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ORIGINAL ARTICLE

High prevalence of seronegative occult hepatitis C in high-risk individuals

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ABSTRACT

Background and Objectives: Routine screening for occult hepatitis C virus (OHCV) is not a standard procedure in medical laboratories, which has resulted in an increased incidence of OHCV among high-risk groups and the general population. The objective of this study was to investigate the molecular epidemiology of (OHCV) in Iranian injecting drug users (IDUs).
Materials and Methods: To determine chronic hepatitis C virus(HCV) and OHCV, plasma and peripheral blood mononuclear cell (PBMC) were collected from 103 (96 (93.2%) males, 7 (6.79%) females) IDUs. Their plasma was tested for Anti-HCV (ELISA). Following RNA extraction from plasma and PBMCs, RT-nested PCR was employed to amplify the 5′ untranslated region (5′UTR) and core regions of the HCV genome in plasma and PBMCs from IDUs. Sequencing of the 5′UTR and core regions, along with phylogenetic tree construction, was used to determine HCV genotypes.
Results: Among the 103 individuals, 12/96 males (12.5%) were positive for both anti-HCV and HCV RNA in plasma, indicating chronic HCV infection. In addition, 18/96 males (18.75%) and 1/7 females (14.28%) were positive for anti-HCV but negative for HCV RNA, indicating evidence of past HCV infection (p = 0.1). Furthermore, 5 individuals, including 4/94 males (4.1%) and 1/7 females (14.28%), were found to be seropositive for HCV (p = 0.77). Meanwhile, 23/103 individuals (22.33%), including 20/96 males (20.8%) and 3/7 females (42.85%), were seronegative for HCV (p = 0.37). HCV genotype 1a was the dominant genotype among IDUs.
Conclusion: In conclusion, a high prevalence of HCV infection was observed among IDUs, underscoring the pressing necessity for the implementation of an efficacious strategy to eradicate HCV transmission in this high-risk population.

Keywords: Hepatitis C; Hepatitis C antibodies; Hepatitis C antigens; Drug users

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INTRODUCTION

According to estimates provided by the World Health Organization (WHO) in 2022, approximately 50 million individuals worldwide are affected by chronic infections of the hepatitis C virus (HCV). WHO estimated that approximately 242,000 people die each year from cirrhosis and liver cancer as a direct consequence of this infection (1). Hepatitis C is characterized by inflammation of the liver, which is specifically caused by the hepatitis C virus (HCV). The virus can cause both acute and chronic hepatitis (2), with the severity of the illness ranging from mild to severe and potentially lasting a lifetime. Furthermore, the illness can result in the development of cirrhosis and liver cancer. HCV is a virus that is transmitted through the blood. It primarily spreads through blood exposure from unsafe injection practices, unsafe healthcare procedures, unscreened blood transfusions, injecting drug use, and unsafe sexual practices that lead to blood exposure (3). The use of direct-acting antivirals (DAAs) is highly effective for curing of individuals infected with HCV, with a cure rate of over 95% (4).

Occult hepatitis C virus infection (OHCI) is defined as the presence of hepatitis C virus (HCV) HCV-RNA in hepatocytes or peripheral blood mononuclear cells (PBMCs) without detectable HCV RNA in serum using conventional polymerase chain reaction (PCR) assays (5). Two categories of OHCI have been identified: seronegative OHCI (negative for HCV RNA and anti-HCV antibodies in serum) and seropositive OHCI (negative for HCV RNA and positive for anti-HCV antibodies in serum), while HCV-RNA is detectable in hepatocytes or peripheral blood mononuclear cells (PBMCs) (6). Observation of OHCI has been documented in patients with chronic liver disease, coinfections, or comorbidities. Furthermore, OHCI has been documented in populations with risk factors, such as IDUs, who are at an elevated risk for the transmission of blood-borne infectious diseases. This indicates that individuals with OHCI may contribute to the transmission of OHCV (7).

The World Health Organization (WHO) has set a goal of eliminating the hepatitis C virus (HCV) by 2030. To achieve this, the WHO aims to diagnose 90% of people living with HCV (PLHCV) and treat 80% of them. This is to reduce the incidence of HCV in high-risk groups, such as people who inject drugs (PWID), dialysis patients, and recipients of unsafe blood transfusions (8). The PWID represent the primary source of hepatitis C virus (HCV) infection, resulting in two distinct groups of people living with HCV (PLHCV). The first group is comprised of individuals who were infected through unsafe medical practices and who have comorbidities such as obesity, alcohol use, and diabetes. The second group is a younger cohort that was infected through injection drug use (IDU) in the recent past (9). The phase was simplified to:" Positive HCV people who don't inject drugs may still spread the virus if they share contaminated instruments. Furthermore, OHCI has been previously documented in this population (10, 11).

