Detection of hepatitis C virus virulence Genes (UTR &NS5) using PCR among patients with kidney failure disease in Wasit province

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Abstract

Hepatitis C virus (HCV) is a single-stranded RNA that causes liver disease and cell death. This study was designed to analyze these genetic regions in patients with renal failure in the dialysis center in Wasit Governorate. 117 blood samples were collected from patients of both gender and of different ages. The blood samples were divided into two groups, the blood group and the serum group. RNA was extracted from blood samples. A qualitative identification of the virus was made using RT-PCR. .Where 28 positive samples out of 47 were isolated. The study showed that out of 28 samples, 3 isolates positive for the UTR gene were identified. A statistical analysis was done for all positive samples according to (gender, age, blood type, place of residence) . The UTR region is considered very safe in the installation of the virus and genetic identification is a great challenge. The UTR region enables the identification of virus genotype. As its identification in infected patients shows that the patient's blood is capable of transmitting infection in a dangerous manner, especially patients in the center of renal failure. With the presence of many equipments used by the workers in the center, like blood washing machines drawing blood and conducting tests. As for the NS5 B

region, it was not obtained from where it is isolated from patients. It is considered a highly variable and non-structural region and a golden region for genotyping analysis

Keywords : HCV, UTR , NS5

Introduction

Hepatitis C virus (HCV) is a single-stranded RNA virus with a length of 50–60 nanometers that causes liver diseases and cell death [1]. (HCV) infection is a blood-borne disease that is spread through direct contact with infectious blood (transfusions) or through indirect contact with contaminated substances (unsafe injections and other medical treatments) . Accounting for the virus's global spreading and causing a severe global health problem in the majority of countries In 70—80 percent of patients, The acute infection of hepatitis C progresses to chronic carriage, with the risk of developing liver disease, Cirrhosis, and cancer. [2].

HCV is a tiny encapsulated virus with a positive-sense single-stranded RNA genome made up of an Open Reading Frame (ORF) with about 9.6kb in length and encodes a single polyprotein that is post-translationally cleaved into three structural proteins (core protein, E1

and E2) and seven non- structural proteins (NS-2, NS-3, NS-4A, NS-4B, NS-5A, NS-5B and P7) [3]. The viral particle is composed of a genetic material (RNA) core, an icosahedral protective shell of protein, and a biological lipid (fatty) envelope. E1 and E2 viral envelope glycoproteins are found in the lipid envelope. [4].

Untranslated regions (UTR) are located at the 5′ and 3′ ends of the RNA and are essential for viral RNA translated and replicated. A ribosome binding site (RBS) or

an internal ribosome entry site (IRES) is found in the 5′ UTR, which starts the translation of a 3,000-amino-acid protein.. [5].

When HCV infects the liver and is not treated. Chronic hepatic inflammation occur and progresses to fibrosis, which can proceed to cirrhosis .At which point people are at risk of liver failure, hepatocellular carcinoma (HCC), and liver-related mortality [6].

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are biochemical surrogate indicators for hepatocyte necrosis and inflammation .They are clinically relevant in detecting hepatic injury in viral hepatitis when AST rise is traditionally thought to be less than ALT elevation [7].

The kidney and chronic hepatitis C virus (HCV) infection have a reciprocal relationship: On the one hand, HCV infection raises the risk of renal impairment (cryoglobulinemia vasculitis, chronic inflammation leading to renal degeneration, diabetes, and vascular disease) as well as morbidity and mortality [8]. Patients who survive acute kidney infection needing a dialysis episode are at a significant risk of death, rehospitalization, and chronic kidney disease progression, which may require long-term dialysis therapy [9]. The most important factor in maintaining homeostasis and improving these patients' quality of life is dialysis frequency or the timing of dialysis initiation. Serum levels of metabolic byproducts such as creatinine, urea, and potassium must be monitored on a regular basis. Venipuncture on a regular basis increases the patient's risk of infection [10].

The main objectives of this study were to identify of Hepatitis C virus among renal failure patients and to determine the virulence gene for this virus depending on following steps:

- Serological – Anti HCV antibodies (IgG) detection by ELISA

- PCR for detects (UTR & NS5) gene .

