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PHYLOGENETIC STUDY OF HEPATITIS C VIRUS IN HEPATITIS PATIENTS IN WASIT PROVINCE, IRAQ

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Abstract

***Background and aims*:** Viral hepatitis represents a health problem that affects millions of people worldwide and is associated with high mortality, except for hepatitis A virus (HAV), all hepatotropic viruses, including hepatitis B, C, D, and E viruses (HBV, HCV, HDV and HEV), can produce chronic infections, whereas HAV causes acute self-limiting hepatitis that normally resolves spontaneously Hepatitis C virus (HCV) is one of the major globally cause of death and morbidity**,** and recent estimates showed an increase in its seroprevalence over the last decade to 2.8%. The whole extent of RNA genome is about 9.6 kb with one open reading frame (ORF) and 5′ and 3′ untranslated regions (UTRs) at both edges, 5′UTR is a more preserved portion of HCV genome, which aided in evolutionary studies and genotyping, the open reading frame encodes a polyprotein, which is comprised of 10 viral proteins named as Core (C), E1, E2, P7, NS2, NS3, NS4A, NS4B, NS5A and NS5B. ***Materials and methods*:** This study was conducted on 85 samples from HCV whom were confirmed to be infected with as they were diagnosed by a through ELISA screen test, RT-PCR and conventional PCR for selected genes. Their ages ranged from (5 - 75) years old during July and September 2022. ***Results*:** The work has been carried out on anti-HCV 85 (100%); sero-positive patients of the two sexes gathered comprising according to presumptive cause of infection 22.4% unknown patients; 35.3% hemodialysis patients; 35.2%; thalassemic patients; and finally, 7% other patients. Real-Time PCR was used to confirmed the serological diagnosis and for measurement of the viral loads (concentrations) in the 85 (100%) of seropositive HCV Ab HCV patients only 54(100%). The results revealed that all thalassemic patients was positive by ELISA technique, while 35.2% were gave positive results; 33.3% hemodialysis patients; 27.7% unknown patients; and finally, 3.8% other patients with HBV and HCV gave positive results. This study showed that 54 samples which were tested by Real time PCR for HCV viral load, then extraction HCV – RNA and amplification of Nonstructural protein 5A (NS5A) gene by using specific primers. Eight samples were positive amplification of NS5A gene, while the remaining was negative. A phylogenetic tree of HCV-NS5A gene revealed samples are related to genotype (4a). ***Conclution*:** The results of this study concluded that dialysis patients have a high degree of risk factors for infection with the virus, through frequent blood transfusion as well as the dialysis machine or through the nursing staff. Also, thalassemia patients were observed to have a

high infection rate.

