Occult Hepatitis C Virus Infection among Hemodialysis Patients: An Iranian Experience

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Abstract
Background: Patients with chronic kidney failure and those undergoing chronic hemodialysis (CHD) treatment are at high risk of infection with hepatitis C virus (HCV). The incidence of occult HCV infection (OCI) in CHD remains controversial and the real burden of HCV in this population may be affected by the rate of OCI. This study evaluates the molecular assessment of OCI in CHD in an Iranian population.

Methods: All subjects on CHD in the South Khorasan province of Iran were invited for participation in the study. Whole blood samples were taken and serological, clinical, and demographic information was recorded. HCV-RNAs were checked in serum and peripheral blood mononuclear cells (PBMCs) using an in-house semi-nested PCR assay. Viral load was determined using a real-time PCR-based quantification kit. Sequencing was performed to determine genotypes.

Results: Overall, 120 cases participated in the study; 57.5% were male and the rest were female. In serum samples, no positive case was found for HCV-RNA. In PBMC samples, 2/120 (1.6%) were positive for HCV-RNA (95% CI, 0.002 to 0.059); the mean age of OCI positive cases was 37.5 ± 19.2 years which was significantly lower than OCI negative cases (P = 0.026). Only one case had detectable viral load which was 49 IU/mL. The only HCV genotype identified was 1a.

Conclusion: This study showed that there is a risk of OCI among CHD patients; the very low and undetectable viral loads of OCI warrant further follow-up molecular testing for earlier diagnosis and treatment in the era of DAA.

Keywords: Dialysis, Genotype, Hemodialysis, Hepatitis C virus, Occult HCV


Introduction
Hepatitis C virus (HCV) infection is still a major health concern around the world. Globally, 71 million people are estimated to have chronic HCV infection with 399,000 HCV-related deaths occurring in 2016, mostly from cirrhosis and hepatocellular carcinoma. The rate of HCV infection is in close association with intravenous drug use, sharing needles or syringes, tattooing, multiple sexual partners, blood transfusion, hemodialysis, and etc. However, with the advent of direct acting antivirals (DAA), there are promising prospects in reduction of HCV burden. In Iran, the prevalence of HCV is estimated at 0.3% among the general population, 6.2% among intermediate risk populations, and 32.1% among high-risk populations, which is lower than other countries.

The newly identified type of HCV infection, the occult HCV infection (OCI), is defined as the presence and detection of HCV-RNA in hepatocytes and/or in peripheral blood mononuclear cells (PBMCs) in the absence of, or undetectable HCV-RNA in serum. OCI can be observed in two types: seropositive (positive for anti-HCV but negative for HCV-RNA), and cryptogenic HCV infection (negative for the both markers). OCI is important in terms of transmission, hepatic diseases, and even extrahepatic tissue damage (lymphoproliferative disorders, glomerulopathies and end-stage renal disease [ESRD]).

Chronic kidney disease (CKD) and ESRD are global major health conditions which are mostly progressive, irreversible conditions. Globally, the incidence of ESRD has been increasing in both developing and developed countries over the last decades. Similarly in Iran, it increased from 467 in 2006 to 507 per one million in 2010.

Dialysis is the most common reliable and accessible therapeutic approach in ESRD which increases survival and life expectancy in patients. Despite the screening tests on blood products and devices, nosocomial infections are one of leading causes of morbidity and mortality in ESRD. HCV is more prevalent in ESRD compared to the general population, with an overall prevalence of 9.9% among chronic hemodialysis (CHD) patients in high- and middle-income countries. According to a recent meta-analysis, the overall prevalence of anti-HCV among CHD patients in Iran was estimated at 8.3% ranging from 0.0% to 31.4%. This is far higher than the 0.3% rate in the
general population of Iran.

The mortality and morbidity of kidney transplant patients positive for HCV are higher than those of non-HCV infected individuals. Although use of DAAs as a new therapeutic regimen has resulted in considerable improvement in management of CHD patients, there are some recent findings on the observation of OCI in those who have developed sustained virologic response after DAA treatment. The global reported prevalence of OCI in CHD patients is extremely variable with a range between zero and 45%, which seems to have been affected by the regional epidemiology of HCV as well as access to new treatments. Note that there is significant controversy on this topic, and information is still limited on OCI in CHD populations.

In the clinical setting, although an OCI infection may lead to milder consequences compared to overt infection, there is the possibility of transmission of OCI to others in dialysis units. Thus, diagnosis, follow-up, and treatment of OCI cases are recommended. Considering the variable global data on OCI and the limited information from Iran, especially eastern Iran, we aimed to evaluate the prevalence of OCI among patients undergoing maintenance dialysis in South Khorasan, eastern Iran.