Material and methods

A sample of 5 ml of venous blood was collect from patients and divided into two parts 2.5ml in gel tube. And 2.5 ml in EDTA tube. The serological tests were done after centrifugation at 2000 rpm for 10 minutes.

❖ **Serological test:**

- ELISA assay for the detection of HCV
- Biochemical test that include urea, creatine, albumin, sugar and total protein
- Electrolytes test that include k, Na , P and Cl
- Liver enzymes test that included AST and ALT

❖ **hematological test:**

- CBC Complete blood count
- Blood group test

❖ **Molecular test:**

- Qualitative HCV RNA.
- HCV genotyping.

Anti HCV antibodies (IgG) detection by ELISA

Any anti-HCV antibodies in the sample will bind to the immobilized antigens after being diluted in a specimen diluent and incubated with a microtiter well coated with recombinant HCV antigen during the first incubation. [11] . The captured anti-HCV antibodies are incubated with hourseradish peroxidase conjugated monoclonal anti-human igG after being washed to remove unbound material. The conjugate will bind to the antibody immobilized in the first step during the second

incubation, and the bound enzyme will be detected using a solution containing tetramethlbwnzidine (TMB) and hydrogen peroxide after the excess conjugate is removed. In the wells containing anti-HCV positive samples, a purple color will appear.[12].

RNA extraction

AccuZolTM Total RNA Extraction Reagent was used to extract the RNA (K-3090, Bioneer) The RNA Extraction Reagent (K-3090) from Bioneer is a ready-to-use reagent for isolating total RNA from a variety of sample materials. The reagent keeps RNA intact during homogenization or lysis because it is a monophasic solution of phenol and guanidinesalt that inhibits RNase activity. AccuZolTM enables high-yield total RNA extraction from even small amounts of starting materials. Reagents required

- AccuZol™ Total RNA Extraction Reagent
- Chloroform - Isopropyl alcohol
- 80% Ethanol (in DEPC-treated water)
- RNase-free water or 0.5% SDS solution

cDNA synthesis

To achieve simultaneous genomic DNA removal and cDNA synthesis, a unique genomic DNA remover is combined with EasyScript First-strand cDNA synthedis supermix. Heat gDNA remover and reverse transcriptase for 5 seconds after cDNA synthesis to inactivate the

RNA , primer and water were mixed and incubated the mixture at 65C for 5 minutes then incubated in ice -8 C for 2 minutes after that other components were added. Mixture was incubated at 25C for 10 minutes after that incubate at 42 C for

30 minutes . Finally to inactivate enzyme , mixture was incubated at 85C for 5 seconds.

Table (1). The content of mixture conventional PCR working for target gen

Reverse transcription	50°C	30:00 min
Initial denaturation	95°C	14:30 min
Denaturation	97°C	00:30 min
Annealing	55°C	01:20 min
Synthesis	72°C	00:15 min
Hold	32°C	01:00 min

50 cycles

RT-PCR Program

The thermal protocol for the Bosphore® HCV Quantification Kit consists of two steps: initial denaturation to activate the Taq DNA Polymerase with hot start property, followed by a two-step amplification cycle and a final hold. At the second stage of the amplification cycle real time

Table (2). Programming the Real-Time PCR Instrument

Total RNA / mRNA	0.1 ng-5 mg/l
Random primer (0.1mg/ml)	1 µl
2× ES reaction mix	10 µl
EasyScript RT/R1enzyme mix	1 µl
gDNA remover	1 µl
RNase-free water	To 20 µl

To begin a Real-Time PCR reaction with the Bosphore® Kits, complete the following steps:

- Choose the right filter pairs (FAM and HEX),
- identify unknown samples, standards, positive and negative controls, assign quantitative values to the standards,
- choose the appropriate thermal protocol.
- Get started with the protocol.