In Iran, there is a paucity of epidemiological data on HCV infection among people who inject drugs (PWID). In Iran, the occurrence rate of Hepatitis C Virus (HCV) showed approximately 0.13% among blood donors and is reported to be below 1% within the broader general population (12). The rate of anti-HCV detection was reported 0.5% among the general population of Semnan city, Iran (13). The objective of this study was to assess the prevalence of HCV/OHCV infection among PWID receiving harm reduction services in the Ahvaz city of Iran.

MATERIALS AND METHODS

Study design. The cross-sectional study was conducted. The data were derived from the 'Ahvaz Behavioral Diseases Counselling Center among Female and Male Injecting Drug Users in Ahvaz, Khuzestan province, Iran, from February 2022 to December 2022.

Inclusion criteria. Drug users who had not been tested for HCV RNA, negative HBsAg, HBV DNA, HIV tests, participant who had not received direct-acting antiviral treatment.

Exclusion criteria. The participants who were positive HBsAg, HBV DNA, anti-HCV tests. Participants under chemotherapy, or who had cirrhosis or hepatocellular carcinoma, were excluded from the study.

Study sample. The descriptive analytical cross-sectional investigation was performed. The following sample size was based on a prevalence of 42% report-

ed by Najimi-Varzaneh et al (2020) (14). Considering a 95% confidence level and an error margin defined d= 0.25 X p included in the study.

The sample size was estimated using the following formula (14):

$$n = \frac{Z^2\,\mathrm{P}(1-\mathrm{P})}{d^2} = 103$$

Z1-$^{\alpha}$/2 =1.96, p=0.42, p-0.58-1 , d= 0.25 X p=105

The demographic characteristics of the participants that have been used in this study, age, sex, the level of education, the duration of drug injecting time, sexual contacts, and marital status, were recorded.

Collection and preparation of the specimens. Approximately 5 mL of peripheral blood was collected from each individual in a sterile Vacutainer tube containing EDTA. Plasma required for HCV antibody screening, alanine aminotransferase (ALT) measurement, and HCV RNA genome extraction was obtained by centrifugation of the blood samples at 2,000 g rpm for 10 minutes. To obtain peripheral blood mononuclear cells (PBMCs), the precipitated blood cells were treated with Ficoll-Hypaque (Lymphodex, Inno-Train, Germany) and centrifuged at 400 × g for 20 minutes at a temperature range of 18-24°C. PBMCs were washed three times with PBS (pH 7.4) by centrifugation at 400 × g for 20 minutes. PBMC pellets were then suspended in PBS, and 200 µL of RNA later (Ambion, Austin, TX) was added. The samples were then kept at -80°C until the RNA extraction process was initiated.

Serological and biochemical tests. The plasma samples were subjected to an anti-HCV antibody test using a third-generation commercial ELISA kit (DIAPRO, Diagnostic, Bio Probes Srl, Milano, Italy) in accordance with the manufacturer's instructions. Furthermore, all patients underwent an assessment of alanine aminotransferase (ALT) levels (Pars Azmon Kit, Iran).

Extraction of viral RNA and cDNA synthesis. Viral RNA was extracted using the High Pure Viral Nucleic Acid Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. The extraction was performed on approximately 3-5 × 10^6 PBMCs using neobar lam. The concentration of the extracted viral RNA from plasma and peripheral blood mononuclear cells (PBMCs) was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA).

A cDNA synthesis kit (Yekta Tajhiz, Iran) was employed to synthesize cDNA according to the manufacturer's instructions. The cDNA was subsequently stored at -80°C for future testing.

RT-nested PCR for the 5′-UTR region. To detect the presence of HCV RNA, Specific primers were employed to amplify and detect the 5'-UTR region of the HCV genome in plasma and PBMCs using RT-nested PCR (Table 1).

The initial round of amplification was conducted using a 25 µl volume comprising PCR master mix, (Amplicon, Denmark), 1 µL (10 pmol/ µL) forward, 1 µL (10 pmol/ µL) reverse primers,300 ng cDNA, and distilled water. In the second round, 1 µL of the first-round product was used as a template, and 9.5 µL of distilled water was added to reach a final volume of 25 µL. The first and second rounds of RT-nested PCR mixture were subjected to a thermocycler (Peqlab, Germany) with the following thermocycling program: initial denaturation at 94°C for 5 minutes; 35 cycles of 94°C for 30 seconds, 62°C for 45 seconds, 72°C for 30 seconds; and a final extension at 72°C for 10 minutes.

