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Molecular study of Hepatitis C virus infection and genotypes among seropositive renal dialysis in Diyala province\ Iraq

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Abstract

Background: Hepatitis C virus (HCV) infection is a major health problem among dialysis patients in developing countries. Geographical distribution of various genotypes of HCV is useful for understanding the epidemiological status, detection of mode and source of infection, designing the program of control, evaluating the response to treatment and development of diagnostic methods and vaccine production.

Objectives: The present study aimed to detect the anti-HCV Ab and to investigate the HCV genotypes and subtypes among renal dialysis patients.

Materials and Methods: The prevalence of anti-HCV antibody among 90 renal dialysis patients was determined by Enzyme Linked Immunosorbent assay (ELISA). Then, HCV specific RNA was detected in those anti-HCV seropositive renal dialysis patients, utilizing conventional PCR, Furthermore, Genotyping the HCV-RNA positive samples was done. Simple statistical analysis was done using SPSS Version 25 and P value was considered significant wherever it is below 0.05. PCR products were sent for Sanger sequencing using ABI3730XL, automated DNA sequencer, by Macrogen Corporation – Korea. The results were received by email then analyzed using geneious software.

Results: The overall prevalence of Hepatitis C antibody positive patients were 32.2%, while the PCR test results were 17.8% in anti-HCV Ab positive. The 5^{-}-UTR gene sequences were submitted to NCBI which gave the published accession number on NCBI, it can be found on the website; https://www.ncbi.nlm.nih.gov/nuccore/LC546841.1/. In order to determine the genotypes of HCV, all of the renal dialysis who was positive for the 5^{-}-UTR gene had the genotype 1a.

Conclusions: The prevalence of HCV was high among renal dialysis in Diyala province/Iraq. HCV genotype 1a was the prevalent in all patients. Further population-based study is required to determine the prevalence HCV genotypes.

Keywords: HCV, genotype,anti-HCV Ab, renal dialysis.

Introduction

Infection with hepatitis C virus is a global health issue. Currently, more than 200 million subjects are infected with the hepatitis C virus. Chronic infection with the virus is associated with deleterious consequences such as liver cirrhosis and hepatocellular carcinoma (Nawfal *et al*., 2019).

Hepatitis C virus (HCV), a member of the Hepacivirus C species, is a small (55–65 nm in size), enveloped, positive-sense single-stranded RNA virus of the family Flaviviridae. The hepatitis C virus is the cause of hepatitis C and the causes of hepatocellular carcinoma (HCC) and lymphomas in humans (Rusyn, and Lemon, 2014; Smith *et al*., 2016). The HCV particle consists of a lipid membrane envelope, two viral envelope glycoproteins, E1 and E2, are embedded in the lipid envelope. They take part in viral attachment and entry into the cell. Within the envelope is an icosahedral core. Inside the core is the RNA material of the virus (Dubuisson *et al*., 2014).

Renal dialysis (RD) is the most frequent mode of renal replacement therapy in end-stage renal disease (ESRD). Patients undergoing RD potentially have an increased risk of exposure to infections especially blood-borne viruses infections. HCV is the most frequent disease resulting as a complication of RD treatment (Nadia and Marwa, 2019). Infections with HCV is well-known and important causes of liver disease in ESRD patients on RD. RD patients are at high risk for HCV infections due to a history of blood transfusion, the high number of blood transfusion sessions, organ transplantation, chronic hemodialysis, drug injection, occupational exposure among healthcare workers, unprotected sex, vertical transmission, the potential for exposure to infected patients and contaminated equipment (Shihab *et al*., 2014). HCV infection is important causes of morbidity and mortality among RD patients and pose problems in the management of patients in the renal dialysis units. The prevalence of viral hepatitis C infections in patients on RD is far higher than the prevalence of these diseases in the general population (Nariman *et al*., 2008).