PCR Technique

According manufacture procedure : All lyophilized primers forward and reverse suspended by suitable double deionized distill water to reach volume .Double deionized water added to give concentration of (100 pmol/µl) (in stock solution), then 10 pmol/µl concentration prepared by resuspend 10 µl of stock solution with 90 µl double deionized water the primer used for this study show in table **4**

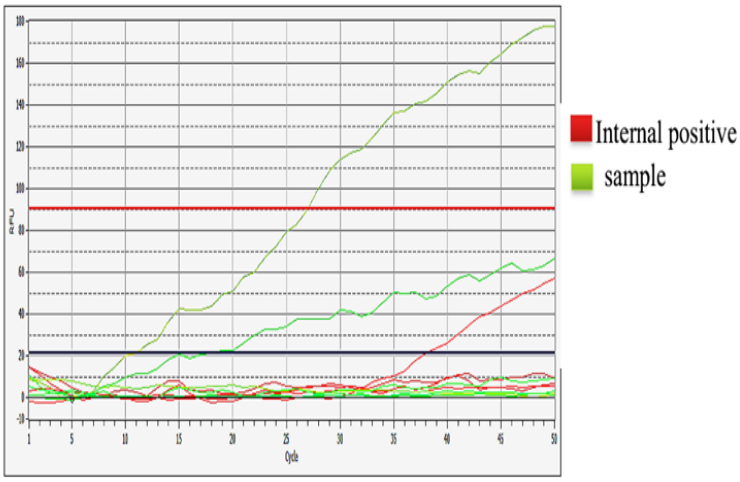

Fig. (1) The Graph of positive HCV obtained by RT-PCR Thermocycler

Several trials of thermal cycles were done using Mastermix Gold Multiplex 20x to optimize the PCR reactions to obtain annealing temperature for the primers. For that annealing temperatures were gradient (50, 52, 54, 56, 58, 60, 62, 64, 66)°C

Table (3). PCR protocol for target genes detection

Step	Temperature	Time	No. of cycles
Initial denaturation	95 C	5 min	1
Denaturation	95 C	30 sec	
Annealing	50 C	30 sec	40 cycles
Extension	72 C	40 sec	
Final extension	72 C	5 min	1

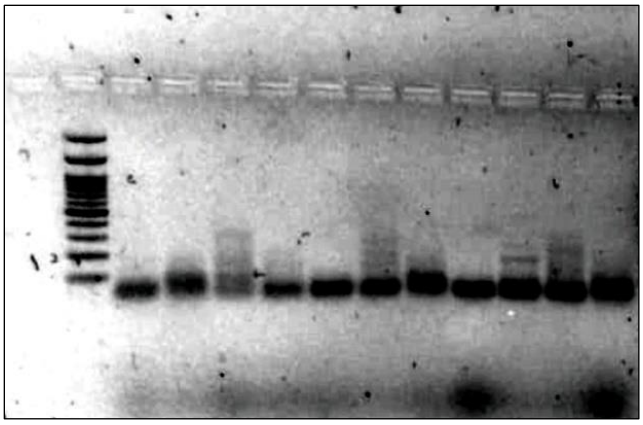

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Fig. (2) 5'UTR gene bond in Agarose Gel

Table (4). primer used for this study

Primer	Type	Sequences	size (bp)	Ref
5'UTR-	F	5'-ACTCCACCATAGATCATCCC-3'	241 bp	[13]
	R	5'-AACACTACTCGGCTAGCAGT-3'		
NS5B	F	5'-CAATWSMMACBACCATCATGGC-3'	826 bp	[14]
	R	5'-CAGGARTTRACTGGAGTGTG-3'		

Statistical analysis

The Chi-squire test was used to statistically analyze all of the evidence using the system SPSS IBM version 20 software. Statistical significanc was defined as a P-value of less than 0.001. [15].

Result

ELISA results The present study was revealed that out of 117 individuals from the patients in kidney dialysis center in Al-Zahra Hospital - Wasit province, 47 (40 %) were positive result for HCV by ELISA III technique.

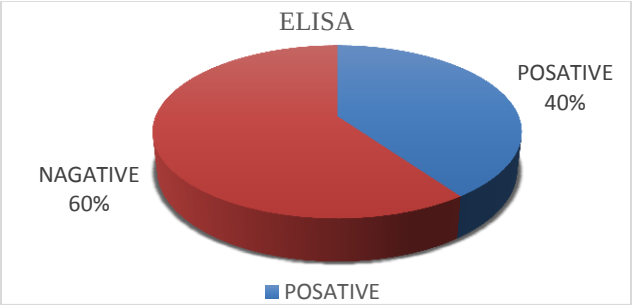

Fig. (3) The distribution of HCV infection according to ELISA technique.

