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Molecular and Immunological Detection of Hepatitis C Virus in Patients with Chronic Renal Failure

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Abstract

Due to its association with hepatocellular carcinoma and being one of the ten most common malignancies worldwide, hepatitis C viral infection has become a severe public health concern. Therefore, establishing an accurate, reliable and sensitive diagnostic test for this infection is strongly advised. Real-time polymerase chain reaction (PCR) has been created to achieve this purpose. The current study was established to investigate the hepatitis C virus among Iraqi patients with chronic renal failure and to detect the virus immunologically by the fourth generation enzyme-linked immunosorbent assay technique and molecularly by real-time PCR. As a result, out of 50 patients with chronic renal failure undergoing dialysis, 39 patients tested positive for anti-HCV IgG by ELISA test and only 9 patients of the total count tested positive and showed growth curves with copy numbers of 1×10^2, 2.5×10^2, 3×10^2, 2.7×10^3, 2.7×10^3, 3.5×10^3, 8.7×10^4, 3.5×10^5 and 5.5×10^6 copies/ml respectively detected for HCV only by real-time PCR. Therefore, real-time PCR is beneficial for precise viral load determination and detection of viral hepatitis in chronic renal failure patients.

Keywords: HCV, Real-time PCR, ELISA, Detection.

التحري الجزيئي و المناعي لفيروس التهاب الكبد الوبائي نمط سي لدى مرضى الفشل الكلوي المزمن

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الخلاصة

تعد العدوى الفيروسية لالتهاب الكبد الوبائي نمط سي مصدر قلق شديد للصحة العامة ، نظرًا لارتباطها بسرطان الخلايا الكبدية وكونها واحدة من أكثر عشرة أورام خبيثة شيوعًا في جميع أنحاء العالم ، يُنصح بشدة بإجراء اختبار تشخيصي دقيق وموثوق وحساس لهذه العدوى. تم إنشاء تفاعل البلمرة المتسلسل بواسطة(Real-Time PCR)لتحقيق هذا الغرض. أُجريت الدراسة الحالية للتحري عن فيروس التهاب الكبد الوبائي نمط سي بين المرضى العراقيين المصابين بالفشل الكلوي المزمن وللكشف عن الفيروس مناعيًا عن طريق اختبارالامتزاز المناعي المرتبط بالانزيم من الجيل الرابع وجزيئيًا بواسطة تقنية تفاعل البوليميراز المتسلسل في الوقت الحقيقي. نتيجة لذلك ، من بين 50 مريضًا يعانون من الفشل الكلوي المزمن الذين كانوا يخضعون لغسيل الكلى ، اتضح انه كان لدى 39 مريضًا نتيجة إيجابية لـ HCV IgG بواسطة اختبار ELISA وكان 9 فقط من المرضى لديهم نتيجة إيجابية من العدد الإجمالي وأظهروا منحنيات نمو مع عدد نسخ (1×10^2, 2.5×10^2, 3×10^2 ,2.7×10^3, 2.7×10^3, 3.5×10^3, 8.7×10^4, 3.5×10^5, 5.5×10^6) نسخ / مل

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على التوالي بواسطة PCR في الوقت الحقيقي. لذلك فإن تفاعل البوليميراز المتسلسل في الوقت الحقيقي مفيد في التشخيص الدقيق لحاملي الفيروس والكشف عن التهاب الكبد الفيروسي في مرضى الفشل الكلوي المزمن.

1. Introduction

Viral hepatitis is a serious public health issue which is linked to hepatocellular carcinoma [1], and is one of the ten most prevalent malignancies globally [2]. Hepatitis C virus is currently classified into 8 genotypes and has 91 subtypes [3], according to the International Committee on Taxonomy of Viruses: HCV Classification Database (Last accessed on 8 March 2022). Chronic HCV infection affects 75-85% of those who have it, with 10-20% developing liver cirrhosis. Rates vary by age, sex, race and immune system status. The global HCV burden is still high, especially in Iraq [4, 5], due to barriers to treatment, high-cost medication, and the potential for re-infection [6]. Hence, a precise diagnosis is necessary to determine whether HCV RNA is present in carriers who appear to have no symptoms.