Based on genetic differences between HCV isolates, the HCV species is classified into six genotypes (1–6) with several subtypes within each genotype (represented by lower-cased letters). Subtypes are further broken down

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into quasispecies based on their genetic diversity. Genotypes differ by 30–35% of the nucleotide sites over the complete genome. The difference in genomic composition of subtypes of a genotype is usually 20–25%. Subtypes 1a and 1b are found worldwide and cause 60% of all cases (Rose *et al*., 2007; Ohno *et al*., 2007; Aygen *et al*., 2017).

The HCV causes both acute and chronic hepatitis, ranging in severity from a mild illness lasting a few weeks to a serious, lifelong illness, and it is the major cause of liver cancer. HCV is a blood borne virus: the most common modes of infection are through exposure to small quantities of blood. So among the risky groups are hemophilia, thalassemia, renal dialysis plus other blood recipients (Duc *et al*., 2019). Globally, an estimated 71 million people have chronic hepatitis C virus infection. A significant number of those who are chronically infected will develop cirrhosis or liver cancer. WHO estimated that in 2016, approximately 399 000 people died from hepatitis C, mostly from cirrhosis and HCC. Unlike hepatitis A and B, there is currently no vaccine to prevent HCV infection (Yu and Chiang 2010; Jawetz *et al*., 2016). For the best of our knowledge no previous study was exploring this line in Diyala province and probably even in Iraq.

Materials and Methods

Study groups

The present study was conducted in Diyala province for the period from 1/ 12 / 2019 to 30 / 1/ 2021. This study included renal dialysis group consist of 90 patients. The patients were attended Ibn-Sina Dialysis Center-Diyala Directorate of Health. Sixty of patients were males and 30 were females. The age range was <10 years to ≥60 years. Special questionnaire was pre-constructed to collect information regarding age, sex, residence, education level and vaccinated with HBV vaccine. The information was collected through short personal interview with patients.

Blood samples collection

Five milliliters of venous blood was collected from each participant using 5 milliliters sterile disposable plastic syringes after cleaning the area of aspiration by 70 % ethyl alcohol. The blood samples were poured in 10 milliliters disposable plastic tubes soon after aspiration. The tubes were left in a rack in upright position for 30 minutes at room temperature for clotting. Thereafter, the tubes were transferred to the laboratory by cool box. At the laboratory, the tubes containing blood samples were centrifuged at 3000-4000 rotation/minute (RPM) for 5 minutes using bench centrifuge for separation of sera. Serum samples were aspirated and poured in new plastic disposable tubes using 250 microliters automatic pipette and disposable tips. Serum samples were divided into aliquots 250 microliter each in Eepindorff plastic tubes. These tubes were arranged in a rack in upright position and kept at -20 C until use.

Detection of serological marker Ani-HCV Ab (serum)

This test was performed using commercially available kit (Dia.PRO, Italy HBs Ag ELISA). Reactive results were indicated by the absorbance reading of 1.1 and above, while the non-reactive results were indicated by the absorbance reading less than 0.9.

Nucleic Acid Extraction

Genomic RNA was isolated from serum samples according to the protocol of QIAamp® MinElute® Virus Spin Kit.

Complementary DNA synthesis

The GoScript™ Reverse Transcription System was a convenient kit that includes a reverse transcriptase and an optimized set of reagents for efficient synthesis of first-strand cDNA optimized in preparation for PCR amplification. RT step included two runs, the first run started with annealing step (70ºC for 5 minute) followed by hold step (4ºC for 10 minute) in 1 cycle. While, the second run started with annealing step (25ºC for 5 minute) followed by extension step (42ºC for 60 minute), enzyme inactivation (70ºC for 15 minute) and finally hold step (4ºC for 10 minute) in 1 cycle.

Primer

Sets of PCR primers for 5¯-UTR region Hepatitis C virus gene has been used in the conventional PCR amplification in order to get PCR products used in the sequencing method for genotyping of the virus and phylogenetic tree analysis (Maryam *et al*., 2018). These primers were used for positive samples detected by ELISA test for detected (Anti-HCV Ab). Primers were provided by (Macrogen/ Korea) and sequences, table (1).

