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ORIGINAL ARTICLE

# Distribution of Hepatitis C Virus Genotypes in Iran: A Population-Based Study

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*Background and Aims:* Iran is a vast country with a population of different ethnic backgrounds. Therefore, a wide variation in the frequencies of hepatitis C virus (HCV) genotypes is expected to occur.
*Methods:* To address this issue, 116 serum samples from HCV RNA-positive carriers representing different parts of Iran were collected from 2000-2005 and were tested by restriction fragment length polymorphism (RFLP) method.
*Results:* 1a, 1b, and 3a were the predominant genotypes circulating throughout the country with an overall prevalence rate of 61.2%, 13.8%, and 25%, respectively. The rate of HCV genotypes did not differ significantly in relation to demographic characters and risk factors.
Sequence analysis of the core region from 16 HCV isolates representative of all genotypes confirmed the RFLP results, except for one sample, which submitted as a new subtype 3.
*Conclusions:* The predominance of genotypes 1a and 3a in our population is in agreement with available data collected from blood donors and patients in Iran. Considering the even distribution of genotype 1a indicates that it has been presented and circulated in our community for a long time.
*Keywords:* Hepatitis C Virus, Genotypes, Iran

## Introduction

Hepatitis C virus (HCV) infection is a global public health problem with an estimated 170 million chronically-infected individuals worldwide. Twenty percent of patients with hepatitis C develop cirrhosis and hepatocellular carcinoma, both of which are fatal [1, 2].

According to Simmonds' nomenclature, HCV variants are classified into six genotypes. Multiple subtypes are also defined based on HCV complete or partial nucleotide sequence data [3, 4].

The HCV genotypes are distributed differently and have different susceptibility to interferon (IFN)-based therapies. Genotype 1 is the predominant variant in developed countries and shows the poorest response to IFN-based therapy. Genotypes 2 and 3 are also common in Europe but less prevalent than genotype 1 and show the best response to IFN therapy [5].

Geographical distribution of subtypes shows significant differences. In North America and Northern Europe, 1a is the most common subtype followed by 2b and 3a [6, 7]. In Southern and Eastern Europe, the most common subtype is 1b, followed by genotypes 2 and 3 [8, 9].

In other parts of the world, genotypes other than 1, 2 and 3 are more common. Genotype 4 seems to

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be confined to the Middle East [10] and Central Africa [11, 12] while genotype 5 has been isolated almost exclusively from South Africa where it predominates, followed by genotypes 1, 2, 3 and 4 [13]. Genotype 6 has been reported from Hong Kong, Vietnam and throughout South East Asia [14, 15].

There are few studies on the prevalence of HCV genotypes in Iran, in most of which much of the information is based on readily accessible samples such as those obtained from screening of individuals in blood banks, and patients with chronic hepatitis or on hemodialysis [16, 17]. These studies do not represent the real prevalence of HCV infection in the general population in this area. We conducted this study to report the genotype and subtype distribution of HCV in a population-based study in Iran.

## Materials and Methods

### *Serum Samples*

To cover the whole country, we studied six regions based on population and geographical zones, the North included Tehran, Guilan, Mazandaran, Golestan and Semnan provinces; the Northwest consisted of West and East Azerbaijan, Ardabil, Zanjan and Qazvin; the West included Kordestan, Ilam, Hamadan, Kermanshah and Lorestan; the Southwest and South consisted of Khouzestan, Bushehr, Hormozgan and Kohkilouyeh Boyerahmad; the East included Sistan Balouchestan, Khorasan and Kerman and the Center consisted of Isfahan, Markazi, Qom and Yazd (Fig. 1).

The study population consisted of 14-25 native people of each region. All cases had a positive test for antibodies to HCV (anti-HCV) detected by third-generation enzyme-linked immunosorbent assay (ELISA) with a commercial kit (Ortho Diagnosis, Raritan, NJ, USA); they were also HCV-RNA positive by reverse transcriptase polymerase chain reaction (RT-PCR) assay. Serum samples were kept frozen and stored at -20 °C until used.