Keywords: HCV, NS5A, Hemodialysis, Thalassemia

Introduction

Inflammation of the liver parenchyma in response to viral infections is called viral hepatitis, The hepatotropic viruses including hepatitis A (HAV), hepatitis B (HBV), hepatitis C (HCV), hepatitis D (HDV), and hepatitis E (HEV) make up the majority of such infections, Globally, millions of people are affected by these viruses annually(1).Viral hepatitis represents a health problem that affects millions of people worldwide and is associated with high mortality, except for hepatitis A virus (HAV), all hepatotropic viruses, including hepatitis B, C, D, and E viruses (HBV, HCV, HDV, and HEV), can produce chronic infections, whereas HAV causes acute self-limiting hepatitis that normally resolves spontaneously(2).Clinical presentation varies from asymptomatic or acute flu-like illness to acute liver failure or chronic liver disease, characterized by jaundice, hepatomegaly and ascites among many other signs, Eventually, this can lead to fibrosis (cirrhosis) of the liver parenchyma and carries a risk of development into hepatocellular carcinoma(3).Hepatitis C virus (HCV) existence was first fully recognized in 1975 when Feinstone*et al*. found that most cases of transfusion-associated hepatitis were not associated with hepatitis A virus or hepatitis B virus (HBV) infections, and thus defined the disease non-A, non-B hepatitis (4).Hepatitis C virus (HCV) is one of the major globally cause of death and morbidity (5, 6). And recent estimates showed an increase in its seroprevalence over the last decade to 2.8%, corresponding to > 185 million infections worldwide (6, 7). Hepatitis C virus is enveloped, small circular, positive-sense and single stranded ribonucleic acid (RNA) virus from genus Hepacivirus, family Flaviviridae with a diameter of 50 nm (8). HCV particle consists of a nucleocapsid-containing the single-stranded RNA genome associated with the viral core protein and a lipid bilayer where the viral envelope proteins (E1 and E2) are assembled as heterodimers (9). In reality, the structure of HCV is more complex and the virus exhibits unusual and striking features (10). Indeed, a hallmark of HCV particles is their association with host cell lipids and lipoproteins, mainly very low-density lipoproteins (VLDL) and low-density lipoproteins (LDL) (11). The whole extent of RNA genome is about 9.6 kb with one open reading frame (ORF) and 5′ and 3′ untranslated regions (UTRs) at both edges (12). 5′UTR is a more preserved portion of HCV genome, which aided in evolutionary studies and genotyping,the open reading frame encodes a polyprotein, which is comprised of 10 viral proteins named as Core (C), E1, E2, P7, NS2, NS3, NS4A, NS4B, NS5A and NS5B(13).The NS5A protein is a membrane-associated phosphoprotein that appears to have multiple functions in viral replication. It is phosphorylated by different cellular protein kinases indicating an essential but still not understood role of NS5A in the HCV replication cycle. In addition, NS5A has been found to be associated with several other cellular proteins (14, 15).Hepatitis C virus (HCV) infection is one of the most common causes of hepatocellular cancer (HCC) is a serious consequence caused by HCV infection, with high death and morbidity rates , HCV-induced HCC develops over time and is influenced by the duration of the infection as well as the viral genotype (16).

Materials and Methods

Specimen collection

This study was conducted on 85 samples from HCV whom were confirmed to be infected with as they were diagnosed by a through ELISA screen test, RT-PCR and convential PCR for selected genes. Their ages ranged from (5 - 75) years old. In the period between july and september 2022, hepatitis patients were collected from haemodialysis Center in Al-Zahra Teaching hospital, Al karama teaching hospital, Thalassemia center in AL-Kutwomenchildren Hospital of Wasit Province Health Directorate.Specimens Collection Five milliliters of venous blood were drawn from each patients groups by medical syringe. The first portion (2.5 mL) was placed in gel tubes and left at room temperature for approximately thirty minutes to coagulate, then centrifuged at 5000 rpm for 10 minutes to separate serum, which was used to measurement HCV Ab by ELISA, The second part (2.5 mL) was placed in EDTA tube then centrifuged at 5000 rpm for 20 minutes to separated the plasma which was used to determine viral load and extraction of Nucleic acid of virus.

Serological Test

Enzyme- linked immune sorbent assay test was used for the detection of HCV Ab in human serum in clinical laboratories and as a first - line screening assay in blood.Serum samples were added according to the designation on the ELISA working sheet (Hightop Biotech /china) .

Molecular Test

The RNA was extracted from 300 µl of plasma in a 50 µl elutionvolume the Quick-RNATM Viral Kit – Zymo (USA) research (catalog No. R1034), Then, the purity and concentrationof RNA were measured by NanoDrop.The NS5A was amplified by semi-nested PCR (snPCR) using primer (Table 1).

Table (1): Primers for NS5A gene region of HCV virus (17)

Species	Gene	Primer	5'-3'	PCR product
HCV	NS5A	F	GGIGARGGIGCIGTICARTGGATGAA	767bp
		R	TRTGRGAIGGRTCIGTIARCATIGA	
		R	TRTGRGAIGGRTCICTIARCATIGA	

According to instruction of the primer synthesizer company, the primers (originally lyophilized), were dissolved in the free ddH2O to obtain a final concentration of 100 µM/µl which served as a stock solution that stored at -20°C. A concentration 10 µM/µl was prepared from the stock primers to be used as a work primer.