Table (1): Primers used for detection of HCV- 5¯-UTR gene

Type of virus	Target gene	Primer	Oligo sequence (5'-3')	Annealing Temperature (oC)	Product size (bp)	Reference
HCV	*5¯-UTR*	Forward	5`-CACTCCCCTGTGAGGAACTACTGTC-3`	58	306	Maryam

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		(outer)				et al., 2018
HCV	5¯-UTR	Reverse (outer)	5`-ATGGTGCACGGTCTACGAGACCTCC-3`			
HCV	5¯-UTR	Forward (inner)	5`-TTCACGCAGAAAGCGTCTAGCCATG-3`			Maryam
HCV	5¯-UTR	Reverse (inner)	5`-GCGCACTCGCAAGCACCCTATCAGG-3`	58	254	et al., 2018

Assay Optimization

After optimizing of primer, PCR detection HCV was performed. To determine the optimum annealing temperature, gradient PCR was set at 58°C.

Synthesis of cDNA amplification and genotyping

Molecular detection of HCV cDNA was set as follows: Nested PCR step using of RT-PCR step products and inner primer sets of HCV was performed as follows: initial activation at 95°C/5 minutes, 40 cycles of 94°C/30 seconds, 58°C/30 seconds, 72°C/30 seconds, and a final extension step of 72°C/10 minutes. All reactions were performed at least twice and in the negative and positive controls. All reactions were performed in duplicate and in the presence of negative and positive controls. The final products were detected by electrophoresis on 2% agarose gel and the size of the PCR products were estimated by the migration pattern of a 1500-bp DNA ladder.

Standard Sequencing

PCR products were sent for Sanger sequencing using ABI3730XL, automated DNA sequencer, by Macrogen Corporation – Korea. The results were received by email then analyzed using geneious software.

Statistical analysis

Analysis of data was carried out using the available statistical package of SPSS-25 (Statistical Packages for Social Sciences- version 25).

Results

Renal dialysis group

Ninety patients under renal dialysis were enrolled. The majority of them were male with 60 years and older. All (100%) of them were vaccinated with HB vaccine, as shown in table (2).

Table (2): Variables of the renal dialysis group.

Variables	**No.**	**%**
Age (Ys)		
<10 years	-	-
10---19	5	5.6
20---29	8	8.9
30---39	9	10.0
40---49	13	14.4
50---59	18	20.0
=>60 years	37	41.1
Gender		
Male	60	66.7
Female	30	33.3
Residence		
Urban	13	14.4
Rural	77	85.6
Level of education		
Illiterate	20	22.2
Primary	33	36.7
Intermediate	31	34.4
Secondary	6	6.7
Vaccination with HBV		
Yes	90	100
No	-	-

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Distribution of serological marker according to study group

The anti-HCV Ab positivity rate among the renal dialysis group were 32.2% with a statistically high difference (P= 0.0001), table (3).

Table (3): Distribution of study group according to serological marker.

Education	Renal Dialysis		P value
	No.	%	
Anti-HCV Ab			
Positive	29	32.2	0.0001*
Negative	61	67.8	

*Significant difference between proportions using Pearson Chi-square test at 0.05 levels.

Molecular detection and sequencing of HCV

Detection and amplifying of HCV (5⁻ UTR) gene by conventional PCR

The detection rate of HCV-5⁻ UTR gene at 254 bp among patients with renal dialysis was 16 (17.8%). So, the detection rate was significantly higher in patients with renal dialysis (P= 0.0001), table (4).

Table (4): Distribution of detected gene (5⁻ UTR) according to study group.

Education	Renal Dialysis		P value
	No.	%	
HCV-5⁻ UTR gene at 254bp			
Detected	16	17.8	0.0001*
Not-detected	29	32.2	
Not done	45	50.0	

*Significant difference between proportions using Pearson Chi-square test at 0.05 level.

The PCR products after amplification using specific primers for (5⁻-UTR) (5-Untranslated Region) gene have been presented to the gel electrophoresis, which showed the bands of 5⁻-UTR gene, as shown in the figures (1.)

