Iranian Journal of Microbiology
Volume 13 Number 5 (October 2021) 703-711

ORIGINAL ARTICLE

Frequency and genotype distribution of hepatitis C virus infection in patients with diabetes type 2 in Ahvaz, Iran

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Received: March 2021**, Accepted:** August 2021

ABSTRACT

Background and Objectives: Diabetes is recognized as a great concern and a public health problem worldwide. Several factors including environmental and genetic factors have been involved. Recently, infectious agents such as hepatitis C virus (HCV) have been reported to be associated with diabetes. Thus, this study was conducted to determine the frequency of HCV infection among patients with diabetes type 2 in Ahvaz city, Iran.
Materials and Methods: A case-control study design was conducted at Ahvaz Jundishapur University of Medical Sciences. A total of 600 study subjects were included in this research. All the patient sera were tested for Anti- HCV antibody, HBsAg, and HIV antibody. The sera of positive Anti-HCV antibody, were assayed for 5'- UTR and core regions of the HCV genome by Nested RT-PCR. Finally, the HCV genotyping was determined by sequencing.
Results: The prevalence of HCV in type 2 diabetes and nondiabetic controls was 2% and 0.33%, respectively. The distribution of HCV genotypes among the HCV-positive patients were 3a (1.66%) and 1a (0.33%).
Conclusion: To control and improve the treatment, the screening of HCV infection with anti-HCV antibody was followed by molecular techniques such as PCR and HCV genotyping which should be implemented for all patients with diabetes type 2.

Keywords: Diabetes mellitus type 2; HCV; Prevalence; Genotype

INTRODUCTION

Diabetes mellitus (DM) is one of the most prevalent chronic metabolic disorders reported in different regions of the world. The clinical course of the disease is represented by the lack of insulin (DM1) or the increase of peripheral insulin resistance (DM2). The clinical signs comprise a rise in blood sugar levels resulting, in polyuria, polydipsia, and weight loss. There are several types of diabetes among which diabetes mellitus types one and two are the most prevalent in different regions of the world (1). In 2013, at least 382 million adults had diabetes (all types) worldwide, and this number rose to 422 million by

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2014 (2).

Approximately 2-5% of the world population and 4.6%-10.0% of the Iranian population are suffering from DM (3). Type 2 DM accounted for 90-95% of all diabetic cases. Several risk factors including environmental, obesity, physical inactivity, aging, genetic predisposition, and viral infections have been associated with diabetes type 2. Among viral infections, HCV infection has become a great concern (4).

Nowadays, chronic HCV infection is considered a systemic disease while it only not affects the liver, but also the other organs. Nearly three-quarters of patients also suffer from extrahepatic manifestations, which is evident even before the diagnosis of chronic HCV infection. Diabetes mellitus type 2 (T2DM) is one of the most common extrahepatic manifestations of chronic HCV infection (5).

HCV genome, is a single RNA strand, with positive sense, genus Hepacivirus and belonging to Flaviviridae family (6). Based on the genomic diversity, HCV has been classified into seven genotypes and over 100 different subtypes (7).

HCV is transmitted via infected transfusion of blood, organs transplantation, surgery, sharing drug injection equipment, sexual intercourse, and tattooing (8). Most individuals newly infected with HCV are asymptomatic (9). Approximately 20% of the infected individuals develop symptoms such as fatigue, abdominal pain, poor appetite, or jaundice which usually last for 4-12 weeks. More than 50% of the infected individuals acquire chronic hepatitis, which may finally lead to severe liver disease, cirrhosis, and the development of hepatocellular carcinoma (HCC) (8). WHO has estimated that about 180 million of the world population are infected with HCV (10).

However, the rate of HCV prevalence varies from 0.5% to 10% in different regions of the world (11). In Iran, the seroprevalence of HCV among blood donors is about 0.13% and in general population is less than 1% (12). Iran is considered as a country with a low frequency of HCV infection (13). Nevertheless, a high rate of 50%-75% of HCV infection has been observed among intravenous drug abusers (IDUs) (14). Overall, it seems the prevalence of HCV is rising in the country (15).

A recent study in Iran indicated the prevalence of HCV genotype 3a among the patients with T2DM while in Egypt, the association HCV genotype 4 has been reported among the patients with T2DM (16) .