The resulting PCR products were analyzed by electrophoresis on a 2% agarose gel, stained with a DNA-safe stain, and visualized under ultraviolet light. The expected lengths of the PCR products for the outer and inner sets were 306 bp and 252 bp, respectively (11).

RT-nested PCR for the core regions. The specific primers were employed to amplify and detect the core region of the HCV genome in plasma and PBMCs using RT-nested PCR (Table 1).

The initial round of amplification was conducted using a 25 µl volume comprising PCR master mix, 12.5 µL (Amplicon, Denmark), 1 µL (10 pmol/L) of each forward and reverse primers, 400 ng cDNA, and distilled water up to 25 µL. In the second round, 1 µL of the first-round product was used as a template, and 9.5 µL of distilled water was added to reach a final volume of 25 µL. The first and second rounds of RT-nested PCR mixture were subjected to a thermocycler (Peqlab, Germany). The thermocycling process consisted of 30 cycles, each of which was conducted under the following conditions: an initial

Table 1. Primers and PCR conditions for 5UTR and Core HCV regions detections

Primers	Sequence	5´UTR HCV Region	Fragment Size	PCR Condition
				94Cº/5 min
BKP-7	F	5´-CACTCCCCTGTGAGGAACTACTGTC-3´	306 bp (11)	35cycles:
BKP-8	R	5´-ATGGTGCACGGTCTACGAGACCTCC-3´		94Cº/30 Sec
				62Cº/45 Sec
1st Round				72Cº/30 Sec
				94Cº/5 min
BKP-9	F	5´-TTCACGCAGAAAGCGTCTAGCCATG-3´	252 bp (11)	35cycles:
BKP-10	R	5´-GCGCACTCGCAAGCACCCTATCAGG-3´		94Cº/30 Sec
				62Cº/45 Sec
2nd Round				72Cº/30 Sec
Primers	**Sequence**	**5´UTR Core HCV Region**	**Fragment Size**	**PCR Condition**
				94Cº/4 min
SC2:		5´-GGGAGGTCTCGTAGACCGTGCACCATG-3´	500 bp (11)	30cycles:
AC2:		5´-GAGMGGKATRTACCCATGAGRTCGC-3´		94Cº/1 min
				55Cº/1 min
1st Round				72Cº/2 min
				94Cº/4 min
S7:		5´-AGACCGTGCACCATGAGCAC-3´	420 bp (11)	30cycles:
584:		5´-CCCATGAGGTCGGCRAARC -3´		94Cº/1 min
				55Cº/1 min
2nd Round				72Cº/2 min

denaturation at 94°C for 4 minutes, followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 2 minutes. The final extension step was performed at 72°C for seven minutes. For the second round, 2 µL of the first-round product was used as a template, 1µL (10 pmol/L) of each forward and reverse primer, PCR master mix, 12.5 µL, 8.5 µL of distilled water to reach a final volume of 25 µL (Table 1). The same program and thermal conditions were followed for the second round. The resulting PCR products were analyzed by electrophoresis on a 2% agarose gel, stained with a DNA-safe stain, and visualized under ultraviolet light. The expected lengths of the PCR products for the outer and inner sets were 500 bp and 420 bp, respectively (11).

HCV genotyping and phylogenetic analysis. The polymerase chain reaction (PCR) amplified products that yielded positive results were subjected to sequencing in both the forward and reverse directions using an Applied Biosystem 3500 instrument manufactured by ABI Scientific in the United States.

The analysis and alignment of both forward and reverse sequences were performed using SnapGene Sequence Alignment Editor version 3.2.1.

The results of 20 samples, comprising 10 partial sequences of the 5'UTR and 10 core regions, were submitted to GenBank to obtain the approved Gene Bank version of accession number. A phylogenetic tree was constructed using the maximum likelihood method for the partial sequences of the 5' UTR and core regions of the HCV genome from the HCV RNA isolates found among IDUs in Ahvaz City. This study compared the analyses of partial sequences of the 5' UTR and core regions with known HCV genotypes from various regions worldwide to assess the prevalence of HCV genotypes in the region. The Kimura 2-parameter distance model was employed in this method, which was executed with 1,000 bootstrap replicates. The aforementioned methods were analyzed using MEGA software version 6.

Statistical analysis. Results were summarized with descriptive statistics (mean, median, and standard deviation for numerical variables, frequencies, and percentages for categorical variables). Confidence intervals (CI) are presented to assess the asso-

ciation between independent and outcome variables. A p-value < 0.05 was considered to indicate statistical significance. The data analysis was conducted utilizing the SPSS software version 21. The data was subjected to analysis using chi square test.