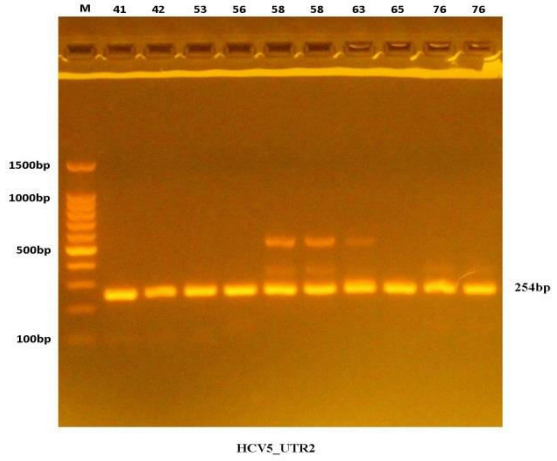

Figure 1: Results of the amplification of HCV5_UTR gene of Serum samples were fractionated on 2% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lanes 41-76 resemble 254bp PCR products.

Sequencing and Genetic diversity of HCV 5⁻-UTR Gene

With regard to the detection of the HCV 5⁻-UTR gene (16) of 5⁻-UTR gene samples showed good sequences. The 5⁻-UTR gene sequences were submitted to NCBI which gave the published accession number on NCBI, it can be found on the website;

https://www.ncbi.nlm.nih.gov/nuccore/LC546841.1/

(Hepatitis C virus subtype 1a ADS8311 5'UTR, LC546833.1; **Hepatitis C virus subtype 1a ADS8317 5'UTR,** LC546834.1 ; **Hepatitis C virus subtype 1a ADS8325 5'UTR,** LC546835.1; **Hepatitis C virus subtype 1a ADS8334 5'UTR,** LC546836.1; **Hepatitis C virus subtype 1a ADS8334 5'UTR,** LC546839.1and **Hepatitis C virus subtype 1a ADS8342 5'UTR,** LC546837.1)

Blast and Alignment of Partial HBV S Nucleotide and Amino Acid Sequences

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In order to determine the genotypes of HCV and the patterns of the observed variations, which including the positions of similarity and differences within this sequences. With regard to the investigated 16 samples of HCV, the sequencing reactions indicated the exact positions after performing NCBI blastn for these PCR amplicons. NCBI BLASTn engine has shown about 99% sequences of similarities between the sequenced samples and this target. NCBI BLASTn engine has indicated the presence of remarkable homology with the expected target that covered a portion of the 5′-untranslated region (5′-UTR) within the Hepatitis C virus (HCV). Based on its high conservative sequences, this targeted fragment was used for genotyping and sub-genotyping of HCV, and the targeted 5′-UTR 254 bp was selected for this purpose. By comparing the observed DNA sequences of these 16 local samples with the retrieved DNA sequences (GenBank acc. MG436881.1), the exact positions and other details of the retrieved PCR fragment were identified (Fig.2).