Chronic renal failure (CRF) is a major public health problem that affects around 10% of the adult population globally. It is a chronic and irreversible disorder characterized by a gradual decrease of kidney function, leading to end-stage renal disease (ESRD) and renal replacement therapy [7]. Due to the intrusive nature of therapy and equipment contamination, hemodialysis patients are at risk of contracting viral diseases [8]. Individuals on hemodialysis are more vulnerable to infections towing to compromised immune systems [9].

HCV research lacks a sensitive and specific technique to assess viral levels in plasma or serum due to its low copy number and diverse genome [10]. Based on conventional reverse transcription polymerase chain reaction (RT-PCR), many researchers have employed variety of techniques to measure the viral load in HCV-infected individuals[11, 12]. Real-time PCR analysis has been successfully used in both scientific and clinical research, and is the most recently produced nucleic acid testing technology [13]. Quantitative nucleic acid testing has become the gold standard marker for clinical choices on antiviral drug usage [14]. Viral nucleic acids are unstable, requiring strict conditions for sample collection, transmission, storage, and processing [15]. RT-PCR is a popular real-time technique for detecting and measuring viral copy number, using a probe tagged with a specific fluorescent dye [16]. The capacity to monitor the real-time progress of tagged target amplification considerably enhances DNA and RNA quantification [17]. Real- time technique uses threshold cycle (Ct) values derived during the exponential phase of PCR to measure the quantity of the target and establish its relative expression levels [18]. In this present study, a highly specific, sensitive real-time one-step RT-PCR approach was adopted for quantifying HCV RNA copies in plasma of renal chronic kidney disease patients undergoing regular dialysis.

2. Material and Methods

This was a case control study which comprised 50 patients all of whom suffered from chronic kidney failure and were undergoing regular weekly dialysis. Thirty-nine (39) samples were collected from patients undergoing dialysis from positive hepatitis C dialysis unit. A volume of 10 ml of blood was obtained from each patient. Half of the blood was immediately submitted to the hospital laboratory for identifying anti-HCV IgG antibody using the fourth-generation enzyme linked immune sorbent assay (ELISA) technique with a cutoff value of > 1.00. (Fortress, Strip Reader, United Kingdom) and the other half of blood was kept frozen at -20°C until the time of its use. Eleven samples were collected from patients from negative dialysis unit. The samples were collected from patients who attended Hamida Al-Misfaa Dialysis Center of Al-Kadhimiyah Educational Hospital as well as the dialysis center of Al-Yarmouk Hospital in Baghdad in Iraq. The samples collection took place between November

2022 and January 2023. The ages of patients ranged between 20 and 84 years. Forty samples from apparently healthy individuals were collected as control. The displayed study involved receiving signed consent from each patient after verbally describing the risks of procedure and why the blood sample was being drawn. Patients with hematological malignancies, anemia and pregnant women were excluded from the research. The hospital's ethics and the College of Science, University of Baghdad Ethics committee approved the research (Ref.: CSEC/0922/0080). Patients were informed of the study goals and advantages, and they received assurances regarding its risks and rewards. Each patient was asked to fill out a questionnaire which included general and specific information and if they agreed with it.

3.1 Real-time PCR Quantitative Detection

The RNA extraction and quantitative detection from blood samples was achieved according to the manual of manufacturer (Bektop Company. Russia). High automated extraction of purified RNA was converted to cDNA by one step reverse transcription which was then amplified by using real-time PCR.

3.2 The Statistical Analysis

The Statistical Analysis System (SAS, 2018) was used to detect the effects of different factors in study parameters and quantitative characteristics including age and disease, dialysis duration and the mean and standard were calculated. The t-test was used to compare between means. Chi-square test was used to compare between percentage (At 0.05 and 0.01 of probability *p*-values). Tables and graphs were used to display each outcome [19].