Ethical considerations. Ethical considerations. This study was conducted with approval ethical code IR.AJUMS.REC.1400.318 by the ethics committee of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran. Informed consent was obtained from patients or their corresponding relatives.

RESULTS

Demographic characteristics: Among 103 IDUs, the age ranged from 21 to 72 years and the average age was 44.58 ± 13.9 years. The level of ALT among the IDUs ranged between 57 and 96 IU/L.

In this study, chronic HCV infection was detected in 12 out of 103 participants (11.65%). This included 12 out of 96 males (12.5%) who tested positive for both anti-HCV and HCV RNA in plasma. No chronic HCV infection was found in the 7 female participants (p=0.7). Additionally, 19 out of 103 participants (18.44%) showed evidence of prior exposure to HCV but no current infection. These individuals tested positive for anti-HCV but negative for HCV RNA in plasma. This group comprised 18 out of 96 males (18.75%) and 1 out of 7 females (14.28%) (p=0.1). Furthermore, 5 out of 103 individuals (4.85%) were found to be seropositive for HCV (positive anti-HCV, negative HCV RNA in plasma, but positive HCV RNA in PBMCs). This group consisted of 4 out of 96 males (approximately 4.16%) and 1 out of 7 females (14.28%) (p=0.77). A further 23 out of 103 participants (22.33%), including 20 out of 96 males (20.8%) and 3 out of 7 females (42.85%), tested seronegative for HCV (negative anti-HCV, negative HCV RNA in plasma, positive HCV RNA in PBMC) (p=0.37). Overall, 54 (52.42%) IDUs had detectable HCV markers. The remaining 49 (47.57%) IDUs showed no detectable HCV markers (negative anti-HCV, HCV RNA in plasma and PBMC) (Table 2). The distribution of HCV markers between males and females was not significantly different (50/96 males, 52.08%; 4/7 females, 57.14%; P=0.83).

Among the IDUs, the prevalence of chronic hepatitis C infection did not differ significantly by sex (p=0.7), level of education (p=0.53), years of abuse (p=0.94), STD status (p=0.69), or marital status (p=0.98). Similarly, past HCV infection prevalence was not significantly associated with sex (p=0.1), level of education (p=0.76), years of abuse (p=0.20), STD status (p=0.88), and marital status (p=0.83). For negative HCV infection, no significant associations were found with sex (p=0.23), level of education (p=0.706), years of abuse (p=0.38), STD status (p=0.97), or marital status (p=0.54). Regarding seropositive HCV status (positive anti-HCV, negative HCV RNA in plasma, positive HCV RNA in PBMCs), no significant differences were observed based on sex (p=0.77), level of education (p=0.94), or marital status (p=0.18), years of abuse (p=0.2) and STD status (p=0.2). Finally, for seronegative HCV status (negative anti-HCV, negative HCV RNA in plasma, positive HCV RNA in PBMC), prevalence was not significantly different by sex (p=0.37), level of education (p=0.19), or marital status (p=0.43). Years of abuse (p=0.0001) and STD status (p=0.003) were also found to be significantly associated with seronegative HCV status.

The 5′ UTR sequences of three non-OHCV samples (OR498610–11, OR498619), four seropositive OHCV samples (OR498613, OR498618, OR498620–21), and two seronegative OHCV samples (OR498615, OR498617) were found to cluster with HCV genotype 1a, which has been isolated from various regions worldwide. However, one non-OHCV sample (OR498612) clustered with HCV genotype 3a.

The accession numbers for the core sequence, three non-OCI samples (OR-751372, OR-751374–75), four seropositive OCI samples (OR-751376–79), and two seronegative OCI samples (OR-751370–71) were found to cluster distinctly with HCV genotype 1a, which was isolated from various regions worldwide. However, one non-OCI sample (OR751373) clustered with HCV 3a isolates (D17763) from Japan. The results are presented in phylogenetic trees (Figs. 1 and 2).

DISCUSSION

Chronic hepatitis C virus (HCV) infection is a major challenge for public health worldwide. The aim of the World Health Organization is to eliminate hepatitis C infection by 2030 (1). Approximately 15-30% of individuals with chronic hepatitis C are at risk of developing cirrhosis within 20 years (14). Salary et

Table 2. Demographic and clinical data for Iranian drug users.