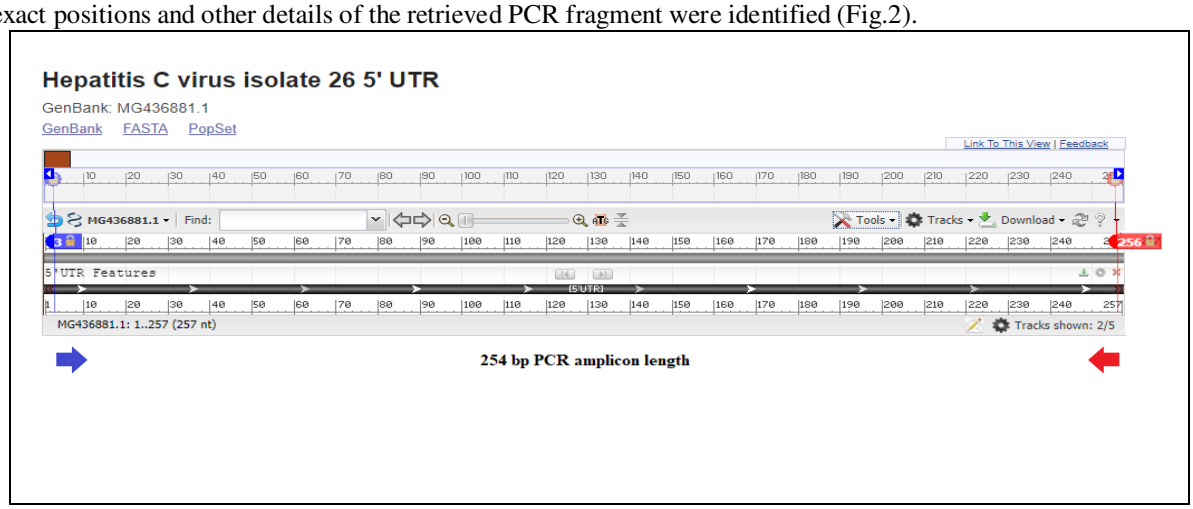

Fig. 2. The exact position of the retrieved 254 bp amplicon that covered a portion of 5′-untranslated region (5′-UTR) locus within the Hepatitis C virus (HCV) genomic sequences (acc. no. MG436881.1). The blue arrow refers to the starting point of this amplicon while the red arrow refers to its endpoint.

After positioning the 254 bp amplicons' sequences within the 5′-UTR of HCV sequences, the details of these sequences were highlighted, starting from the position of the forward primer to the position of the reverse primer within the HCV sequences (Table 5).

Table 5: The position and length of the 254 bp PCR amplicons used to amplify a portion of the 5′-untranslated region (5′-UTR) within the Hepatitis C virus (HCV) genomic sequences. The amplified sequences were extended from 3 to 256 of the NCBI reference DNA sequence (GenBank acc. no. MG436881.1). the grey color refers to both forward and reverse primers sequences.

Amplicon	Reference locus sequences (5′ - 3′)	length
HCV 5′-UTR DNA sequences	TTCACGCAGAAAGCGTCTAGCCATGGCGTTAGTATGAGTGTCGTGCA GCCTCCAGGACCCCCCCTCCCGGGAGAGCCATAGTGGTCTGCGGAA CCGGTGAGTACACCGGAATTGCCAGGACGACCGGGTCCTTTCTTGG ATAAACCCGCTCAATGCCTGGAGATTTGGGCGTGCCCCCGCAAGAC TGCTAGCCGAGTAGTGTTGGGTCGCGAAAGGCCTTGTGGTACTGCCT GATAGGGTGCTTGCGAGTGCGC*	254 bp

*[1] **Notice;** the reverse primer was placed in a reverse complement mode.

The alignment results of the 254 bp samples revealed the detection of only two mutations in comparison with the referring sequences of the GenBank acc. MG436881. These two mutations were represented by two nucleic acid substitutions detected in the majority of the investigated samples.

The sequencing chromatogram of DNA sequences as well as its detailed annotations were documented and the pattern of these variants within the amplified sequences was shown (Fig.3). At the 141th position of the PCR amplicon, a nucleic acid substitution of thymine (T) to cytosine (C) was detected in almost all investigated samples, T141C. At the 162nd position of the same targeted amplicons, another two nucleic acid substitution was observed, which was generated by the substitution of Guanine (G) with cytosine (C) in all investigated samples, G162C.