A total of 116 serum samples were collected from chronic HCV carriers from 2000-2005. All participants were also HCV-RNA positive and

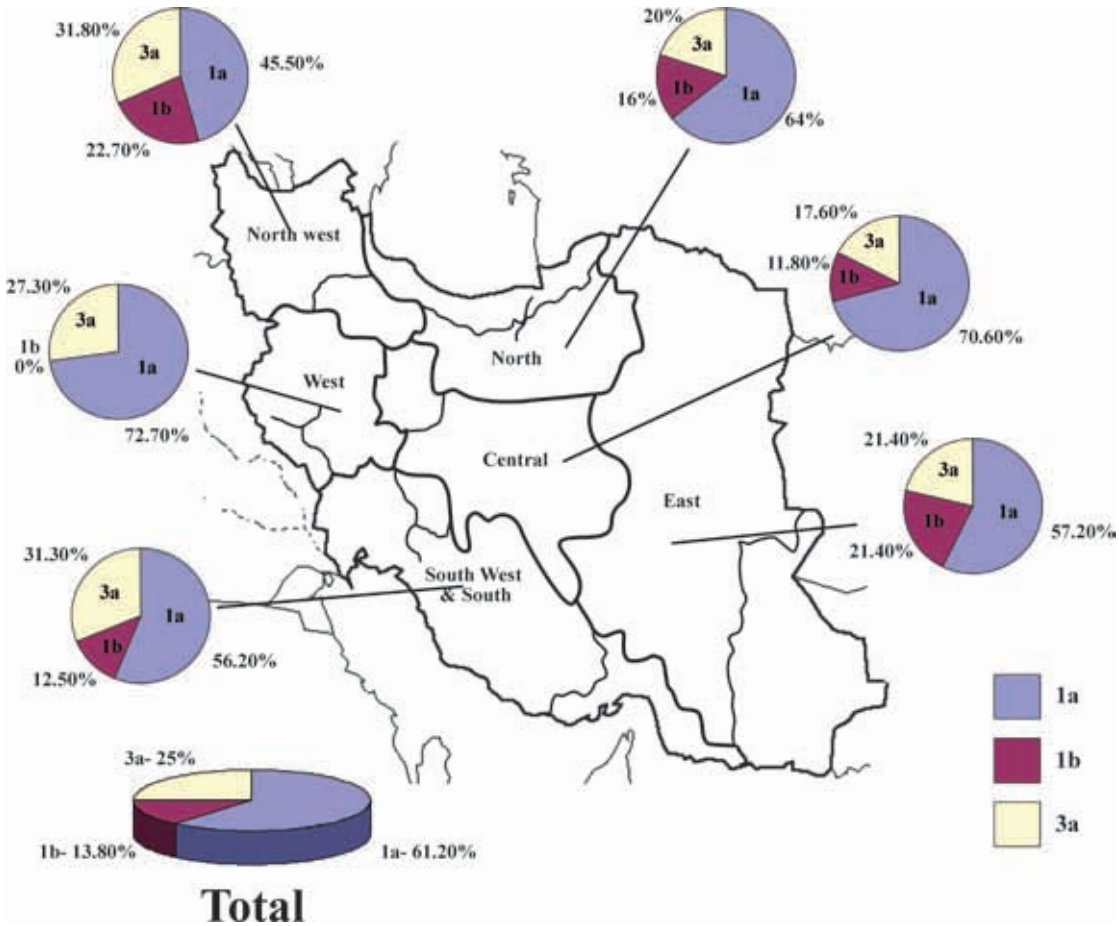


**Figure 1.** Distribution of HCV genotypes in the various Iran regions. The distribution is even in various regions (P=0.51).

represented six different regions of Iran.

A questionnaire consisting of demographic information and risk factors was completed for each subject.

### HCV-RNA extraction and cDNA synthesis

RNA was extracted from 250 µl of each serum using Trizol LS reagent (Life Technologies, Gibco-BRL) according to the manufacturer's instruction. The reaction for synthesis of cDNA included addition of 7 µl of RNA with 200 U of AMV reverse transcriptase (Promega, USA), AMV reverse transcriptase 5× reaction buffer, 10 mM of the dNTPs, 40 U random hexamer and 20 U of RNase inhibitor (Promega, USA).

### PCR amplification

Samples were amplified with nested PCR using the 5' untranslated region (5'UTR) primers (Table 1) according to Bukh [18]. Samples with a 235-bp band in agarose gel were considered as HCV-RNA positive. The guidelines of Kwok and Higuchi [19] were strictly followed to avoid carryover contamination and appropriate negative controls for RNA extraction; cDNA synthesis and PCRs were routinely included in each PCR round. To validate results, a negative control was included from the extraction step for every four samples and another negative control was also added from reverse transcription step. A high viral load serum was included from RNA extraction step as the positive control.