In order to convert the RNA to cDNA, PrimeScriptTM RTreagent kit (Takara, Cat. # RR037A) was used. PCR have performed in a 25 µl and this volume composed of 3 µlcDNA 10 µl master mixPCR (Intron, Korea), 1 µl of each forward and reverse primer andthen the volume completed to 25 by adding nuclease-free water. The programming conditions were as follow: 4 min of 95°C; which followed by 45 cycles of 15 s of 95°C, 25 sec of 48°C, 72°C for1 min. 2% of agarose then

were used to visualize the amplified region of DNA. The NS5A gene was sequenced by the Macrogen Company using their ABI 3730xl genomic analyzer (Applied Biosystems, US). The (NCBI) BLASTN program was used to analyzethe results (Table 2).

Table (2): Components of PCR's master mix and amplification procedures to detect NS5A genes (final volume 25 µl)

Phase	Tm (°C)	Time	Cycles
Initial Denaturation	94°C	3 min.	1 cycle
Denaturation -2	94°C	15 sec	45 cycle
Annealing	48°C	30 sec	
Extension-1	72°C	1 min	
Hold	4°C	∞	

Statistical analysis

Data were entered and analyzed using the software program Statistical Package for Social Sciences (SPSS) version 26. All numerical variables were represented by means (a measure of central tendency) and standard deviation (a measure of dispersion) while categorical variables were presented by frequencies and percentages. The Chi-Square test and Fisher´s Exact test (when more than 20% of cells have expected frequencies < 5) were used accordingly to assess the presence of an association between categorical variables. The independent samples t-test was used to assess the mean differences of numerical continuous variables. Considering a P-value is equal to or less than 0.05 a significant (**18**).

Results

Serological results

The work has been carried out on anti-HCV 85 (100%); sero-positive patients of the two sexes gathered comprising according to Presumptive cause of infection as: 19 (22.4%) unknown patients; 30 (35.3%) hemodialysis patients; 30 (35.2%); thalassemic patients; and finally 6 (7%) other patients (Table 3).

Table (3): Descriptive data of patients who diagnosed as hepatitis C by ELISA

ELISA test		
Item	**Frequency**	**Percent**
HCV Ab	85	100
Total	85	100

P value =***0.002; *Significant at level of P < 0.05

Molecular diagnosis for HCV

Viral load results for HCV

The molecular quantitative technique with Real-Time PCR was used to confirmed the serological diagnosis and for measurement of the viral loads (concentrations) in the 85 (100%) of seropositive

HCV AbHCV patients only 54 (100%)The results revealed that all thalassemic patients was positive by ELISA technique, while 19 (35.2%) were gave positive results; 18 (33.3%) hemodialysis patients; 15 (27.7%) unknown patients ; and finally 2 (3.8%) other patients with HBV and HCV gave positive results (Table 4).

Table (4): Descriptive data of patients who diagnosed as hepatitis B or C by real time PCR

Item	diagnosis	N	Mean	SD	P value
Viralload copies /ml	HCV	54	739756.5	1.3	0.001
Viral load Iu /ml	HCV	54	5928726.9	2.6	0.001