Liver transplantation may be required as a result of HCV infection (16). Moreover, the HCV infection may contribute to extrahepatic manifestations which involve rheumatologic, dermatologic, renal, hematologic, and endocrine abnormalities like diabetes mellitus (11, 17). There are several reports on the prevalence of HCV infection among patients with diabetes mellitus in Iran, the results show great heterogeneity between 0-2.5% (18).

Nowadays, the number of diabetic patients is increasing; it is estimated that the cases may rise from 171 million in 2000 to 366 million by 2030 which indicates a global threatening health problem reaching pandemic levels by 2030. On the other hand, the number of people with diabetes is also rising from 84 million to 228 million in developing countries (5). Thus, evaluation of viral agents associated with the diabetic need to investigate among the diabetic population. There are some reports describe that HCV may alter glucose homeostasis through direct or indirect mechanisms, such as the TNF-α pathway, which induce the destruction of insulin signaling pathways and subsequently result in the development of insulin resistance (19). Immune-mediated pathogenesis or direct cytotoxic effects of HCV on pancreatic islet cells results in dysfunction of β cells and declines the insulin production (20). Concerning the significance of these consequences, this study was conducted to determine HCV genotypes among diabetic patients >40 years old in Ahvaz city, Iran. Ahvaz city is the capital of Khuzestan Province, located in the southwestern region of Iran.

MATERIALS AND METHODS

Ethical approval. This study with registration number D-9215 was approved by the ethics committee of Ahvaz Jundishapur University of Medical Sciences, Iran. All experiments were performed in compliance with relevant laws and institutional guidelines and under the ethical standards of the Declaration of Helsinki. Ethical consent was obtained from each participant registered in this study. Blood samples were collected only from those patients who were interested to donate their samples voluntarily. The information of each patient under the study was preserved confidentially.

Specimen collection. The case-control study was carried out for 300 confirmed type 2 diabetic outdoor

patients and 300 non-diabetic individuals who regularly attending for a check-up at the Endocrinology Clinic of the Golestan hospital, and Jahad Clinic from October 2014 through March 2015. The control group was matched for age and sex with the patient group. In this study, cases and controls were defined as follows:

Cases. Known, diagnosed diabetic patient. All the T2DM participants were under antidiabetic agent consumption.

Controls. Non-diabetic, voluntary counseling with fasting blood sugar (FBS) <100 mg/dl or Random blood sugar (RBS) <126 mg/dl, with no overt liver disease.

All the patients were aged > 40 years, and they were asked to fill in a questionnaire regarding age, sex, surgery, family history of diabetes, history of blood transfusion, tattooing, dental service, intrafamilial hepatitis, and intravenous drug use. All the participants were confirmed to have diabetes based on 2 fasting plasma glucose levels > 126 milligram per deciliter (mg/dl) and 2-hour post-prandial blood glucose levels 200 mg/dl, respectively.

Laboratory testing. About 5 ml of the blood sample was collected from each individual 12 hrs after fasting and centrifuged to collect the serum from the blood. The biochemistry tests including fasting blood sugar and liver enzymes tests (AST, ALT) were carried out for all the patients using BT 3000 autoanalyzer. All the sera were tested for HBsAg, Anti-HCV antibodies, Anti-HIV antibodies, using a fourth-generation ELISA (Diapro, Italy) according to the manufacturer's instructions. The positive samples for the anti-HCV antibody were tested for the detection of 5'UTR and Core regions of the HCV genome by Nested RT-PCR.

RNA extraction and cDNA synthesis. The total RNA was extracted from individuals positive for anti-HCV positive using the high pure viral nucleic acid kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instruction, followed by preparation of cDNA (Fermentas company).

Nested RT-PCR for 5' untranslated region (UTR). The following primers from the 5'UTR of Hepatitis C virus genome were used in nested RT-PCR:

BKP-7,-CACTCCCCTGTGAGGAACTACTGTC (nucleotides 38 to 62) as the outer sense,

BKP-8,ATGGTGCACGGTCTACGAGACCTCC (nucleotides 319 to 343) as the outer anti-sense;BKP-9,TTCACGCAGAAAGCGTCTAGCCATG (nucleotides 63 to 87) as the inner sense;BKP-10,GCGCACTCGCAAGCACCCTATCAGG (nucleotides 292 to 314) as the inner anti-sense primer (21). For the first round: The reaction mixture containing 2.5 µl 10× PCR buffer with $MgCl_2$ (Roche), 0.5µl dNTP mix (0.2 mM), 1 µl of BKP-7 and BKP-8 primers (10 pmol), 0.2 µl (1 unit) Taq polymerase, 5 µl of template and water up to 25 µl. The PCR was performed on Techne Thermocycler (UK) for 35 cycles. Cycling conditions were as follows: 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec with a final extension at 72°C for 10 minutes. The second round was carried out like the first round with the inner set of primers (BKP-9 and BKP-10) with the same PCR mixture and program. PCR product was subjected to electrophoresis on a 2% agarose gel, stained with DNA safe stain, and observed under ultraviolet light. The first-round PCR product was 306 bp for the outer set, and the second product was 254 bp for the inner set.