3. Results and Discussion

3.1 Immunological Investigation

In 2019, Nguyen *et al*. investigated that the incidence of anti-HCV antibodies is greater in dialysis patients than in healthy people, indicating that dialysis patients may be more vulnerable to HCV infection [20]. In this paper, hepatitis C virus IgG antibody rates were investigated clinically by commercial immunological tests for measuring HCV antibody levels using the fourth generation ELISA. Several commercial immuno-enzymatic assays have been adapted for determining anti-HCV IgG. Ward *et al.* first described a modification of a second-generation anti-HCV ELISA [21]. Coppola *et al.* had also employed the 3rd-generation ELISA technique as HCV 3.0 ELISA test (Ortho-Clinical Diagnostics) kit in a more recent study [22]. Results in Table 1 below show that the hepatitis C virus IgG antibody rates were highly significant among 61-80 years old when compared to the controls. The mean age of patients and control was 52.08 ±1.96 and 44.24 ±2.32 respectively with t-test=6.214. In all age categories, there was a significant relationship between IgG and cases ($P \leq 0.01$), *P*-value=0.010. Forty-eight percent of patients were males and 52% females. In 2012, Al-Assaf conducted a study that assessed serum concentrations of immunoglobulins IgG, IgM and IgA in HCV patients with renal failure. The results showed that serum IgG increased in patients of positive and negative groups compared to healthy individuals [23]. In 2012, Saleh resulted the difference according to gender of the two groups which might be explained by the fact that males are more likely than females to come into touch with HCV risk factors which may enhance the liver damage caused by HCV [24]. In this investigated study, Table 2 shows that out of 50 patients with chronic renal failure undergoing dialysis, 39 patients tested positive for anti-HCV IgG by ELISA test and 11 were diagnosed as negative. There was a highly significant difference between the two results ($P \leq 0.01$), *P*-value=0.0001. Al-Azzawi *et. al.* investigated that there are several immune response indicators such as IL-6, IFN, and TNF- in chronic hepatitis C patients who demonstrated a highly significant immune response between pre- and post-treatment patients [25]. Also anti-inflammatory cytokines

such as interleukin-10 are essential for the body protection and immunological tolerance against HCV infection [26, 27].

Table 1: The distribution of all cases according to age groups in patients and control

Factor		Patients (No.=50)	Control (No.= 40)	*P*-value
Age groups: No (%)	20-40 yr.	12(24%)	16(24%)	1.00 NS
	41-60 yr.	23(46%)	18(28%)	0.071 NS
	61-80 yr.	15(30%)	6(8%)	0.010 **
	P-value	0.140 NS	0.050 *	---
	Mean ± SE of Age (year)	52.08 ±1.96	44.24 ±2.32	
	T-test	6.214 **		
	P-value	0.010		
* (*P*≤0.05), ** (*P*≤0.01).				

Table 2: Distribution of sample study according to anti HCV IgG

Factor		Patients (No=50)	Control (No= 40)	*P*-value
Anti HCV IgG No (%)	Positive	39(78%)	0(0%)	0.0001 **
	Negative	11(22%)	40(100%)	0.0001 **
P-value		0.0001 **	0.0001 **	---
** (*P*≤0.01).				

4.2 Molecular Investigation

A total of 50 CRF patients undergoing weekly dialysis and 40 apparently healthy individuals were molecularly detected with HCV. Real-time PCR was used for amplification of the virus gene in the mentioned cases by using specific ready-to-use primers. The results in Table 3 shows the distribution of sample study according to HCV real time -PCR. It was found that there was a significant relationship (*P*≤0.05) between positive cases and healthy group, (*P*-value =0.0481 and 0.0001 respectively). Also, there was highly significant difference according to negative cases of patients compared to control (*P*=0.0466 and *P*=0.0001 respectively). The anti-HCV was discovered with 78% in serum, resulting in a significant rise when compared to the control group participants who in the same test had a negative result. The results of real-time PCR for HCV testing revealed a percentage of 18% of the virus genome. Whereas Jumaa and Ahmed's research resulted HCV percentage of 99% in serum of patients compared to the control cases [28]. In our investigation, individuals were considered as "chronic carriers" because anti-HCV seroconversion happened within the previous 6 months when the antibodies of anti-HCV and hepatitis C viral RNA were detected. according to World Health Organization [WHO] 2022, it is widely recognized that 20 to 30% of HCV-exposed people remove the virus on their own. Their anti-HCV reactivity then gradually diminishes, perhaps leading to seroreversion [29]. It was examined that 30 samples collected from individuals with HCV infection who were negative for HCV RNA by real-time PCR, tested positive for HCV antibody when the procedure was conducted under proper conditions to avoid minimum contamination, due to high sensitivity of real-time PCR which can detect HCV RNA levels as low as 2000 copies/ml [30]. It has its limitation and strict processes are required to avoid any false positive results as even a little contamination can affect the results [31]. The quantity viral genome of HCV was determined by visible rise in

fluorescence dye (ROX – FAM) as the reaction progressed (Growth Curve) till the end of 50 cycles (Figure 1).