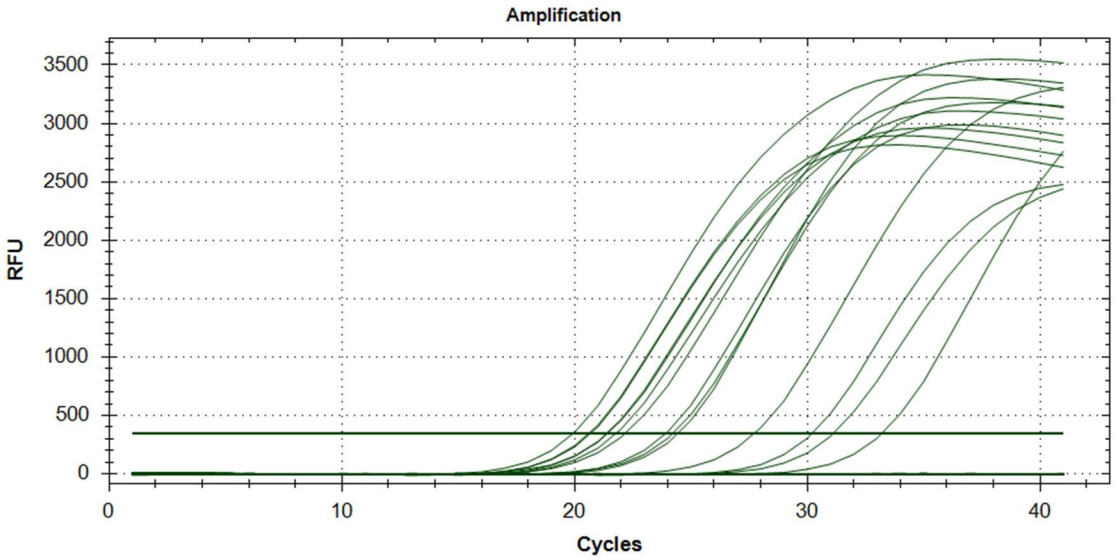

Figure (1): Amplification plot for RT-qPCR viral load (HCV)

Conventional PCR detection for HCV- RNA

This study showed that 54 samples which were tested by Real time PCR Technique for HCV viral load, Then extraction HCV – RNA and amplification of Nonstructural protein 5A (NS5A) gene by using specific primers. Eight samples were positive amplification of (NS5A) gene, while the remaining was negative (Table 5).

Table (5): Distribution of HCV RNA conventional PCR technique amongpatients

Gender	No. of positive samples by Real time PCR	No. of positive samples by conventional PCR
Males	29(53.7%)	5(62.5%)
Females	25(46.3%)	3(37.5%)
Total	54(100%)	8(100%)

The amplification of the NS5A gene has been done successfully as the electrophoresis result showed sharp band at 725bp (Figure 2).