Parameter	Positive Anti-HCV, Positive HCV RNA 12/103 (11.65%)	Positive Anti-HCV, Negative HCV RNA 19/103 (18.44%)	Negative anti-HCV, Negative HCV-RNA 49/103 (47.57%)	Seropositive OHCV 5/103 (4.85%)	Seronegative OHCV 23/103 (22.33%)
Sex	P=0.7	P =0.1	P=0.23	P=0.77	P=0.37
Male	12/96 (12.5%)	18/96 (18.75%)	43/96 (44.79%)	4/96 (0.04%)	20/96 (20.8%)
Female	0/7 (0.0%)	1/7 (14.28%)	6/7 (85.7%)	1/7 (14.28%)	3/7 (42.85%)
Level of education	P=0.53	P=0.76	P=0.706	P=0.94	P=0.19
Under diploma	11 (31.4%)	5 (10.2%)	24 (68.6%)	2 (5.7%)	4 (11.4%)
Diploma	13 (26.5%)	5 (10.2%)	28 (57.1%)	2 (4.1%)	14 (28.6%)
Upper diploma	5 (26.3%)	2 (10.5%)	11 (57.9%)	1 (5.3%)	5 (26.3%)
Years of abuse:	P=0.94	P=0.20	P=0.38	P=0.2	P=0.0001
>3years	26 (28.3%)	12 (13.0%)	57 (62.0%)	4 (4.3%)	19 (20.7%)
<3years	3 (27.3%)	0 (0.0%)	6 (54.5%)	1 (9.1%)	4 (36.4%)
STD	P=0.69	P=0.88	P=0.97	P=0.2	P=0.003
Yes	6 (25.0%)	3 (12.5%)	14 (58.3%)	1 (4.2%)	6 (25.0%)
No	23 (29.1)	9 (11.4%)	49 (62.0%)	4 (5.1%)	17 (21.5%)
Marital Status	P=0.98	P=0.83	P=0.54	P=0.18	P=0.43
Single	14 (27.5%)	6 (11.8%)	31 (60.8%)	4 (7.8%)	10 (19.6%)
Married	11 (28.9%)	5 (13.2%)	25 (65.8%)	0 (0.0%)	8 (21.1%)
Divorced	4 (28.6%)	1 (7.1%)	7 (50.0%)	1 (7.1%)	5 (35.7%)

al. (15) reported that 21.6% of males and 8.7% of females with chronic HCV infection in the age range of 40-74 years were at risk of developing liver cancer. Shiffman et al. (16) reported that 50% of injecting drug users had HCV, and that 25% of these individuals were under 25 years old. Injecting drug users are less likely to seek healthcare for HCV treatment (16). Of the sampled individuals, plasma from 12 out of 96 males (12.5%) tested positive for current HCV infection (defined by both anti-HCV and HCV RNA in plasma), while zero out of 7 females showed current infection (p=0.7 for sex difference). Additionally, plasma from 18 out of 96 males (18.75%) and 1 out of 7 females (14.28%) tested positive for anti-HCV but negative for HCV RNA. These results indicated prior exposure to HCV but no current infection (p=0.1 for sex difference). Among the total participants, 5 individuals (4.85%) were seropositive for OHCV (4 males, 1 female), and 23 individuals (22.3%) were seronegative for OCI (comprising 20 males and 3 females). Overall, 54 (52.42%) injecting drug users (IDUs) had detectable HCV markers. The prevalence of HCV markers in our study is consistent with findings from other studies among IDUs: ranging from 26% to 47.7% in different regions of Iran (17, 18), 44.82% in people who inject drugs across 22 countries in the Eastern Mediterranean region (19) and a reported rate of 61.01% HCV infection among people who inject drugs (PWIDs) globally, based on an assessment of 16 studies involving 38,952 participants (20). Overall, this survey found a high prevalence of OHCV markers among IDUs, with 28 out of 103 individuals (27.18%) testing positive. This group comprised 5 individuals who were seropositive for OHCV and 23 who were seronegative for OHCV. The prevalence of OHCV among injecting drug users (IDUs) has been reported in Portugal at 20.8% (21) and in Egypt at 42% (22).