**Table 1.** Sequence of primers used for HCV RNA RT-PCR assay.

| Region | Primer Name | Sequence | Position |
|---|---|---|---|
| 5'UTR | C1 sense | 5' AGC GTC TAG CCA TGG CG T | -268 to -251 |
| | C2 antisense | 5' GCA CGG TCT ACG AGA ACCT | -4 to -22 |
| | C3 sense | 5' GTG GTC TGC GGA ACC GG | -199 to -183 |
| | C4 antisense | 5' GGG CAC TCG CAA GCA CCC | -26 to -43 |
| Core | F1 | 5'ACT GCC TGA TAG GGT GCT TGC | -54 to -34 |
| | R1 | 5' ATG TAC CCC ATG AGG TCG GC | 410 to 391 |
| | F2 | 5' AGG TCT CGT AGA CTG TGC A | -22 to -4 |
| | R2 | 5'CAC GTA AGG GTA TCG ATG AC | 383 to 364 |

### Digestion of PCR products with Restriction Fragment Length Polymorphism method (RFLP)

The RFLP method was carried out using the 5'UTR nested-PCR products as described by Pohjanpelto, *et al.*, [20] with minor modifications.

The products of the second round of PCR (174 bp) were digested with ScrFI/HinfI, MvaI/ HinfI and MvnI restriction enzymes (Roche, Germany) for two hours at 37 °C and visualized after electrophoresis on 3% agarose gel by 100 V for one hour by ethidium bromide staining. Molecular weight markers (100-bp plus, Fermentas) and undigested PCR products were included in each analysis. The genotypes were evaluated based on fragmentation pattern of the amplified DNA. The RFLP method in the 5'UTR (position 199 to 43) was applied for HCV genotype on all samples (Fig. 2).

### Amplification and cloning of the core region

To confirm the PCR-RFLP results, the core region of HCV from 16 randomly selected samples representative of all genotypes were amplified using the modified primers (Table 1) of Lole [21].

The products from the second round of nested PCR (360 bp) were cloned using TOPO TA Cloning kit (Invitrogen, USA) into the plasmid PCR [21]. The DNA from two clones of each sample was purified using the QIAprep Spin Plasmid kit (Qiagen, Germany) according to the manufacturer's instructions.

### Sequencing and phylogenetic analysis of the core region and statistical analysis

To get consensus sequences, the purified PCR products (plasmids) were sequenced (Primm, Italy) in both directions. The basic local alignment search tool (BLAST) was used to search the public domain nucleotide database maintained by National Center for Biotechnology Information (NCBI). Multiple sequence alignments were carried out with ClustalX (v 1.81). Evolutionary distances between sequences were determined with the Kimura two parameters method of the MEGA package (v 4) [22], and the computed distances were used to construct the phylogenetic tree by the neighbor-joining (NJ) method [23].

Isolates sequenced in the study were aligned with some representative sequences for each major genotype and subtype selected from the LANL database (www.hcv.lanl.gov). The association between risk factors and demographic characteristics with genotypes in each region was calculated using $\chi^2$ or Fisher's exact test (SPSS, v 11.5).

## Results

The studied sample consisted of 93 men and 23 women with mean age of 36.7 and 41.2 years, respectively. Our results showed that 1a, 1b and 3a were the most prevalent genotypes in Iran (Fig. 1)