Nested RT-PCR for the core region. The samples positive for the 5'UTR region were again tested for the HCV core region of the HCV genome by nested RT-PCR. The following specific primers, including SC2: GGGAGGTCTCGTAGACCGTGCACCATG, AC2: GAGMGGKATRTACC-C ATGAGRTCGGC, S7: AGACCGTGCACCATGAGCAC and 584: CCCATGAGGTCGGCRAARC were used (22). To do this, 3 µl of the template with the same amount of PCR reaction mixture as described previously was subjected to a thermocycler for 30 cycles. The cycling conditions were accomplished as follows: 94°C for 4 min; 30 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 2 min, and final elongation at 72°C for 7 min. The expected PCR product for the outer set and the inner set was 500 bp and 420 bp, respectively. PCR product was subjected to electrophoresis on a 2% agarose gel, stained with DNA safe stain, and observed under ultraviolet light.

HCV genotyping/sub-typing using nucleotide sequencing. The PCR product of 6 samples that were positive for 5'UTR and core region were sequenced (Bioneer Company, South Korea).

GenBank accession numbers. The nucleotide sequence data of core protein were deposited in the GenBank database and given accession numbers.

Phylogenetic analysis. To clarify the relationship between the HCV isolates, phylogenetic analysis was performed based on the core region. HCV nucleotide sequences were aligned by Muscle (Mega 6.0 software). The Maximum likelihood method was constructed for the HCV core region with parameters such as Genetic distances by Kimura two-parameter, with the site heterogeneity gamma and invariant sites, phylogenetic distances by the nucleotide substitutions model. The bootstrap probability at a branching point was calculated with 500 pseudo-replicate datasets.

Statistical analysis. The statistical analysis was performed using SPSS (Version 19) software for the comparisons of variables using 2 ×2 tables with x^2 test. The p-value of ≤0.05 was considered as the level of significance.

RESULTS

In this study, a total of 600 participants (300 subjects with diabetics and 300 nondiabetic controls) were included. Of the diabetic subjects, 112 (37.3%) were males and the rest of 188 (62.7%) were females. On the other hand, 112 (37.3%) males and 188 (62.7%) females were included from nondiabetic controls. The ratio of females to males was 1.67:1. The patient ages ranged from 40 to 70 with the mean age of 42.93 ± 8.37 years. The mean duration of diabetes was 9.26 ± 6.04 years. 30 (10%) subjects were treated with insulin. ALT level was raised in 66.66% of the positive cases, as compared to 23.8% of the seronegative patients. The prevalence of HCV in diabetes and non-diabetic controls was found out to be 2% and 0.33%, respectively (P<0.05). There was a statistically significant difference in the distribution of HCV among diabetic and non-diabetic controls. 6 (2%) patients showed positive results for the Anti- HCV antibody and both HCV 5' UTR and core regions by nested-PCR. Table 1 shows demographic data among diabetic patients. 254 patients showed seronegative for Anti- HCV antibody. The distribution of HCV genotypes were 5 (1.66%) 3a, 1 (0.33%) 1a among diabetic patients and 1 case (0.33%) with 3a genotype among control group.

All the positive samples for HCV infection displayed negative results for Anti-HIV antibody and HBsAg tests. Table 2 demonstrates the distribution of HCV infection and the risk factors in the diabetic group. Figs. 1 and 2 show the nested RT-PCR amplification of the hepatitis C virus and Fig. 3 shows the nucleic acid identities of the Iranian and other isolates.