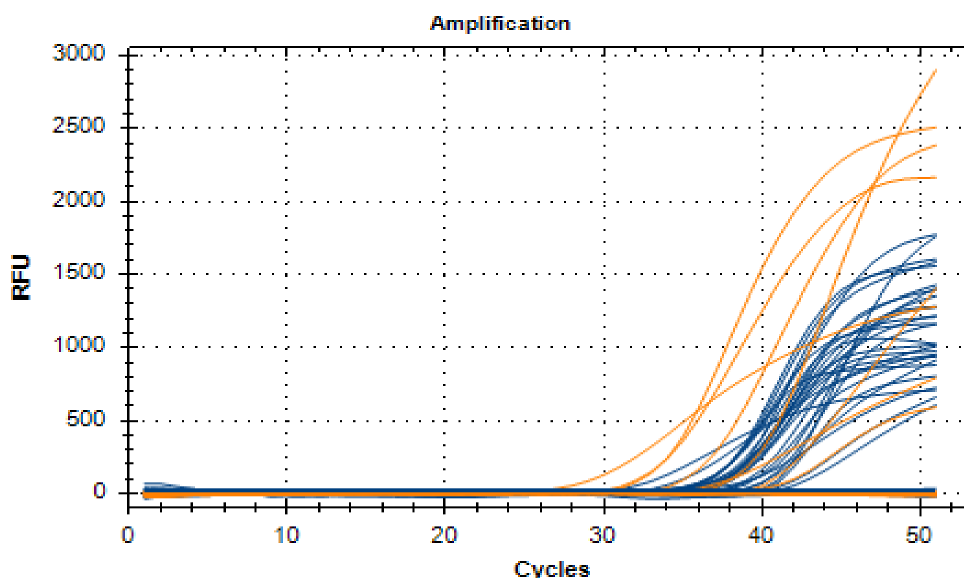

Figure 1: HCV Real-Time PCR Curves

The threshold level, known as cycle threshold (Ct), refers to the cycle at which the fluorescence crosses a specific level. It depends on the amount of viral RNA present. Real-time PCR software uses this Ct (of the standard samples). Hence, in order to precisely calculate the viral load of unknown samples, a standard curve must be created [32]. Same prospect was used by Mohammed and Bassim when HBV was detected by real-time PCR [33]. In the present study, HCV copy number was measured by an equation according to the kit leaflet for the positive detected samples, linear runs from 1×10^2 to 3.7×10^5 copies/ml. Nine PCR positive samples were detected, three of them copy number resulted as 1×10^2 , 2.5×10^2 , 3×10^2 copies/ml each and other three cases were 2.7×10^3, 2.7×10^3, 3.5×10^3 copies/ml respectively. Two single cases were measured as 3.5×10^5, 8.7×10^4 copies/ml. The last case was measured as 5.5×10^6 copies/ml. Test results larger than 100.000.000 copies/ml should be recorded as greater than 100.000.000 copies/ml. A study of HCV detection of CRF patients undergoing regular dialysis resulted in early hepatitis C screening. Although this therapy may help patients with CRF, it has to be shown in randomized controlled studies.

Table 3: Distribution of sample study according to HCV Real time -PCR

Factor		Patients (No=50)	Control (No= 40)	*P*-value
HCV Real-time PCR: No (%)	Positive	9(18%)	0(0%)	0.0481 *
	Negative	41(82%)	40(100%)	0.0466 *
P-value		0.0001 **	0.0001 **	---
* ($P\leq0.05$), ** ($P\leq0.01$).				

4. Conclusion

Real-time PCR showed to be a beneficial technology for precise determination of viral amounts in renal failure patients' blood, as evidenced by its detection sensitivity. More exact viral load categorization may be useful for initiating and monitoring therapy, as well as distinguishing between inactive carriers and chronic hepatitis C patients.

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