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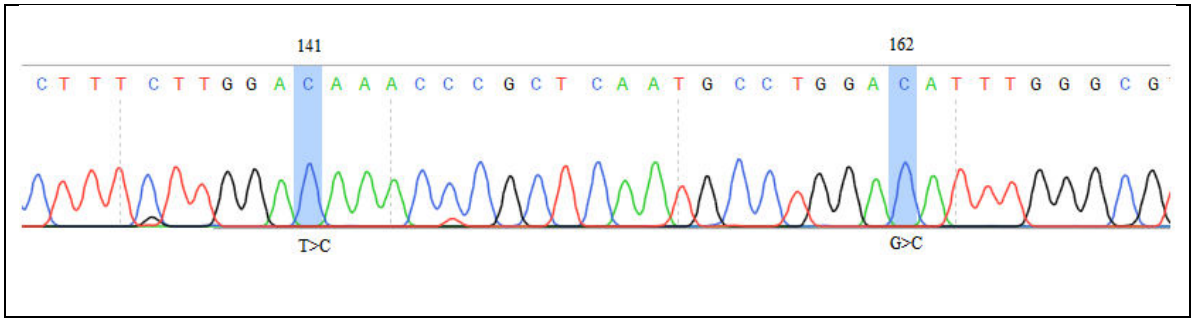

Fig. 3. The chromatogram profile of the observed genetic variants of the 5′-untranslated region (5′-UTR) within the Hepatitis C virus (HCV) local isolates. Each substitution mutation is highlighted according to its position in the PCR amplicon. The symbol ">" refers to "substitution" mutation.

To summarize all the results obtained from the sequenced 254 bp fragments, the exact positions of the observed mutations were described in (Table 6).

Table 6. The pattern of the observed mutation in the 254 bp of the 5′-untranslated region (5′-UTR) amplicons in comparison with the NCBI referring sequences (GenBank acc. no. MG436881.1). The symbol "#HCV UTR" refers to the "sample" code.

Sample No.	Native	Allel	Position in the PCR fragment	Position in the reference genome	Locus	Variant summary	Novelty
All samples except 21 HCV UTR1	T	C	141	143	5′-UTR	MG436881.1;g.143T>C	novel
All samples	G	C	162	164	5′-UTR	MG436881.1;g.164G>C	novel

Analysis of Phylogenetic Tree

A comprehensive phylogenetic tree was generated in the present study, which was based on the observed nucleic acid variations detected in the investigated HCV UTR samples. This phylogenetic tree was contained all these 16 samples, alongside with other relative viral reference HCV sequences. A total number of the aligned nucleic acid sequences in this comprehensive tree were 113. This comprehensive tree indicated the presence of only one type of organism, HCV, which represents the only incorporated organism within the tree. Thus, the only variation seen in the currently constructed tree was only concerned with the genotyping or sub-genotyping of HCV sequences. Based on the 5′-UTR sequences, the investigated 16 samples of HCV were clustered into two adjacent clades (Fig. 4). However, there was no deviation from HCV as both clades were only minor variations within the sequences of this viral sequence.