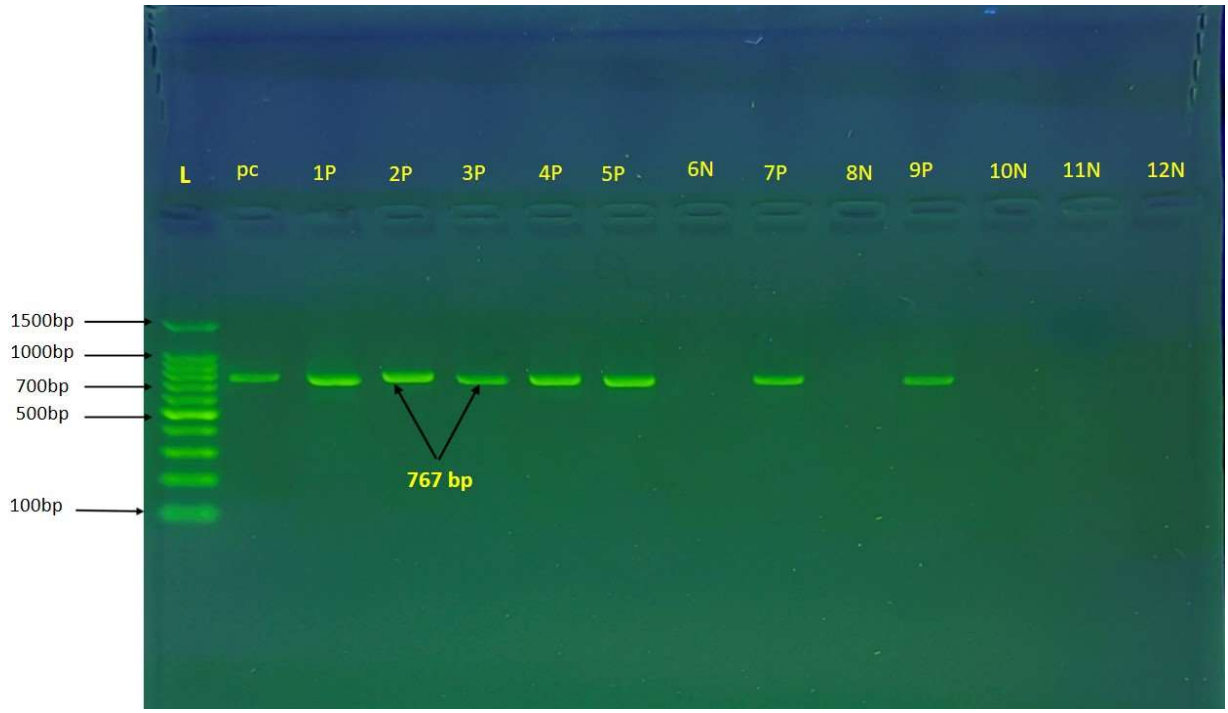

Figure (2): PCR product the band size 767 bp. The product was electrophoresis on 2% agarose at 5 volt/cm2. 1x TBE buffer for 1 hour; L: DNA ladder (1500-100); (PC) positive control, (P) positive sample, (N) negative sample

Phylogenetic tree

A phylogenetic tree generated by (MEGA) software version 6.0 using the Nonstructural Protein 5A (NS5A) gene. To showed identical between Iraq and the isolates of the world. Hierarchical cluster analysis determine the following clusters: large cluster divided into several neck: first root including Hepatitis C virus subtype 4a isolate Cyprus (ID:HQ537008.1), which has a 99% similarity to Hepatitis C virus subtype 4a isolate Portugal (ID: ON06818.1), also has two root: first including Hepatitis C virus subtype 4a isolate (ID: DQ988079.1) the identical 100% it is close to USA (ID: JX463528.1), and two root: including Hepatitis C virus subtype 4a isolate Australia (ID: KU871289.1) and Japan (ID: LC368356.1) the identical 99%), The last cluster is divided into two branches the first branch divided Iraq1 including Hepatitis C virus the identical 99%, the second branch divided into branch the first Iraq2 including Hepatitis C virus the identical 99%, the second branch Iraq2, Iraq (ID:OQ446441) including Hepatitis C virus the identical 99%. Following correspondence from the NCBI, the NS5A gene was registered, given an accession number, and became a reference for Iraq, the Middle East, and the rest of the world. After the validation steps have been completed the sequences got reference ID. A phylogenetic tree of HCV-NS5A gene revealed samples are related to genotype (4a) in figure (3).