Table 1. Demographic data among diabetic patients

	Type 2 DM patient (n=300) Frequency (%)	**Type 2 DM patient (n=300) Mean ± standard deviation**
Gender	112 (37.3)	
Male	188 (62.7)	
Female		
Age		
Mean age (yr)		42.93 ± 8.37
AST		23.7 ±13.2
ALT		19.91 ±13.5
BMI (kg/m2)	7 (2.33)	
<18.5	150 (50)	
18.5-24.9	130 (43.33)	
25-29.9	13 (4.33)	
>29.9		
HCV	294 (98)	
Negative	6 (2)	
Positive		
Viral genotypes	1	
Genotype 1	5	
Genotype 3		

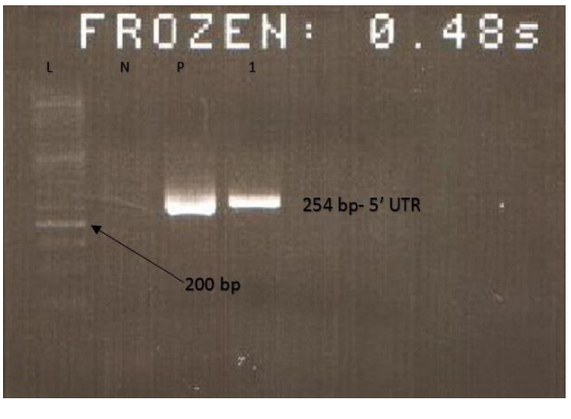

Fig. 1. The nested RT-PCR RT-PCR amplification of the hepatitis C virus with the following primers from 5' UTR. L, 50-bp DNA ladder; –, negative control; +, positive control; 1, amplified product (254 bp) on 2% agarose gel electrophoresis

Table 2. Frequency distribution of HCV risk factors in the diabetic group

Risk factors for HCV	HCV +ve Frequency (%)	Diabetes (n=300) HCV –ve Frequency (%)	P value
Blood transfusion			0.00
Yes No	1 (0.33)	4 (1.36)	
Surgery	5 (1.66)	290 (96.66)	
			0.00
Yes	1 (0.33)	94 (31.33)	
No	5 (1.66)	200 (66.66)	
Drug addiction			0.00
Yes No	2 (0.66)	1 (0.33)	
Hospitalization	4 (1.33)	293 (97.66)	
			0.00
Yes	2 (0.66)	140 (46.66)	
No	4 (1.33)	154 (51.33)	
Tattoo			
Yes	0 (0)	2 (0.66)	NA
No	6 (2)	292 (97.33)	
Dental services			
Yes	0 (0)	184 (61.33)	NA
No	6 (2)	110 (36.66)	
Contact with jaundiced			
Yes	0 (0)	20 (6.66)	NA
No	6 (2)	294 (98)	

NA:not applicable.

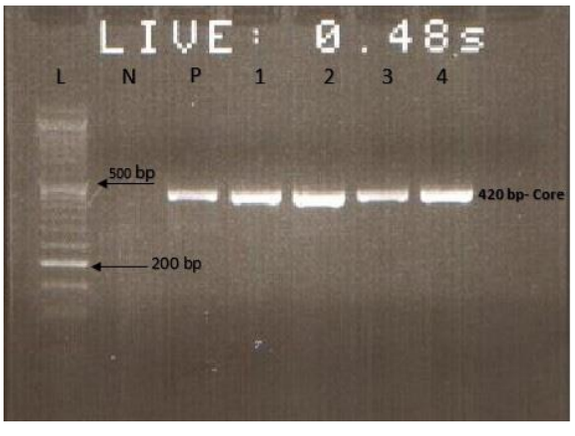

Fig. 2. The nested RT-PCR RT-PCR amplification of the hepatitis C virus with the following primers from the core region. L, 50-bp DNA ladder; –, negative control; +, positive control; 1-4 amplified product (420 bp) on 2% agarose gel electrophoresis.