HCV infections Sero-positivity

The infections with HCV among males (53.19 %) higher than females 22 (46.81) . and The highest percentage In urban area was 35 (74.47 %) and the lowest Ratio was In rural area was 12 (25.53 %) The highest infections Ratio recorded was 25 (53.19%) & 18 (38.3%) at age groups 26-45 and > 46 years respectively, while the lowest infection percentage was recorded at the age groups 5-25 years , it was 4 (8.51 %)

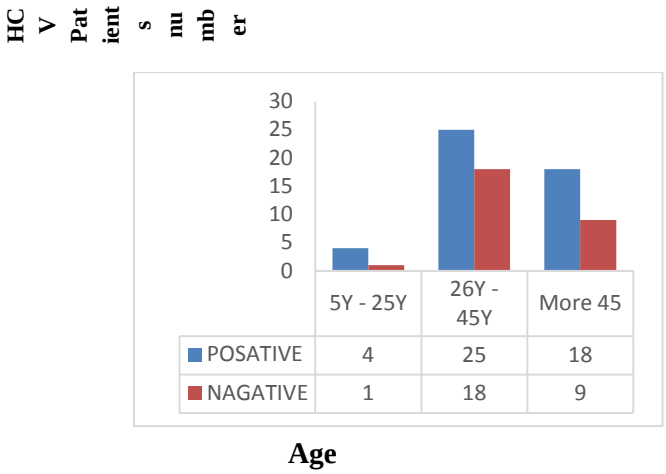

Fig. (4) Distribution of HCV infections according to patients. age groups

The results showed that the highest percentage for HCV was among patients of blood group O 27 (57.45 %), followed by blood group B & A, 8 (17.02 %) ,8 (17.02 %) respectively while the lowest percentage was among patients of blood group AB 4(8.51 %)

HCV genes results

The study showed the detection of the UTR gene Was occurred only 3 (10.71%) patients out of 28 patients, while the NS5 gene gave a negative value for all samples and the results were distributed according to gender, blood type, living and age. Table show in table (6)

Discussion

The results of the ELISA and Real-Time PCR techniques were similar in terms of detecting the prevalence of HCV infections in males versus females. This conclusion was consistent with other research . which found that there were 25 males (53.19%) and 22 females (46.81%) for a variety of reasons. the possibility of males being exposed to infection more than females, especially through the use of razors, frequent travel, and occupational hazards [16]. Other studies have linked

the causes of this variation to genetic factors such as the willingness of either gender to be infected depending on the virus genotype. Other research has linked the origins of this variation to genetic factors relating to the willingness of either gender to Be suspected depending on the genotype of the virus. [17]. The majority of blood donors are men, and it is uncommon to see a woman donate blood, which is due to prevalent Social habit and traditions. Furthermore, guys are far more likely than females to utilize drugs or enter prison, and many males have several sexual encounters.

According to the ELISA technique, HCV

HCV prevalence is higher in people between the ages of 26 and 45 than other . The major source of infection in this population is exposure to various medical devices used for treating and donating blood. and this finding was in agreement with [18]. who notes HCV prevalence in the general population in the United States. The death due to liver failure was the reason for the decrease in infections in age groups older than (45 years) . In contrast, the causes for the decrease in infections in the age groups below this age group are improved blood transfusion conditions and the use of sterile apparatus, as well as the absence of numerous channels of HCV acquisition in children, such as sexual contact and occupational dangers.

According to the current study, the prevalence of hepatitis C virus is concentrated in densely populated areas, such as the governorate center (Al Kut), and this finding is consistent with [19]. finding that hepatitis C virus infection is higher in urban areas than in rural areas.

The fact that the urban population is more developed and that people have a good standard of living and deal more with razors and tattoos, as well as the abundance of hospitals and health centers in urban areas with an increase in

pollution in these areas, may explain the pattern of hepatitis C prevalence in urban areas over rural areas

HCV prevalence was found to be higher in patients with blood group O, which is expected given the high demand for blood group O, which is the most common blood group in the general population, and lower in patients with blood group AB, which agrees with [20].