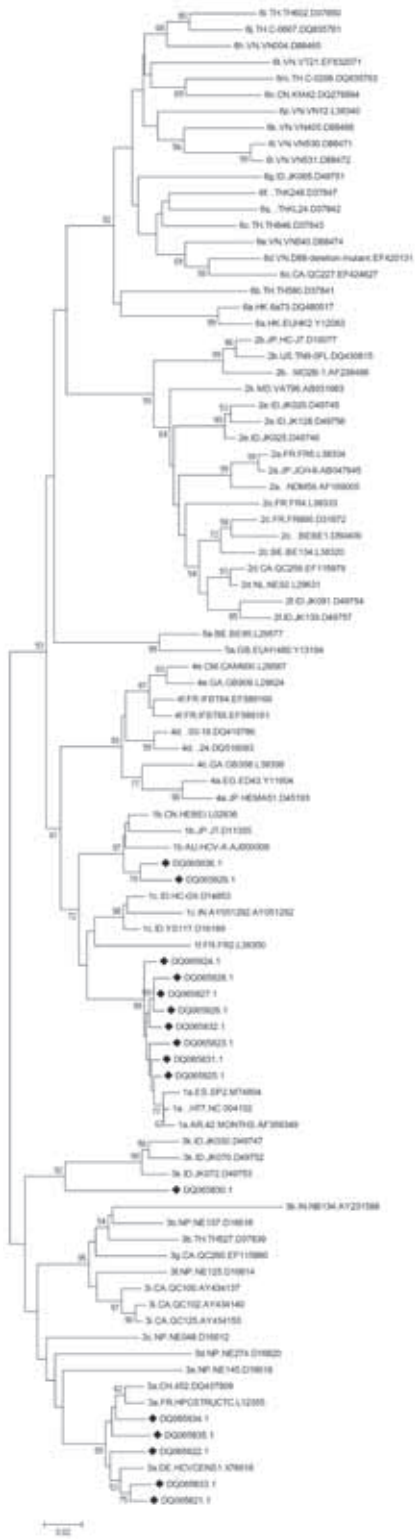

**Figure 2.** Phylogenetic analysis of core sequences (nt 22 to 364) from 16 HCV isolates. The isolates obtained from the North, Northwest, West, Northeast and East, Southwest and South, and Center of Iran. Sequences for each major subtype were obtained from the GenBank database for analysis. The genotypes and accession numbers of the reference sequences are indicated in the tree.

but with different rates in the six studied regions. However, the difference was not statistically significant (P = 0.51).

1a was the most frequent genotype found in all regions, with the highest rate of 72.7% in the West and the lowest rate of 45.5% in Northwest of Iran. Genotype 3a was more prevalent in Northwest (31.8%) but its rate was 17.7% in Central region. The frequency of genotype 1b was 22.7% in Northwest; it was not detected in the West region.

Considering genotypes 1a and 3a, demographic data revealed that genotype 1a was less prevalent (52.3%) in 30-49 year age group, while genotype 3a was the highest (34.1%) in this group (Table 2).

Genotype 1b showed an increasing rate from a minimum of 7% in younger groups (age 13 and less) to 24% in age 50 years and older (P = 0.07).

Genotype 1a showed a similar rate (62%) in both sexes, while 3a and 1b genotypes were of higher rates in males and females (30%) (P = 0.003).

As Table 2 shows, genotype 1a (71.4%) and 3a (28.6%) had the highest prevalence rates in ethnic groups of Kurd, Lur and Arab habitants of West and Southwest of Iran (P = 0.351).

**Table 2.** Distribution of HCV genotypes by demographic variables in HCV-positive individuals in Iran.

| Demographic Variable | Status | No. tested | Genotype No (%) | | | P value |
|---|---|---|---|---|---|---|
| | | | 1a | 1b | 3a | |
| Age (years) | <30 | 43 | 29 (67.4) | 3 (7.0) | 11 (25.6) | 0.07 |
| | 30-49 | 44 | 23 (52. 3) | 6 (13.6) | 15 (34.1) | |
| | >50 | 29 | 19 (65.5) | 7 (24.1) | 3 (10.3) | |
| Sex | Male | 93 | 56 (60.2) | 9 (9.7) | 28 (30.1) | 0.003 |
| | Female | 23 | 15 (62.5) | 7 (30.4) | 1 (4.3) | |
| Ethnicity | Fars | 82 | 51 (62.2) | 11 (13.4) | 20 (24.3) | 0.351 |
| | Kurd, Lur, Arab | 14 | 10 (71.4) | 0 (0) | 4 (28.6) | |
| | Turk | 20 | 10 (50.0) | 5 (25.0) | 5(25.0) | |
| Marital Status | Single | 49 | 30 (61.2) | 3 (6.1) | 16 (32.7) | 0.06 |
| | Married | 63 | 39 (61.9) | 11 (17.9) | 13 (20.6) | |
| | Widow | 4 | 2 (50.0) | 2 (50.0) | 0 (0.0) | |
| Family Size | <4 | 20 | 9 (45.0) | 6 (30.0) | 5 (25.0) | 0.134 |
| | 4-6 | 52 | 31 (59.6) | 7 (13.5) | 14 (26.9) | |
| | >7 | 44 | 31 (70.5) | 3 (6.8) | 10 (22.7) | |
| Level of Education | Illiterate | 12 | 5 (41.7) | 6 (50.0) | 1 (8.3) | 0.001 |
| | Primary | 40 | 25 (62.5) | 5 (12.5) | 10 (25.0) | |
| | Secondary | 50 | 36 (72.0) | 1 (2.0) | 13 (26.0) | |
| | Diploma and higher | 14 | 5 (35.7) | 4 (28.6) | 5 (35.7) | |
| Total | | 116 | 71 (61.2) | 16 (13.8) | 29 (25.0) | |

The widows showed to be the most infected group with the genotype 1b (50%) in comparison with single (6.1%) and married groups (17.9%); 3a was the most prevalent genotype in single groups (32.7%) (P<0.06).

