that used in this study were purchased from metabion company [Table- 1].

Amplified PCR product run on 2.5% agarose gel. 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 isolates were genotyped using universally accepted method of Ohno *et al* [17,18]. Briefly, 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 primers 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] [18,19] and primer sequences are shown in Table- 1. The genotype specific band was visualized on a 2.5% agarose gel by ethidium bromide and UV light [Figure- 1].

Table 1: PCR, sequencing, and genotyping Oligonucleotide primers [modified from Ohno et al., 1997 13]

Primer	Sequence [5'-3']	PCR round
Sc2	GGGAGGTCTCGTAGACCGTGCACCATG	1st round PCR
Ac2	GAG[AC]GG[GT]AT[AG]TACCCCATGAG[AG]TCGGC	1st round PCR
S7	AGACCGTGCACCATGAGCAC	2nd -round PCR for sequencing
A5	TACGCCGGGGGTCA[TG]T[GA]GGGCCCCA	2nd -round PCR for sequencing
Mix 1		
S7	AGACCGTGCACCATGAGCAC	2nd -round PCR for genotyping
S2a	AACACTAACCGTCGCCCACAA	2nd -round PCR for genotyping
G1b	CCTGCCCTCGGGTTGGCTA[AG]	2nd -round PCR for genotyping
G2a	CACGTGGCTGGGATCGCTCC	2nd -round PCR for genotyping

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G2b	GGCCCCAATTAGGACGAGAC	2nd -round PCR for genotyping
G3b	CGCTCGGAAGTCTTACGTAC	2nd -round PCR for genotyping
Mix2		
S7	AGACCGTGCACCATGAGCAC	2nd -round PCR for genotyping
G1a	GGATAGGCTGACGTCTACCT	2nd -round PCR for genotyping
G3a	GCCCAGGACCGGCCTTCGCT	2nd -round PCR for genotyping
G4	CCCGGGAACTTAACGTCCAT	2nd -round PCR for genotyping
G5a	GAACCTCGGGGGGAGAGCAA	2nd -round PCR for genotyping
G6a	GGTCATTGGGGCCCCAATGT	2nd -round PCR for genotyping

Direct Sequencing of HCV core gene was performed in both directions by ABI 3130 genetic Analyzer instrument[Applied Biosystem , Foster City, Calif].
The basic local alignment search
tool [BLAST] was used to search the public domain nucleotide database maintained by National Centerfor Biotechnology Information [NCBI] [20].
Data are presented as percentage [%] or number of patients. Chi-Square and Fisher's Exact tests were carried out by SPSS statistical package version 19.0 for windows. *P* values less than 0.05 were considered significant.

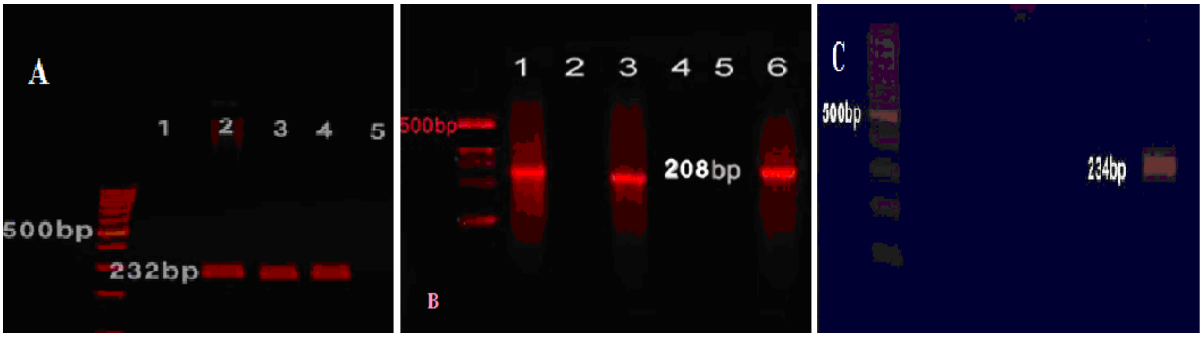

Fig: 1. 2.5% agarose gel electrophoresis of the PCR products of the HCV DNA from different genotypes. DNA marker 500 bp [lane M]; A: Genotype 3a; B: Genotype 1a; C: Genotype 1b.