OHCV has been reported in various high-risk groups worldwide. In Egypt, it was found in hemodialysis patients at 5% (23). In Taiwan, a prevalence of OCI was reported in 11.7% treatment-naive HCV Ab-positive and 5.6% in HCV Ab-negative patients (24). In Egypt, OHCV was found in patients with lymphoproliferative disorders (37.5%) (25) and in patients with blood-related malignancies (32%) (26). In Iran, a prevalence of 7.9% was noted in liver transplant patients with cryptogenic cirrhosis (27). Furthermore, among HCV patients who achieved sustained virological response (SVR) in Egypt, OHCV cases ranged from 5% to 50% (23, 26, 28, 29).
Phylogenetic analysis of the 5'UTR revealed that out

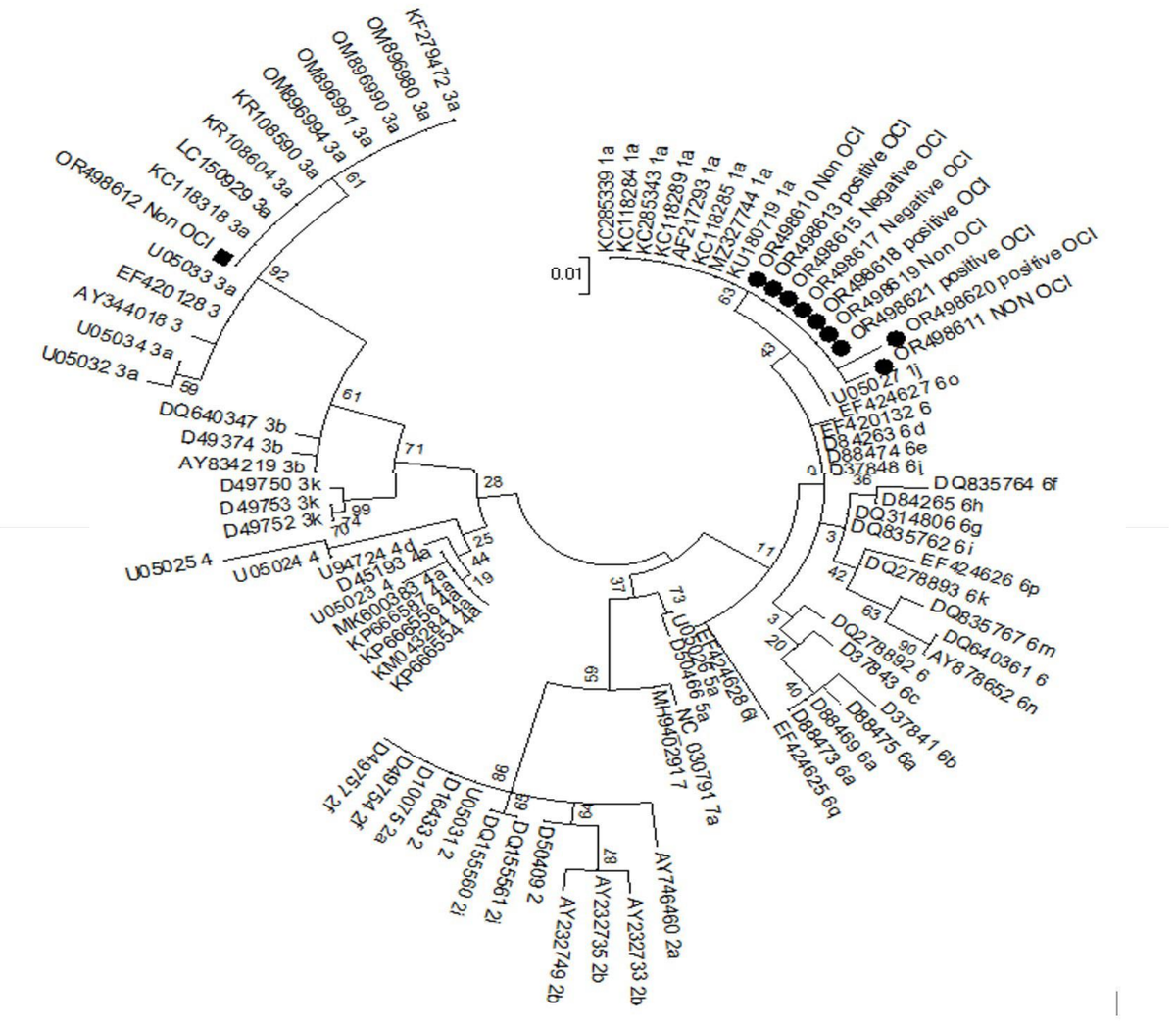

Fig. 1. A phylogenetic tree was constructed using the maximum likelihood method to compare partial sequences of the 5'UTR region of HCV isolates from IDU individuals in Ahvaz city with 10 HCV genotypes. The corresponding accession numbers were obtained from GenBank, and these genotypes were collected from various regions across the globe. The results of the phylogenetic tree showed that all 9 HCV isolates (OR498610-11, OR498613, OR498615, and OR498617-21) from Ahvaz city, identified by black circles, were clustered with HCV genotype 1a isolates from different regions of the world. One HCV (OR498612) isolate was clustered with an HCV 3a (KC118318) isolate from Iran as well as other HCV 3a isolates from various regions around the world. The accuracy of the phylogenetic tree was assessed by performing 1000 bootstrap replicates. The scale bar was set to 0.001.