The family size did not have a significant association with HCV genotype distribution, although genotype 1a had the highest (70.5%) prevalence rate in families with seven or more members and 1b had the highest rate in families with less than four members.

There was a significant difference in the distribution of genotypes considering the level of education-the highest rate of genotype 1a (72%) was in those with secondary school, 1b (50%) in illiterates and 3a (35.7%) in those who completed the high school (P<0.001).

There was no difference in the genotype distribution considering the factor of traveling and job (data not shown).

The rate of HCV genotypes in relation to the studied risk factors (Table 3), including history of blood transfusion, intravenous drug abuse (IVDU), dental visit, laboratory work, surgery, liver disease, alcohol consumption and endoscopic procedures, did not differ significantly.

**Table 3.** Distribution of HCV genotypes by risk factors in HCV-positive individuals in Iran.

| Risk factors | Status | No. tested | Genotype No (%) | | | P value |
|---|---|---|---|---|---|---|
| | | | 1a | 1b | 3a | |
| History of transfusion | Yes | 62 | 36 (58.1) | 10 (16.1) | 16 (25.8) | 0.67 |
| | No | 54 | 35 (64.8) | 6 (11.1) | 13 (24.1) | |
| History of IVDU | Yes | 16 | 12 (75.0) | 0 (0.0) | 4 (25.0) | 0.24 |
| | No | 100 | 59 (59.0) | 16 (16.0) | 25 (25.0) | |
| History of dental visit | Yes | 74 | 46 (62.2) | 11 (14.9) | 17 (23.0) | 0.76 |
| | No | 42 | 25 (59.5) | 5 (11.9) | 12 (28.6) | |
| Laboratory work | Yes | 17 | 11 (64.7) | 1 (5.9) | 5 (29.2) | 0.65 |
| | No | 99 | 60 (60.6) | 15 (15.2) | 24 (24.2) | |
| History of Drug using | Yes | 15 | 12 (80.0) | 0 (0.0) | 3 (20.0) | 0.21 |
| | No | 101 | 59 (58.4) | 16 (15.8) | 26 (25.7) | |
| History of surgery | Yes | 58 | 39 (67.2) | 8 (13.8) | 11 (19.0) | 0.32 |
| | No | 58 | 32 (55.2) | 8 (13.8) | 18 (31.0) | |
| History of liver disease | Yes | 15 | 8 (53.3) | 3 (20.0) | 4 (26.7) | 0.62 |
| | No | 101 | 63 (62.4) | 13 (12.9) | 25 (24.8) | |
| History of alcohol Consumption | Yes | 21 | 16 (76.2) | 0 (0.0) | 5 (23.8) | 0.1 |
| | No | 95 | 55 (57.9) | 16 (16.8) | 24 (25.3) | |
| History of endoscopy | Yes | 19 | 15 (78.9) | 3 (15.8) | 1 (5.3) | 0.08 |
| | No | 97 | 56 (57.7) | 13 (13.4) | 28 (28.9) | |

All the sequenced samples were submitted to the gene bank NBCI and except for one, the sequencing data from 15 samples, confirmed the PCR-RFLP results.

Phylogenetic analysis showed that our 16 Iranian isolates were grouped with genotypes 1 and 3 (Fig. 2). Eight cases including DQ065824, DQ065832, DQ065827, DQ065826, DQ065828, DQ065831, DQ065825, and DQ065823 were identified as genotype 1a; five cases including DQ065835, DQ065834, DQ065821, DQ065833, and DQ065822 as 3a subtype; and two cases including DQ065829, and DQ065836 as 1b by PCR-RFLP as well as phylogenetic analysis. One case (DQ065830) identified as 1b by PCR-RFLP, was however grouped in a separate branch close to 3h and 3k subtypes by the phylogenetic analysis (Fig. 2).

## Discussion

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 [24, 25].

Several environmental, pathological, genetic and immunological factors may contribute to the profound differences in disease progression observed in HCV-infected subjects. Therefore, long-term prospective studies in various population groups are still needed to provide reliable data on the clinical significance of HCV genotypes.

The objective of the present study was to determine the distribution of HCV genotypes and their association with sociodemographic characteristics and a number of risk factors in a representative population of HCV-infected subjects from different regions in Iran.