RESULTS

The distribution of the HCV genotypes in this study population is not in accord with the other known geographic distribution of HCV genotype in Iran. Viral load of all 180 cases was estimated using 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 [Fig. 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% [21].

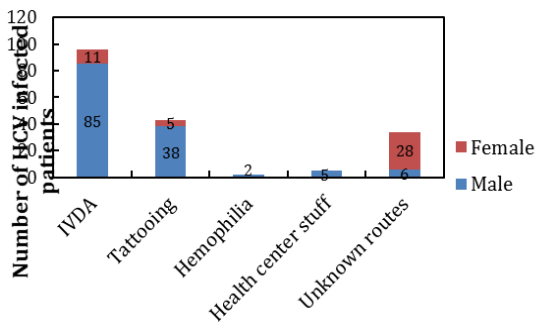

Fig: 2. Routes of infection in HCV infected patients

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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 Fig. 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. 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 [22]. Prevalence of identified genotypes was determined for different age-groups of studied patients. The highest rate was observed in 31 to 50 yr old [Table 3]. Additionally, the results of direct sequencing strongly confirmed RT-PCR results [Figure 4].

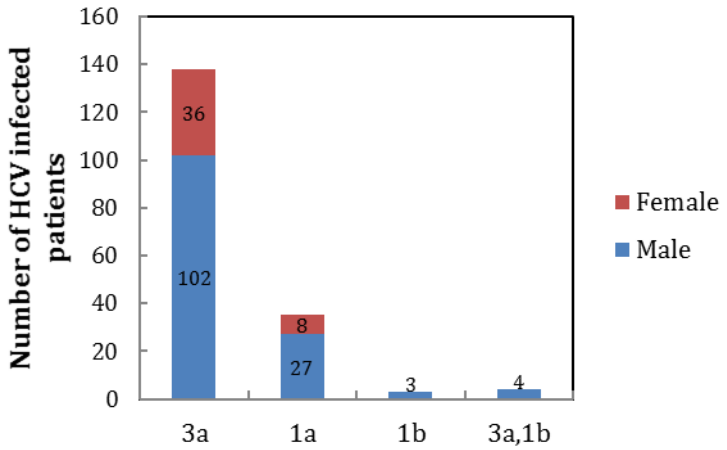

Fig: 3. HCV genotype distribution in Kermanshah province

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

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Table 3: Frequency of HCV positive and there genotypes in different age groups

Age	Number of patients	Prevalence Genotype
0-10	0	-
11-20	2	1a
31 -40	98	3a
41- 50	46	3a
51-60	23	1a
61-70	8	1a
71-80	3	1a

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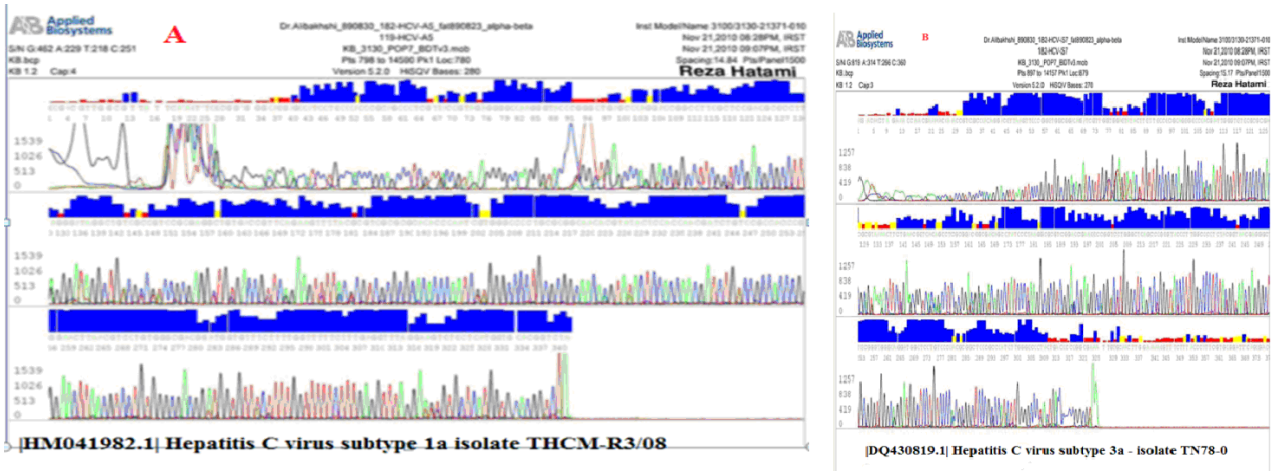

Fig. 4: Direct sequencing results of Core gene in HCV genotypes . A: |HM041982.1| Hepatitis C virus **subtype 1a** - isolate THCM-R3/08. B: |DQ001257.1| Hepatitis C virus **subtype 3a** - isolate MOI257

DISCUSSION AND CONCLUSION

Hepatitis C Virus [HCV] is one of the main causes of acute and chronic hepatitis that is distributed worldwide with prevalence varying between different countries from 0.2 up to 40% [2].