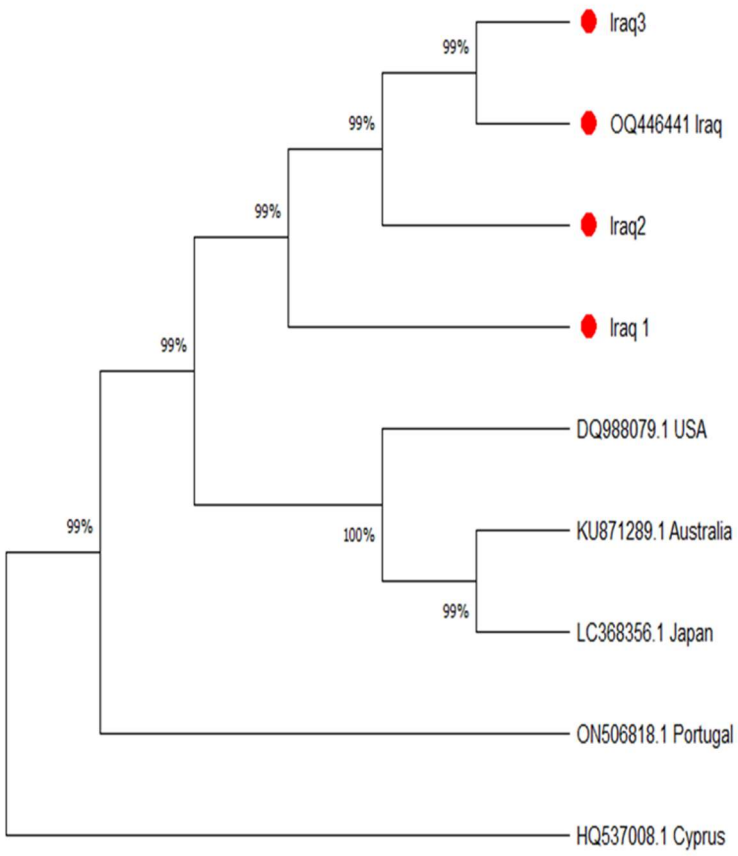

Figure (3): Neighbor-joining tree Hepatitis C virus of NS5A gene

Table (6): Homology sequence Identity (%) of local Hepatitis C isolates and NCBI Blast Hepatitis B isolates using NCBI- BLAST alignment tool

No	Access No. ID	Country	Source	Compatibility
1	-	Iraq 1	Hepatitis C virus	99%
2	-	Iraq 2	Hepatitis C virus	99%
3	-	Iraq 3	Hepatitis C virus	99%
4	**OQ446441**	Iraq	Hepatitis C virus	99%
5	**DQ988079**	USA	Hepatitis C virus	100%
6	**KU871289**	Australia	Hepatitis C virus	99%
7	**LC368356**	Japan	Hepatitis C virus	99%
8	**ON506818**	Portugal	Hepatitis C virus	99%
9	**HQ537008**	Cyprus	Hepatitis C virus	99%

Discussion

Infection with HCV is a common public health issue especially in developing countries such as Iraq. Such an infection is associated with deleterious consequences predisposing to liver cirrhosis and hepatocellular carcinom. The results of this study agreementwith previous studies done by Salman *et al.* The anti-HCV Ab positivity rate among the renal dialysis group were 32.2% with a statistically high difference (P= 0.0001) (**19**). The study done by Hussein *et al.* a total of 255 hemodialysis patients with positive HCV-Ab results were referred for further evaluation. HCV-Ab positivity was confirmed again by ELISA (**20**). The results of this study disagreement , Jalil *et al.* (2022) showed that 23% of patients were anti-HCV positive and 77% were anti-HCV negative using ELISA technique (**21**). Infection rates varied between countries and came at different rates, due to the most important reasons, the most important of which are frequent blood transfusions, injecting drug use, preventive measures used in dialysis units, surgeries, especially organ transplants, and hand tools for workers in dialysis units, as previous studies recorded. The most common method of transmission of infection was from the patient's nursing staff, and they agreed that the most important way to avoid this infection is washing hands, so it is necessary to take preventive measures to reduce the spread of infection among dialysis patients, and the reason may be due to the different examination method used (22). Hepatitis C is the most common chronic blood borne infection **(23)**. Reverse transcription real time polymerase chain reaction (RT-qPCR) was performed for direct and rapid detection of hepatitis C virus infection using one step technique **(24)**. Similar transmission models, HCV and HBV co-infection is prevalent, in this study, HBV were detect in hemodialysis patients with chronic HCV patient, viral interference has been described in patients with dual HBV and HCV infection **(25)**.The current results of this study are in agreement with previous studies done byJasim *et al.*, 2021 The extracted RNA from the 17 (Egyptian samples) and 89 (Iraqi samples) positive samples had tested by the RT-PCR, and the results showed 9 and 39 samples Egyptian and Iraqi respectively only were positive to HCV (**26)**. The current study revealed that 86% of study patients were detected with positive RT-PCR assay, HCV–positive patients were significantly older from HCV-negative ones ($p<0.001$). In addition, those results indicate that higher prevalence of anti-HCV or HCV RNA were significantly associated with longer duration of transfusion ($p<0.003$ and $p<0.001$, respectively **(27)**. Al Kubaisy *et al*. (2006) Iraqi children with thalassemia showed a higher percentage than that recorded in other countries, such as 40.7% in Jordan, 40% in Saudi Arabia, and 14% in Turkey **(28)**.