Our study showed that 1a subtype was the most frequent genotype (61.2%) with almost an even distribution in all the studied regions of Iran (Fig. 1). This is in accordance with reports on HCV genotypes in blood donors [16] and patients [26, 27] and also with Ramia [28] who suggested two main patterns for the distribution of HCV genotypes in the Middle East-one peculiar to the Arab countries where genotype 4 predominates, and another pattern characteristic for non-Arab countries (like Iran) where genotype 1 predominates [28].

The high frequencies of genotypes 1a (61.2%) and 3a (25%) are different from our neighbor countries, where the predominant genotype is 1b in Turkey [29, 30], type 3 in Pakistan [31], type 4 in Iraq [32] and 1b in Russia [9, 33]; the distribution in Iran

is more similar to European countries [33-35] like Spain with a frequency of 64% for 1a followed by genotype 3 (20.9%). This may indicate that possibly, HCV transmission has in part occurred via receiving blood or blood products from Europe and North American countries in the past. This is more emphasized by previous studies on HIV-1 infection in hemophiliacs in Iran by Sarami's group [36] who reported receiving of infected blood factors imported from Europe and North America as the main route of virus transmission in Iran. However, more information are needed for confirming the route of HCV transmission in Iran. Because of similar routes of HIV and HCV transmission, this scenario may be a possible explanation. However, since blood safety is improved nowadays, it is more likely that the major route of transmission has been switched to other modes.

The similar distribution of our HCV genotypes to that of Europe and North America, may indicate that IVDU is the major route of HCV transmission in our country, since this route is recently considered as the core of HCV transmission in epidemics in Europe. Genotypes 1a and 3a are also nominated as the IVDU-associated genotypes in Europe and North America [37]. This is more likely since IVDU is now considered as the most important source of fuelling new HIV infections in Iran [36] which is in agreement with a recent report [27] on distribution of HCV genotypes in patients in Iran which also found a similar pattern of HCV genotypes in IVDUs in both Europe and the USA with Iran.

In fact, traveling cannot be the source of the infection in our country, since in those who had neither traveled to any other country nor even been outside their region, and in those with no history of receiving any blood or blood products, the 1a and 3a isolates seem to be local, and thus, their origin as well as their subsequent spread of the genotypes need to be carefully investigated. This is in agreement with the results from Alavian [38] who reported that no apparent risk factors could be demonstrated in 24.5% of the positive cases of blood donors in Iran.

Correlations of HCV genotypes with demographic and epidemiological variables show that genotype 1a is evenly distributed in both sexes, different age groups and various ethnic groups all over the country, indicating that this genotype has been present and circulated in our community for a long time. This is confirmed by presence of the highest prevalence rate (65.5%) of genotype 1a in the oldest group (≥50 years), the homogenous socioeconomic pattern of the community with no history of immigration and also the absence of infections by more distant HCV 1a genotype (Table 2).

Considering the association of the higher levels of HCV-RNA in patients with genotype 1a to the likelihood of development of a more serious disease [39] and a lower response to antiviral treatments [40], it is speculated that these could be the major factors contributing to the current circulation and maintenance of genotype 1a as well as substitution of other genotypes by this genotype in our population. Yet, this remains to be proven.

We found no association between genotypes and gender, place of residence, age, and exposure to a set of risk factors such as IVDU, blood transfusion, history of dental visit, lab work, liver disease, alcohol intake, past surgery. This is similar to reports from Alaska [39] and Italy [41] where the distribution of HCV genotypes was unrelated to demographic variables.

Using the HCV 5'UTR, despite some mistyping in the annotation of subtypes, has proved to be effective in discriminating the major genotypes [42, 43]. However, sequencing other viral regions is a better tool for more accurate subtyping and molecular epidemiological studies [44]. In spite of using 5'UTR analysis in this study; sequencing techniques revealed a new subtype for the first time tentatively named as subtype 31 [45]. The report on non-typeable HCV samples in Iran [27] may indicate the need for sequencing which may find more new subtypes.

The possibility of identifying more genotype 3 variants in Iran cannot be ruled out since variants other than subtype 3a and 3b have also been reported from countries such as Bangladesh, Pakistan, Thailand, Nepal, Vietnam and India [21]. Therefore, conducting a multicenter study representing large numbers of isolates for all provinces of Iran seems essential to provide more accurate countrywide data.

We also concluded that RFLP is satisfactory for routine and quick genotyping of isolates and that sequencing of the core region is more suitable for further classification of the virus into subtypes.

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