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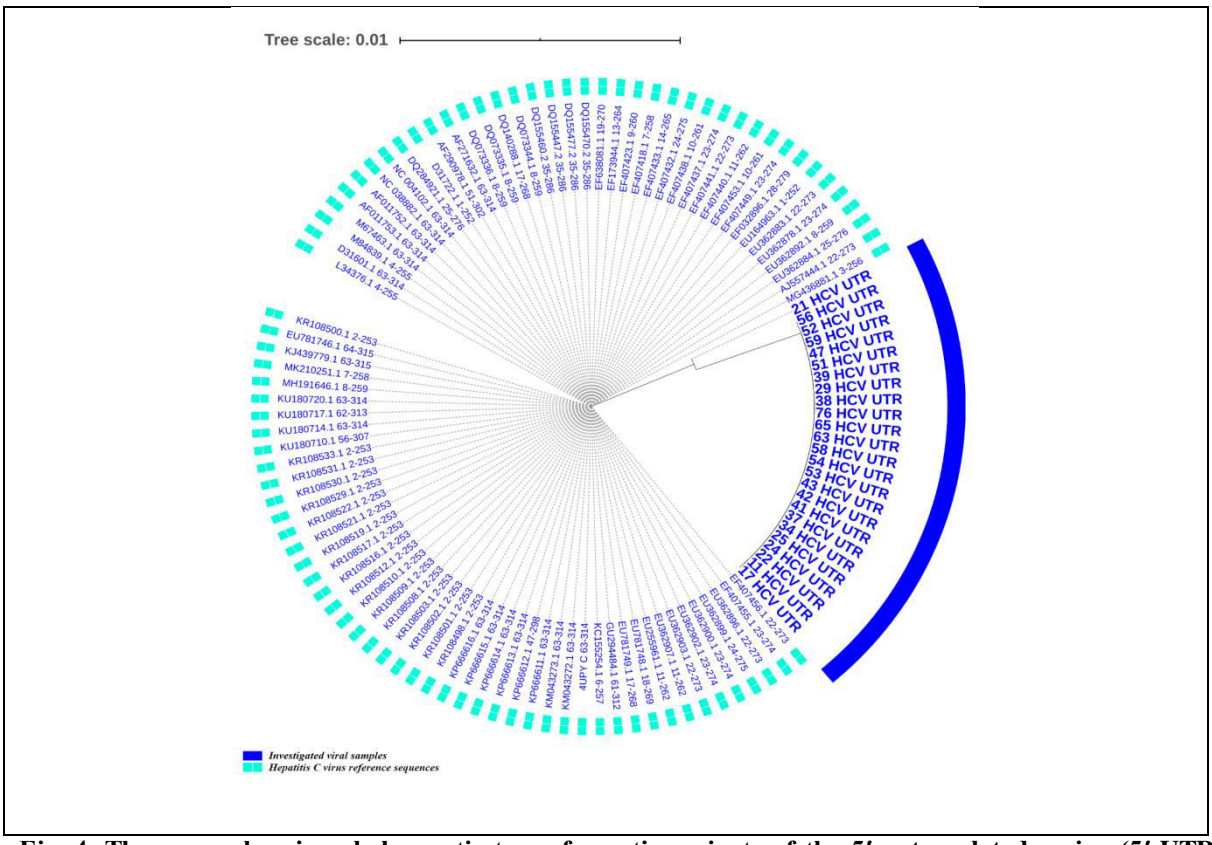

Fig. 4. The comprehensive phylogenetic tree of genetic variants of the 5′-untranslated region (5′-UTR) within the Hepatitis C virus (HCV) local isolates. The dark blue color refers to the analyzed variants, while light blue color refers to the related referring Genbank acc. no NCBI deposited sequences of the HCV. The number "0.01" at the top portion of the tree refers to the degree of scale range among the comprehensive tree categorized organisms. The symbols #HCV UTR refers to the code of the investigated samples.

One of these clades was made from only one sample, 21 HCV 5-UTR. The reason behind this clade was attributed to the presence of only one nucleic acid substitution (G162C) within this viral sample. Whereas the other close clade was made of the other 15 samples, which was characterized by the presence of two nucleic acid substitutions (T141C and G162C). However, both clades were suited beside each other. It was found that these two clades were clustered beside the GenBank acc. no. MG436881.1, and AJ557444.1, which were belonged to Iranian and English isolates of HCV sequences, respectively. Unfortunately, the genotype of deposited Iranian HCV sequences of MG436881.1 was not shown and the results of this accession number were not published yet. Meanwhile, valuable data was observed from the deposited English HCV isolate AJ557444.1. These data entailed that the genotype of HCV was 1a. Furthermore, other related GenBank acc. no., such as the American highly related HCV isolates EU362884.1 and EF407456.1, for our investigated samples that suited beside our investigated samples were also indicated the same observed 1a genotype. For this reason, it was clearly shown that all our investigated HCV samples belonged to genotype 1a. This observation indicated a distinct role of the generated phylogenetic tree in the accurate detection and genotyping of HCV samples.

Discussion

The implementation of blood borne disease transmission control protocols in hemodialysis units have been shown to decrease the prevalence of HCV in patients with renal dialysis (Mohammed, 2014).The present study, high light prevalence of anti-HCV Ab infections in patients on renal dialysis and determinant the prevalence genotyping of HCV in renal dialysis, there is no study done in Diyala about this subject previously.