HCV genome 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 [23,24].The importance of HCV genotyping as an epidemiological marker has been clearly shown, particularly in tracing the source of infection and elucidating the possible modes of transmission. Also The identification of genotypes is clinically important, and treatment protocols recommend that genotype information be obtained in order to delineate the duration and type of the medication to be used[14]. Based on sequence variation in both the coding and non-coding regions, several classification 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-specific primers, that is widely acceptable [18,19].

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. In the United States and Europe, 1a and 1b subtypes are the predominant genotypes. HCV genotype 3a is more common among the abusers of intravenous drug in the United States and Europe [25]. In Middle East genotype 4 of HCV is common [24]. 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 [26]. In comparison with other studies made in Iran ,it can be understood that the most common genotype of other provinces is type1. this genotype is uncommon in our region. The present study in Western Iran, indicate that type 3a of HCV is most prevalent in Kermanshah Province, Iran, which is different from other reports around the world. Moreover, it seems that there is a similarity between the pattern of genotype in Illegal drug users in kermanshah province when compared with other regions of IRAN. Interestingly, the results of RT-PCR in this study was confirmed by sequencing method. For this purpose, we were use of a sequencing technique for HCV genotyping consists of PCR amplification of core region of, ABI 3130 Genetic Analyser system..

CONFLICT OF INTEREST

Authors declare no conflict of interest.

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REFERENCES

[1] Agnello V, Ábel G, Elfahal M, Knight GB, Zhang QX. [1999] HCV and other Flaviviridae viruses enter cells via low density lipoprotein receptor. Proc Natl Acad Sci USA. 96:12766 – 12771.

[2] Frank C, Mohamed MK, Strickland GT, Lavanchy D, Arthur RR. [2011] Evolving epidemiology of hepatitis C virus. Journal of Clinical Microbiology and Infection 17(2): 107–115.

[3] Poynard T Mathurin P, Lai CL, Guyader D, Poupon R, Tainturier MH, Myers RP, Muntenau M, Ratziu V, Manns M, Vogel A, Capron F, Chedid A, Bedossa P. [2003] A comparison of fibrosis progression in chronic liver diseases , J Hepatol 38:257–265.

[4] Pybus OG, Drummond AJ, Nakano T, Robertson BH, Rambaut A. [2003] The epidemiology and introgenic transmission of hepatitis C virus in Egypt. a Bayesian coalescent approach. Mol Biol Evol 20: 381-387.

[5] Op De Beeck A, Cocquerel L, Dubuisson J. [2001] Biogenesis of HCV envelope glycoproteins. J Gen Viral 82 :2589 – 2595.

[6] Pawlotsky JM. [2003] Hepatitis C virus genetic variability; Pathogenic and clinical implications. Clin Liver Dis 7: 45-66.

[7] Fried MW. [2004] Viral factors affecting the outcome of therapy for chronic hepatitis C. Rev Gastroenterol Disord. 4 [Suppl 1]: S8-S13.

[8] Kanai K. [1992] HCV genotypes in chronic hepatitis C and response to interferon. Lancet 339(8808):1543.

[9] Driesel G, Wirth D, Stark K, Baumgarten B, Sucker U, Schreier E. [1960] Hepatitis C virus [HCV] genotype distribution in German isolates: studies on the sequence variability in the E2 and NS5 region. Arch Virol. 139[3-4]: 379-88.

[10] McHutchison JG, Gordon S, Schiff ER, Shiffman ML, Lee WM, Rustgi VK, Goodman ZD, Ling MH, Cort S, Albrecht JK. [1998] Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. Hepatitis Interventional Therapy Group. N Engl J Med 339(21):1485-92.