of ten isolates (OR498610-11, OR498613, OR498615, OR498617-21), eight were identified as HCV genotype 1a: four non-OHCV isolates (OR498610-12, OR498619), four seropositive OHCV isolates (OR498613, OR498618, OR498620-21), and two seronegative OHCV isolates (OR498615, OR498617). These eight isolates clustered with other global HCV genotype 1a isolates. One HCV isolate, OR498612, clustered with HCV 3a (KC118318) from Iran and other worldwide samples. Analysis of core sequences showed nine of the ten HCV strains clustered closely with HCV genotype 1a, distinct from other known genotypes. These included three non-OHCV strains (OR-751372, OR-751374-75), four seropositive OHCV strains (OR-751376-79), and two seronegative OHCV strains (OR-751370-71). One additional HCV infection (OR751373) demonstrated clusters with HCV genotype 3a isolates from various regions. Further evaluation of the full sequences of all nine HCV isolates is necessary to ascertain if these represent a novel form of the virus.

The prevalence of HCV genotypes 1a, 3a, and 1b

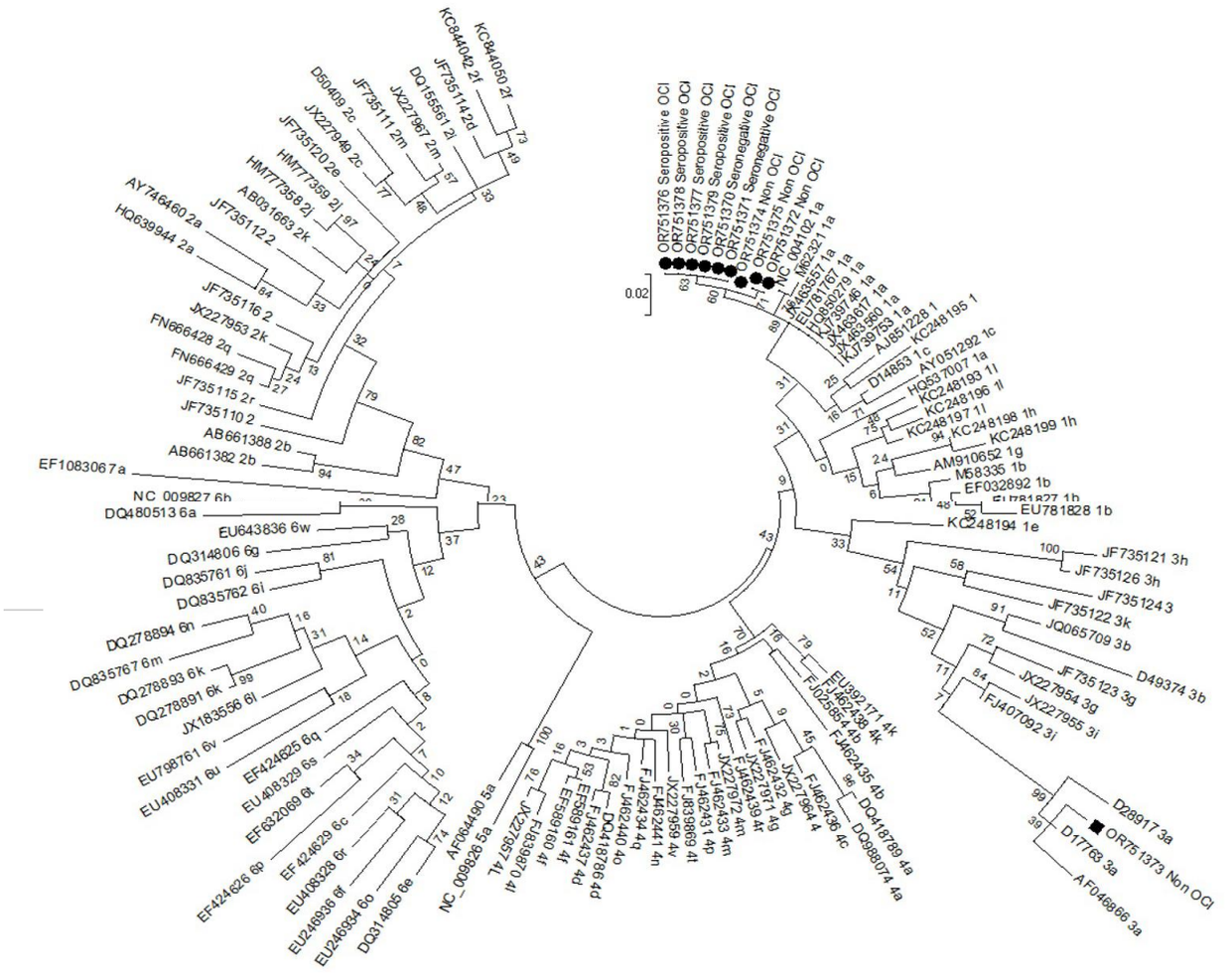

Fig. 2. A maximum likelihood method was used to construct a phylogenetic tree for comparing partial sequences of the core region of HCV isolates from individuals who IDUs in Ahvaz city with 10 distinct HCV genotypes. The accession numbers were obtained from GenBank. The genotypes were collected from various regions around the world.

The phylogenetic tree shows the grouping of all nine HCV isolate accession numbers (OR751370-72, OR751374-79) from Ahvaz city, identified by black circles, which were distinct from other HCV genotype 1a isolates from various regions of the world. Additionally, an HCV isolate (OR751373) marked with a black square was clustered with an HCV 3a isolate (D17763) from Japan and other HCV 3a isolates from around the world. The accuracy of the phylogenetic tree was assessed by conducting 1000 bootstrap replicates. The scale bar was set at 0.02.

was found to be dominant in Iran (30, 31). In Taiwan, the genotype is 1a and 1b (32).

The present study is limited to the investigation of partial sequences of the 5′ untranslated region (5′UTR) and core regions of the hepatitis C virus (HCV) genome. Therefore, further analysis of the complete 5′UTR and core regions is warranted. To detect low copy numbers of the HCV genome among injecting drug users, real-time PCR quantification should be employed. In Iran, the prevalence of HCV infection among injecting drug users is estimated to be approximately 47.7% (18), whereas the prevalence in the general population is around 1% (11).