Material and Methods
Study Settings and Patients
This was a cross-sectional study conducted between July 2018 and May 2019 in the hemodialysis units in the South Khorasan province of Iran. An informed consent was signed by the patients, and all of them completed a questionnaire including all related risk factors and demographic information. All the patients had been previously checked for anti-HCV (anti-HCV; Hepanostika HCV ultra, Beijing United Biomedical Co. LTD, Beijing, China) and the corresponding data were collected.

Sampling
A general call was sent out to all dialysis centers of the region, explaining the goals and details of the plan. According to the health statistics of the province, there were totally 150 patients undergoing maintenance dialysis in the province. Whole blood samples were taken from the participants, and a questionnaire was completed. PBMCs were isolated using a Ficoll-Histopaque density gradient. Isolated PBMC and plasma contents were aliquoted into two tubes; one was used fresh for RNA isolation and the other was persevered at -70°C until further experiments.

RNA Extraction and cDNA Synthesis
The tubes containing the PBMC were centrifuged and the cell pellets underwent RNA isolation using a Triazole approach. The RNA from serum samples was isolated using viral RNA extraction kit, according to the manufacturer’s instructions (Viral DNA/RNA extraction kit, FAVORGEN Biotech Corp, Ping-Tung, Taiwan). The purity of obtained RNAs was checked by a BioPhotometer (Nanodrop 1000), and the integrity was assessed by resolving the RNA on agarose gel (Figure 1); the ratio of 260/280 was ≥1.6. The RNA products (About 4 µg total RNA) were prepared for synthesis of cDNA using a random hexamer primer and applying the First Strand cDNA Synthesis Kit according to the kit’s instructions (Thermo Fisher Scientific, Massachusetts, USA).

Detection of HCV-RNA
Utilizing an alignment of 5’-UTR region of all known HCV genotypes, a semi-nested primer set was designed which allowed for detecting all HCV genotypes. The primers were as follows: HCF1: 5’-CGGTGAGTACCCGGAAAT-3’, HCF2: 5’-GCCCTTGTTGACGCGGATCTGAT-3’, HCR: 5’-ATGTACCCCATGAGRTCGG-3’. The first PCR reaction was performed in a final volume of 40 µL containing 4 µL of reverse transcription solution, 2X PCR master mix (Red, Amplicon), and 20 pmole of outer primers (HCF1 and HCR). The PCR program began at 95°C for 5 minutes, followed by 45 cycles including 94°C for 20 seconds, 54°C for 20 seconds, and 72°C for 50 seconds. Then, an extra extension step was applied for 5 minutes in order to produce full-length 595 base-pair strands. For the nested run, 2 µL of the first PCR product was applied in a reaction with 25 µL final volume using inner primers (HCF2 and HCR) under conditions similar to the first one (The annealing temperature was 56°C, and extension was performed for 30 seconds) for amplification of 474 bp segments of 5’-UTR region of HCV-RNA. The sensitivity of designed primers was checked on approved HCV genotypes 1a, 1b, 2a, 2b, 3a, 4a, and 5a that were kindly donated by Dr. M.H Karbalaie Niya, Firouzgar hospital.

The designed primers were checked to detect HCV genotypes including 1a, 1b, 2a, 2b, 3a, 4a, and 5a. One positive sample with a known concentration was serially diluted and then forwarded for determining the lower limit of detection (LLOD) for the designed primers. The result showed that this semi-nested PCR had an LLOD of 14 IU/mL (Figure 1).

Determination of HCV Viral Load
Viral load was determined using RoboGeneR HCV RNA Quantification kit (AJ Roboscreen GmbH, Germany), as described by the instructions of the kit (The lower limit of detection for the kit was 16.6 IU/mL).

HCV Genotyping
Sequencing of 5’-UTR region was performed to determine the genotypes. For this purpose, the semi-nested PCR products were subjected to a Gel/PCR purification kit according to the manufacturer’s instructions (Favorgen Biotech). The sequence of purified PCR products was
determined using BigDye terminator assay (Metabion international AG, Planegg/Steinkirchen, Germany). The raw sequence data were analyzed in BioEdit software, after which Mega 7 software was employed for construction of the phylogenetic tree using the attribute sequence of all HCV genotypes.