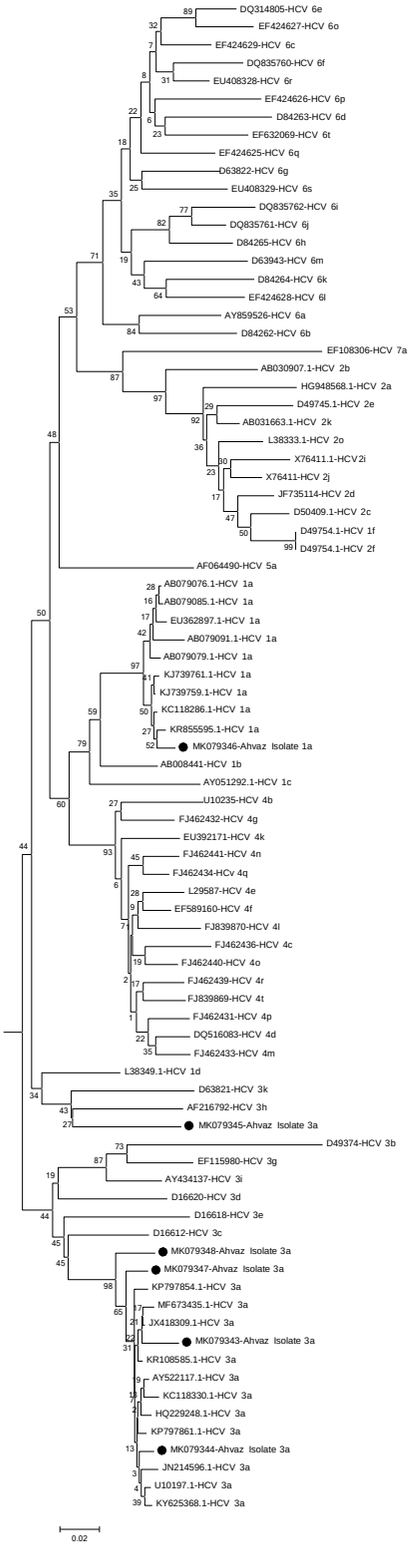

Fig. 3. Phylogenetic tree Maximum likelihood method was constructed for the core region of the HCV genome isolated from patients with diabetes mellitus in Ahvaz city. The sequences of HCV core region of HCV genome with

accession number: MK079343, MK079344, MK079345, MK079346, MK079347, and MK079348 were compared with different genotypes (1-7) and relevant subtypes retrieved from GenBank. The isolated HCV genotypes 1b and 3a with black circles are in cluster with other HCV 1b and 3a isolated from different regions of the world. The Maximum likelihood method was done with under the Kimura two-parameter substitution model with the site heterogeneity gamma and invariant sites, phylogenetic distances by Kimura two-parameter model using MEGA 7 software (https://www.megasoftware.net/). The scale bars represented the frequency of nucleotide substitutions. The accuracy of the tree was assessed by 500 bootstrap replicates.

DISCUSSION

Several studies have shown that the prevalence of HCV among diabetic patients is significantly higher than in non-diabetic patients (23, 24). Remarkably, the high rate of HCV infection is found more among the diabetic mellitus males over 40 years of age with abnormal liver enzymes (25). Recent data also suggest three times higher prevalence of type 2 diabetes in HCV seropositive patients (26).

In the present study, 2% of HCV patients were associated with type 2 diabetes which was 6.66% and 3.33 times higher than the rate of 0.33 and 0.6% HCV infection in the control group and general population in Iran, respectively (27). Solomon et al. have reported the prevalence of HCV infection among the individuals with type 2 diabetes which was 3.17 times higher than the population in Ethiopia (28).

Mehta et al. have described the risk of developing diabetes, in chronic HCV patients over 40 years of age, which increases up to 3.77 times more in the non-diabetic group in the United States (29). Our finding is in concurrence with the results reported by Solomon and Mehta (28, 29). The prevalence of HCV infection has been described in different continents in Table 3 (4).

Furthermore, uncontrolled group studies were reported in Pakistan, Sobia et al. 2007, Nigeria, James et al. 2009, Japan, Michiaki et al. 2003, Japon, Fukui et al. 2003, Pakistan, Qureshi et al. 2002, Italy, Sangiorgio et al. 2000, United kingdom, Gray et al. 1995, Taiwan, Chen et al. 2006 and Maryland, Howard et al. 2003 have exhibited that the prevalence of HCV in different diabetic groups varied from 5.1-36% with $p< 0.05$ (11, 30-37). In contrast, some studies have reported low prevalence of HCV infection in patients with type 2 diabetic (38-41).