[11] Corbet S, Bukh J, Heinsen A, Fomsgaard A. [1960] Hepatitis C virus subtyping by a core-envelope 1-based reverse transcriptase PCR assay with sequencing and its use in determining subtype distribution among Danish patients. J Clin Microbiol 41:1091-100.

[12] Nolte FS, Green MN, Fiebelkorn KR. [2003] Clinical evaluation of two methods for genotyping hepatitis C virus based on analysis of the 5'noncoding region. J Clin Microbiol 41:1558-1564.

[13] Bukh J, Purcell RH, Miller RH. [1992],Sequence analysis of the 5' noncoding region of hepatitis C virus. Proc Natl Acad Sci USA. 89:4942-4946.

[14] Okamoto H, Sugiyama Y, Okada S, Kurai K, Akahane Y, Sugai Y, Tanaka T, Sato K, Tsuda F, Miyakawa Y, Mayumi M.

[15] [1992] Typing hepatitis C virus by polymerase chain reaction with type-specific primers: application to clinical surveys and tracing infectious sources. J Gen Virol 73:673-679.

[16] Davidson F, Simmonds P, Ferguson JC, Jarvis LM, Dow BC, Follett EAC, Seed CRG, Krusius T. [1995] Survey of major genotypes and subtypes of hepatitis C virus using RFLP of sequences amplified from the 5' noncoding region. J Gen Virol 76: 1197-204.

[17] Nakao T, Enomoto N, TakadaN, Takada A, Date T. [1991] Typing of hepatitis C virus genomes by restriction fragment length polymorphism. J Gen Virol 72:2105-2112.

[18] Liew M, Erali M, Page S, Hillyard D, Wittwer C. [2004] Hepatitis C genotyping by denaturing high-performance liquid chromatography. J Clin Microbiol 42: 158-163.

[19] Margraf RL, Erali M, Liew M, Wittwer CT. [2004] Genotyping hepatitis Cvirus by heteroduplex mobility analysis using temperature gradient capillary electrophoresis. J Clin Microbiol. 42,4545-4551.

[20] Hu YW, Balaskas E, Furione M, Yen PH, Kessler G, Scalia V, Chui L, Sher G. [2000] Comparison and application of a novel genotyping method, semiautomated primer-specific and mispair extension analysis, and four other genotyping assays for detection of hepatitis C virus mixed-genotype infections. J Clin Microbiol 38:2807-2813.

[21] Ohno T, Mizokami M, Tibbs CJ, Ohba K, Suzuki K, Nouri Aria KT, Williams R. [1994] New genotype of hepatitis C virus in South Africa. J Med Virol 42:409–413.

[22] Tamura K, Dudley, Nei M, Kumar S. [2007] Molecular evolutionary Genetics Analysis [MEGA] software version 4.0. Mol Biol Evol 24(8): 1596-1599.

[23] McOmish F, Chan SW, Dow BC, Gillon J, Frame WD, Crawford RJ, Yap PL, Follett EA, Simmonds P. [1993]Detection of three types of hepatitis C virus in blood donors: investigation of type-specific differences in serologic reactivity and rate of alanine aminotransferase abnormalities. Transfusion 33(1):7-13.

[24] Cochrane1A, Searle1B, Hardie A, Robertson R, Delahooke T, Cameron S, TedderRS, Dusheiko GM, Lamballerie XD, Simmonds P. [2002] A genetic analysis of hepatitis C virus transmission between injection drug users. J Infect Dis 186: 1212-1221.

[25] Bartenschlager R, Lohmann V. [2000] Replication of hepatitis C virus. J Gen Virol 81:1631-41.

[26] Simmonds P, Holmes EC, Cha TA, Chan SW, McOmish F, Irvine B, Beall E, Yap PI, Kolberg J, Urdea MS. [1993] Classification of hepatitis C virus into six major genotypes and a series of subtypes by phylogenetic analysis of the NS-5 region. Journal of world virology 74:2391-2399.

[27] Qu D, Li JS, Vitvitski L, Mechai S, Berby F, Tong SP, Bailly F, Wang QS, Martin JL, Trépo C. [2003] Hepatitis C virus genotypes in France: comparison of clinical features of patients infected with HCV type I and type II. J Hepatol 21:70-75.

[28] Taherkhani R, Farshadpour F. [2015] Epidemiology of hepatitis C virus in Iran. World J Gastroenterol 21(38):10790–10810.