Statistical Analysis
All the information was recorded and analyzed using IBM SPSS version 25 (IBM Corp., Armonk, NY, USA). Descriptive statistics and mean values were obtained. Estimation of continuous and quantitative variables was performed using chi-square and Mann-Whitney U tests. Also, the risk factors were analyzed using chi-square tests.

Results
Baseline Information and Risk Factors
Overall, there were 150 patients with CHD maintenance in the South Khorasan province. Among them, 30 cases did not participate in the study; the reasons were long distance to the sampling centers, having a respiratory illness, being away on a trip, unwillingness to participate, and old age, fatigue, weakness and lethargy. Totally, 120 cases were willing and available to participate in the study; 57.5% were male and the rest were female. The mean age of the patients was 59.5 ± 14.1 years which varied from 21 to 88 years; the range and median were 67 and 61 years, respectively.

According to the information obtained from the questionnaires, hypertension was the most prominent risk factor observed among the HD patients (67.5%). Other conditions were as follows: diabetes (50.8%), history of blood transfusion (47.5%), and history of endoscopy (45%), history of seizures (7.5%), smoking (5%), acupuncture (4.2%), non-injecting addiction (5.8%), and imprisonment (1.7%). The mean length of dialysis during the lifetime of patients was 3.1 ± 3.1 years which varied from 1 month to 17 years; the range and median were 16.1 and 3.1 years, respectively.

Detection of OCI
Among the cDNA templates of serum samples, no positive case for HCV-RNA was found (95% CI, 0.00 to 0.031, Clopper-Pearson method). In the PBMC samples, two were found positive for HCV-RNA, one of whom was anti-HCV positive. Overall, 2/120 (1.6%) were found positive for OCI (95% CI, 0.002 to 0.059, Clopper-Pearson method); their mean age was 37.5 ± 19.2 years. Regarding gender, one was male and the other OCI-positive case was female (1.4% vs 2%), and both were married and had academic education level. Both OCI-positive cases had been undergoing HD three times per week for 2 years and 6 years, respectively. Totally, four patients had a history of kidney transplantation, one of whom (25%) was OCI-positive (P = 0.066). Regarding the low prevalence of OCI detected, there was no significant association with the risk factors. In terms of dialysis duration, the rate of OCI was 0.00, 1.5% and 2.5% among those with less than one year, 1-5 years and more than 5 years of dialysis, respectively. This result showed an increased pattern of OCI proportional to the duration of dialysis; however, due to the small number of cases positive for OCI, this correlation was not statistically significant (P = 0.806).

Viral Load of OCI and Genotypes
The viral load was determined through RoboGeneR HCV

![Figure 1. A) The product of RNA extraction was resolved on 1% agarose gel determining the integrity of RNA obtained. B) The result of LLOD determination was revealed after serial dilution of a sample with known concentration and implementation of PCR with the semi-nested PCR. Lane 1 is a 100 bp DNA size marker, lane 2: Positive control, lane 3: Negative control, lane 4: A known sample with 1.4 × 10⁷ IU/mL, lane 5: 1.4 × 10⁶, lane 6: 1.4 × 10⁴, lane 7: 1.4 × 10³, lane 8: 1.4 × 10², lane 9: 1.4 × 10¹, lane 10: 14, lane 11: 1.4, and lane 12: 0.14 IU/mL.](image-url)
RNA Quantification, as described by the kit's instructions (The lower limit of detection for the kit was 16.6 IU/mL). Of the two samples, only one had a detectable viral load which was 49 IU/mL. Sequencing of 5′-UTR region of HCV revealed that isolates of the current study belonged to the genotype 1a (Figure 2). The evolutionary history was inferred using the Maximum Likelihood method based on the Kimura 2-parameter model applied using the MEGA7 software.

Discussion
Kidney transplant patients and those on CHD are amongst the most significant groups in terms of HCV-associated clinical consequences. Nevertheless, there is still not enough clinical and epidemiological data on OCI among CHD groups. Furthermore, the global prevalence of HCV infection is extremely variable according to varied rates of reported OCI. Thus, lack of evidence on OCI in CHD groups leads to an underestimation in the real burden of HCV in these populations.

This study showed 1.6% prevalence of OCI in subjects with CHD in the South Khorasan province, eastern Iran. There has been no report from eastern Iran, and this is the first assessment of OCI in the region. Meanwhile, there have been limited studies from other regions of Iran: 0% in a report by Eslamifar et al on 70 HD patients in Tehran, and 3.03% among 200 HD patients in an earlier study in Tehran. These two reports are in line with the present study and support the fact that Iran has a very low prevalence of HCV. Nevertheless, most recently, an Iranian assessment reported 11.3% OCI among 515 cases on CHD. This study is in apparent contradiction with previous Iranian studies as well as ours, which can be explained based on the large sample size, inclusion criteria, and probably regional diversity of the samples.