Genotyping detection

The ability to determine the clinical course and outcome of antiviral therapy is becoming more essential as hepatitis C virus genotypes/subtypes are identified [21]. Given that genotypes are used to make treatment decisions for people with chronic HCV, it is essential to get the genotype and subtype correct. The choice of the genomic region to be investigated is important for identifying HCV genotypes/subtypes [22]. HCV sub classification was dependent on different sections of the HCV genome, including the 5′ UTR, NS5. In this connection, the current research was carried out. and aimed to determine the prevalence of hepatitis C virus genotypes in serum samples of people with hepatitis C virus in the dialysis center in Wasit provence . The study included 28 patients who have antibodies against the hepatitis C virus. The 5-UTR is a highly conserved and frequently targeted region for the detection of important genotypes, according to Baclig and collaborators [23]. It is important to note that the 5′ UTR's high sequence conservation makes it difficult to differentiate all genotypes and subtypes. Genotyping of the NS5B region by sequence analysis has been found to agree well with genotyping of the 5′ UTR, with the added benefit of better subclassification. [24] . Dialysis is one of the most significant risk factors for HCV infection.[25]. (CRF). Cannulation of the fistula for venous access,

contaminated blood state, and direct contact with contaminated items from infected patients. [26].

The most common source of hcv infection is blood transfusions. Protocols for dealing with body fluids, sterilization of dialysis machines and other used devices, and training of health care providers are all important steps in preventing hepatitis C virus transmission in hospitals. , Patients with test positive for anti-HCV antibodies should be isolated if they are being moved or transported. Despite the use of effective monitoring and safe medical practices to reduce the risk of infectious transmission of infection among dialysis patients, rare outbreaks continue to occur in dialysis units. [27].

Patient care is affected by differences in hepatitis C virus genotypes, which are linked to

a variety of conditions. These findings also have much effects. In molecular epidemiological investigations, such as tracking HCV strain distribution and identifying risk factors for transmission, as well as looking at clinical differences between people infected with different strains of the hepatitis C virus. [28].

NS5B amplification was done for 28 samples And we have negative results [29]. And we Use passive voice device settings and we chose the samples that had a higher percentage of cDNA than the rest. We did not obtain any samples, and this reason is likely for the type of patients, as the study was designed on patients with renal failure who undergo hemodialysis 4 times a month and very few patients infected with the virus [30]. And the process of hemodialysis positively affects blood purification It is a virus and also positively affects its inhibition in the blood, so we see that the liver enzymes of people infected with the virus are within their normal limits [31]. In addition to the fact that the virus is in the chronic

Table (5). the distribution of UTR & NS5B genes among patient gender. region. age and blood group

GENDAR	MALE	%	FEMALE	%				
UTR	1	3.57	2	7.14				
NS5B	0	0	0	0				
REGION	CENTER	%	OUT SIDE C	%				
UTR	2	7.14	1	3.57				
NS5B	0	0	0	0				
AGE	5 _ 25	%	26 _ 45	%	MORE 45	%		
UTR	0	0	2	7.14	1	3.57		
NS5B	0	0	0	0	0	0		
ABO	A	%	B	%	AB	%	O	%
UTR	1	3.57	0	0	1	3.57	1	3.57
NS5B	0	0	0	0	0	0	0	0

phase and satisfactory molecular results are usually obtained in the case of the chronic, the study was the first work dealing with Kidney's failure patients in Wasit Governorate..

CONCLUSIONS

Serological tests (ELISA III) are reliable to detect exposure to HCV, and Real-Time PCR technology is required to detect current infection. Blood transfusion, dialysis and medical staff were among the main causes of viral hepatitis infection in Wasit . A high prevalence of hepatitis C virus was detected among males, age group (26-45) years and patients with blood type O. The infection ratio with hepatitis C virus was higher in urban areas than in rural areas of the governorate .

Hemodialysis patients are still at risk of infection with hepatitis C virus through the hemodialysis machine as well as the medical equipment used in the dialysis procedures.

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