The reasons for this discrepancy between antibody-positive as well as HCV RNA negative cases presented, is that the HCV might be existing in peripheral blood mononuclear cells (PBMCs) in such cases and not in serum or plasma as has been indicated *via* (**29**) who detected HCV-RNA in PBMCs in 10.5% out of 38 plasma viremia negative, and that the spontaneous viral clearance occurred in twenty percent of individuals exposed to the virus (**30**). Therefore the presence of anti-IgG reflect resolved infection. Albeldawi *et al*. (2010) mentioned that ELISA is the most accurate serological marker for diagnosis of HCV infections but it still gave false positive and false negative results and cannot discriminate between the past and ongoing infections (**31**). The HCV infection might be the major risk factor for the liver fibrosis in transfusion-dependent thalassemia.

Furthermore, the excess liver iron is identified as one of the co factors for the development of advanced fibrosis in the patients experiencing HCV infection (**32**). The patients experiencing HCV have mild to moderate elevated hepatic iron concentrations and often have extreme hepatic iron overload **(33)**. The results of the current study showed agreement with the researchers' studies (Smith *et al.,* 2014). This study conducted of patients with HCV the result shows subtyping include Hepatitis C virus subtype 4a.For the NS5A region, all of these considerations required the creation of a reliable, easy genotyping and resistance profiling technique. Smith et al. discovered that the phylogenetic tree derived from the NS5A HCV sequences is completely aligned with that of the whole virus coding sequences **(34).** Di Stefano *et al.,* 2021In the present study, GT 4 subtypes were assessed in 17 HCV GT 4-infected patients from Saudi Arabia. The most common subtype was GT 4a, and the other identified subtypes were GT 4o and GT 4d. Interestingly, two patients appeared to be infected with recombinant virus (4a/GT 4o/GT 4a), and one was infected with an unclassifiable virus, which may potentially represent a new, previously unseen, subtype **(35)**.

The PCR we used amplifies a portion of NS5A (domain I) containing resistance-associated substitutions (RASs) associated to viral failure when employing NS5A inhibitors such daclatasvir, ledispasvir, ombitasvir, or velpatasvir. Currently, there is no clear and commonly acknowledged strategy for interpreting the presence of RASs at the start of treatment or in the case of virological failure **(36)** with the findings of all publications on HCV resistance, in vitro as well as in vivo. The list of mutations noted in prior reviews is fascinating because they were discovered in phase II and III studies, which are more typical of real-world clinical practice **(37)**. As a result, any new HCV infected patient may use our proposed protocol to evaluate both subtype and NS5A polymorphism. Recently, the NS5A region was identified as a possible option for HCV genotyping and subtyping **(38).** NS5A gene sequencing was recently used to record an HCV transmission outbreak in a Dutch haemodialysis facility **(39)**. A phylogenetic analysis of the NS3, NS5A, and NS5B genes, as well as Sanger sequencing, recently identified two episodes of HCV transmission in two healthcare settings: one case of patients with acute HCV infection who had been diagnosed with onco-hematologic disease, and the other case of patients with acute HCV infection who had been diagnosed with onco-hematologic disease **(40)**, and a second case in which -thalassemia was diagnosed in patients with acute HCV infection **(41)**. Few studies have reported the use of HCV genotype and phylogenetic analyses combined to investigate HCV infection associated with nosocomial exposure **(42)**. Meaning the mosaic structure was robustly inferred, we recognize that the exact recombination patterns cannot be revealed without full-genome sequencing followed by a detailed recombination analysis, which may represent a possible limit of the study. Likewise, the evolutionary history of the unclassifiable sequence may be revealed after full-genome sequencing and further analyses. This study further analyzed naturally occurring mutations in the NS3,NS5A, and NS5B regions associated with drug resistance **(35)**.

Conclusion

The data given in this study demonstrated that HCV prevalence in hemodialysis centers and thalassemia patientsare relatively high and also suggested that the main risk factor appears to be the amount of time receiving hemodialysis that treatment, pointing to that nosocomial transmission

of HCV and at a lower rate in some patients who acquired the infection through dental clinics or marking, as well as barbershops.

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