The prevalence reported in the present work has strong consistency with the rate of HCV in Iran, as well as the study region. The prevalence of HCV is estimated at 0.3% among the general population in Iran, while in the general population of the study region, the prevalence of anti-HCV has been reported at 0.2%. On the other hand, among the 41 CHD cases of this province, the rate of HCV viremia was 2.4% at 2014. These findings support the low prevalence of OCI observed in this study. In other countries, higher prevalence rates of OCI have been reported compared to Iranian studies which seems to be highly associated with the higher prevalence of overt HCV infection and the rate of anti-HCV in those regions. In Spain, the rate of OCI across CHD groups was reported from 15% to 45%. Spain is one of most HCV-prevalent regions in the European Union, with the prevalence rate in the adult population estimated at 2.64% in 2011. In Egypt, which is the most prevalent HCV region in the world with a national estimation of 14.7%, the rate of OCI among CHD in different centers was reported at 3.7% out of 81, 4.8% out of 62, and 23% out of 40, though further large sample studies are still warranted in terms of OCI. These high rates of OCI suggest the need for strict following and preventive control programs among the CHD units. Furthermore, these findings represent an implicit association between the rates of HCV in each region with the rates of OCI observed among high risk groups.

Among the two OCIs identified, one had 49 IU/mL viral load while the other had no detectable viral load, which is in accordance with most previous studies, although some studies have found higher titers. This observation can be explained by the probable underlying mechanisms of OCI: low levels of infected hepatocytes, specific CD markers in immune responses, and memory T cell activation, which all can be involved in the suppressed status of HCV replication to a very low or undetectable levels. It has been determined that low copies of HCV as few as 20 copies can be transmitted and be infectious, where HCV can be potentially infectious in surfaces for at least 16 hours. On the other hand, a few studies determined OCI as a replicating form of HCV with the capability of transmission and infection. Previous molecular epidemiological studies have shown nosocomial...
transmission of HCV in dialysis units. Primarily, this can be due to underlying diseases including diabetes and CKDs resulting in weakened cellular immunity.

Furthermore, the operation of hemodialysis includes events that increase the risk of exposure to bloodborne pathogens which constantly occur during the patient’s lifetime: blood transfusion, equipment, medication re-use, surgical operations, contaminated surfaces, gloves, patient trays, fistula lavage sinks, etc.

Although blood transfusion was recorded among 47.5% of the studied participants, it was not associated with the incidence of OCI; this can be due to the small number of OCI observed in the current study. However, blood transfusion has been previously associated with higher prevalence of OCI. Among the risk factors, hypertension was the most prominent risk factor observed among the studied CHD patients (67.5%), followed by diabetes (50.8%), blood transfusion (47.5%), and history of endoscopy (45%). Furthermore, some of these conditions have been previously described to be in association with other bloodborne pathogens in the region of study.

The isolates identified in the study belonged to the genotype 1a. The HCV genotype 1a has been determined as the most prevalent HCV genotype in Iran followed by genotype 3, as with most parts of the world. Thus, our result is consistent with previous regional and global studies. In the region of the study, namely the South Khorasan province, previous studies have determined the HCV genotype 3a followed by 1a as the most prevalent.

The discrepancy with the results of those studies can be due to the population type and the time of studies; all those studies were among prisoners and hemophiliacs implemented before the advent of DAAs treatment. Nevertheless, one study conducted in Iran revealed the very low dominance of the 1a, while instead 1b was most prevalent among Azerbaijani patients which is highly in line with former Soviet Union regions.

In conclusion, this study showed that there is a risk of OCI among CHD patients, and the very low and undetectable viral loads of OCI suggest that more follow-up molecular actions are required for monitoring, diagnosis, and treatment of HCV-infected patients.

Authors’ Contribution
MZ: get the idea, designed, supervised the study and critical reviewing of manuscript. ST: performed sampling, collection of data, experiments and data analysis. DJ: designed and performed experimental procedures and drafting manuscript.

Conflict of Interest Disclosures
All the authors have stated there is no conflict to declare.

Ethical Statement
This project was approved by the research ethics committee of Birjand University of Medical Sciences (BUMS), with the ethical code number IR.BUMS.REC.1397.85.

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