Table 3. Global anti-hepatitis C virus prevalence and number of infected individuals (all ages)

Continent	Anti-HCV prevalence (%)	Anti- HCV infected (millions)
Africa	2.9	26.9
Middle East	2.7	12.7
America	1.3	12.4
Asia	2.8	111.6
Australasia	1.8	0.5
Europe	1.8	13.4

The prevalence of HCV genotypes has been reported in patients with diabetic type 2. In the present research, the most prominent HCV genotypes were 5 (83.33%) 3a, followed by low detection of 1 (16.66%) 1a which were found in patients with diabetes type 2. In the study conducted by Memon MS et al. the association of HCV genotypes 3 was detected in the patients with type 2 diabetes which is in agreement with our results (26). Farshadpour et al. have detected 1.98% HCV infection with genotype 3a among the patients with type 2 diabetic mellitus (DM) which was in agreement with our findings (4). About circulating of HCV genotypes, several reports have been revealed that the genotype 1a, genotype 3a is the most dominant genotypes in different regions while genotypes 2 and 4 are infrequent in Iran (4, 18).

Aging (≥40 years) in HCV seropositive patients was significantly associated with type 2 diabetes mellitus as compared to the younger age group (≥18 to 40 years) (42). It seems Advancing age, increased weight, and HCV genotype 3 are independent predictors of type 2 diabetes in HCV seropositive patients (26).

In the present study, the phylogenetic analyses for the core region showed that the core sequences identified in the Iran/2015 isolates (Accession NO. MK079343, MK079344, MK079345, MK079347 and MK079348) belonged to the genotype 3 and subtype a. The strain with accession No. MK079346 belonged to the genotype 1 and subtype a. Based on the result of BLAST X, there were 99% amino acid identity among the MK079344 and MK079345 with KP797861.1 Ireland isolate and AF216793.1 Switzerland isolate respectively. MK079347 and MK079348 Ahvaz isolates were 99% identical with the KP797854.1 Ireland strain and also there was

99% amino acid identity among the MK079343Ahvaz isolate with the JX418309.1 Italy isolate. Additionally, the tree indicated that the MK079346 Ahvaz isolate was 99% identical to KR855505.1 isolate from Australia (Fig. 3).

So far, it is not clear whether all the known seven HCV genotypes can be associated in patients with type 2 diabetes mellitus. To manifest this issue, comprehensive investigations are required.

Although the exact cause of increased number of HCV infections in patients with diabetes is not clear, there are two probabilities; the first possibility is due to the immune-compromised state as a result of diabetes. The second possibility might be due to the direct and/or indirect effect of HCV infection on glucose metabolism (26, 28).

Experimental researches have shown that the HCV core protein can modify the metabolic profile of the infected cells which leads to the development of type 2 diabetes mellitus (43, 44). This hypothesis is also supported by experimental data derived from transgenic mice infected with hepatitis C core protein which might result in induced insulin resistance directly, before to the development of steatosis or fibrosis (45). In individuals with chronic hepatitis C (CHC) infection, it has been observed that the insulin signaling in the infected liver cells are altered via dysregulation in IRS-1 tyrosine phosphorylation and phosphatidylinositol 3kinase activation, which results in insulin resistance (46). The proinflammatory cytokine, TNF-α maybe mediate in this process. TNF-α is upregulated in patients with CHC and interrupts the insulin signaling via reduced-tyrosine phosphorylation of IRS-1 and decreased ability of IRS-1 to associate with the insulin receptor (47). Furthermore, some evidence suggests that the hepatitis C virus may further alter insulin signaling by upregulating expression of the protein suppressor of cytokine signaling 3, resulting in decreased activation of downstream components of insulin receptor signaling (IRS), and altered expression of sterol regulatory binding protein 1c, which is important in de novo lipogenesis (48, 49). The current study demonstrated that the HCV infection could be treated successfully with DAA (direct-acting antiviral) (5, 26). Thus, the early detection of HCV infection in new cases of diabetes by the serological and by high molecular means such as RT-PCR or Real-time PCR might help to stop the further damage to liver cells by HCV component and to rehabilitate patients to normal health.

CONCLUSION

In this study, it is indicated that there is an association between HCV infection and diabetes mellitus. 2% of patients with type 2 diabetes mellitus suffered from HCV infection. The distribution of HCV genotypes were 1.66% 3a, 0.3 1a. Regarding the outcomes of HCV infection and to improve the quality of treatment, it is strongly suggested that the serological and molecular screening of HCV should be implemented for all the new cases of diabetic patients to rule out HCV infection.

ACKNOWLEDGEMENTS

This project with registration number D-9215 was financially supported by Diabetes Research Center, Health Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran. The authors are also grateful to Jahad Clinic staff, Imam Khomeini Hospital Laboratory, staff in the Diabetes Clinic of Golestan Hospital, and diabetic patients for their collaboration during sample and data collections.

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