The prevalence of anti-HCV Ab among renal dialysis in our study was 29(32.2%). This result was higher than in others results reported in Iraq such as in Nineveh governorate (25.8%) by Mohammed, (2014), in Baghdad (13.4%) by Nariman, et al. (2018).While, the present result was lower than the Baghdad by Nadia and Marwa, (2019) which was (46.36%).

Previous studies conducted in Saudia Arabia and Ahfaz- Iran reported that the prevalence of HCV among renal dialysis according to ani-HCV Ab were 49.9% and 25.3% (Maryam, *et al*., 2018; Ghanim, 2019).

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This difference in results may be attributed to contaminated equipment, surfaces, and HCV infected patients in addition to contaminated healthcare worker hands that are considered among the most common modes of transmission of nosocomial infections. This difference may also be attributed to inadequate hematological investigations such as PCR for HCV mainly on blood and blood products as the mean number of blood transfusions in HCV infected group is significantly higher than the non-HCV group.

The genetic fragment 5′ untranslated region (5′UTR) was target for the detection of HCV as it was characterized by highly conserved sequences for this organism (Kabakçı *et al*., 2014). The HCV 5′ untranslated region (5′UTR), responsible for the initiation of viral translation via an internal ribosome entry site (IRES), has been previously described to contain specific nucleotide substitutions when cultured in infected lymphoid cells. Sequence variability in this region has important implications for structural organization and the function of the IRES element and could correlate with HCV RNA concentration (Barría *et al*., 2009).

The 5'-UTR was selected because of its importance as an essential component of the Internal Ribosome Entry Site (IRES) that regulates Cap-independent translation of HCV as we have showed above.

In current study, conventional PCR test was conducted for detected of 5'-UTR on anti-HCV Ab positive, it was 16(17.8%). The present result was lower than other results were reported by other researchers in Iraq, 13(21%) was reported in study conducted in Nineveh and 79(31%) was reported in study conducted in Baghdad (Mohammed, 2014; Nawfel *et al*., 2019).

Nucleotide substitution within the HCV 5`-UTR may influence the viral translation and its sensitivity to the antiviral action of interferon. As well, reduced molecular stability of RNA, which may ultimately reduce binding affinity to ribosomal proteins (Mostafa *et al*., 2009; Hemeida *et al*., 2011; Radwa et al., 2018; Michael and Gesche, 2020).

The fact of the identity of all investigated samples was determined as they belong to the same viral identity, whether in terms of the viral HCV organism or genotype 1a. However, this notion provided a further indication of the viral identity and genotyping of these local studied samples. HCV genotyping distribution has an important influence clinically in the morbidity, total costs and duration of HCV treatment (Cuypers *et al*., 2015). Therefore, for better understanding of the HCV epidemiology as well as the prevalence of its genotypes pattern, performing HCV genotyping studies in our country is very important. In study was conducted in Erbil Province: Kurdistan/Iraq**,** this study gives different estimation of HCV genotypes distribution among infected HCV patients in Kurdistan from prevalent distribution in Iraq and Middle East Arab countries, but comparable to global distribution. Was reported (37%) genotype 1 was the most frequent genotype detected followed by 3 (27.3%), 4 (20%) and 2 (2.4%), while mixed genotypes were detected in 13.3% (Dlshad *et al*., 2018). While, in study was conducted in Baghdad by Waqar *et al*, (2018) reported another results, Genotype HCV-1b showed higher prevalent (52.9%) among the recipients of anti-D Ig therapy while genotype HCV-3a (6.6%) was the lowest.

In Istanbul, Turkey, study was aimed to determine the distribution of HCV genotypes in patients with HCV showed Genotype 1a (82.5%) was dominant genotype (Nuran *et al*., 2018).

This 5′-UTR -based comprehensive tree has provided an extremely inclusive tool about the high ability of such 5′-UTR sequences to efficient identification HCV samples using this genetic fragment. This, in turn, gives a further indication of the power of the currently utilized 5′-UTR specific primers to discriminate among the currently investigated isolates.

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