No significant associations were observed between chronic hepatitis C infection, past HCV infection, negative HCV status, seropositive OHCV, or seronegative OHCV and sex, level of education, or marital status among IDUs. Years of drug abuse and history of sexually transmitted diseases (STDs) were also not significantly associated with these infection categories, except in the case of seronegative OHCV, where significant associations were found for years of drug abuse ($p = 0.0001$) and STDs ($p = 0.003$). Ryan et al. (2024) reported that the prevalence of 314

314 active hepatitis C infection among female IDUs in Madrid, Spain, was 62/314 (19.7%), compared with 252/314 (80.3%) among male IDUs; this difference was not statistically significant (p = 0.1) (33). Scarpetta et al. (2025) reported that the prevalence of HCV infection among 92/162 (56.79%) IDUs was 38/79 (48.10%) in San Francisco, USA, and 54/83 (65.06%) in Montreal, Canada; this difference was statistically significant (p=0.003) (34). Direct-acting antiviral medications (DAAs) have the potential to cure over 95% of individuals infected with hepatitis C, yet access to both diagnosis and treatment remains limited (35).

According to the analysis of the WHO expert committee, four groups of communities are at risk of HCV infection: slums, injecting drug users, prison populations, and family members of HCV-infected persons. These groups should be screened with serological and molecular HCV tests (35).

The World Health Organization announced that HCV infection should be eliminate by 2030 (36). Therefore, screening for occult HCV infection is essential. Detection of HCV in PBMCs from high-risk groups such as injecting drug users, recipients of blood transfusions or transplanted organs, people with glomerular nephropathies, people on hemodialysis or kidney transplants, patients with hepatitis B infection, cryptogenic cirrhosis, hematological or lymphoproliferative disorders, and people with HIV infection is recommended. This will facilitate the implementation of further preventive measures.

CONCLUSION

The HCV infection rate observed in this study is higher compared to other regions in Iran. While the seronegative occult HCV infection rate was higher than in other Iranian regions, the seropositive occult HCV rate was low.

To fully understand these strains, a comprehensive evaluation of the entire HCV genome in isolates is essential. Therefore, effective prevention of HCV transmission necessitates the implementation of screening measures. This includes testing for anti-HCV antibodies and molecular detection of HCV RNA in both serum and PBMCs for high-risk groups, particularly injecting drug users (IDUs). Furthermore, it is recommended to determine the HCV genotype in the plasma and PBMCs of positive IDUs. These measures will aid in the development and implementation of targeted preventive strategies.

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