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FREQUENCY OF HEPATITIS C VIRUS INFECTION AMONG BETA-THALASSEMIA PATIENTS IN BAGHDAD CITY, IRAQ

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Abstract. Patients with thalassemia major are at high risk of Hepatitis C virus (HCV) due to the blood transfusion from donors infected by HCV. The purpose of this study was to study the correlation between some hematological parameters and liver function tests with viral hepatitis and to evaluate the ELISA and RT- PCR techniques for the diagnosis of HCV in thalassaemic patients. Blood samples from eighty patients with ß-thalassemia were subjected to the following tests: PCV, Hb concentration, liver function tests (GPT, GOT, Alkaline phosphatase). Serum samples from all patients were tested for antibodies to hepatitis C virus using ELISA test and for HCV RNA by the real time polymerase chain reaction (RT-PCR). It was shown that 67.5% of study patients were detected with a range of PCV 15-20%, whereas 100% of patients were detected with a range of Hb (5-9 g/dL). In addition, 98.8% of patients were detected with elevated liver enzymes. It was shown that 100% of patients revealed with positive ELISA anti-HCV antibodies in their serum specimens. Positive RT-PCR assay was detected in 86% of study patients. ELISA test was revealed with 14% false positive results. Thalassemia patients should be aware of the risks of viral infection during their blood transfusions, and those with chronic infections should beware of transmitting the infection to uninfected individuals.

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Keywords: *Thalassemia, HCV, Liver function tests, ELISA, PCR.*

1. INTRODUCTION

Beta thalassemia is defined as a group of recessive genetic disorders of hemoglobin synthesis that caused health disorders of prominent importance in the tropical part, especially in low-resource countries, as it is in Iraq [1]The annual total rate of symptomatic persons is estimated to be about 1 in 100,000 cases worldwide. Three types of thalassemia have been described: thalassemia major, thalassemia intermediate and thalassemia minor. It has been observed through studies that the first two years of life for people with thalassemia major are usually characterized by severe anemia, which requires regular blood transfusions for patients with thalassemia [2]. One of the most important human viruses in thalassemia patients is the hepatitis C virus, a virus with a positive sense of the single-stranded RNA genome. It has been proven that this virus spreads between humans through contact with blood. It was noted that most individuals have mild symptoms if any, however, this virus continues to be present in the liver in about 85% of infected **patients [3]. Frequent and regular blood transfusions in** patients with beta thalassemia is an important factor for the risk of infection with HCV, especially if routine detection of this virus is not carried out in the blood of donors. The risk of infection in thalassemia patients is a sign of the risk of transfusion-borne infection because of their exposure to frequent blood transfusions. It has also been proven through studies that if the incidence rate is low in patients with beta thalassemia, this means that the risk to community health will be little [4]. Studies on this subject indicate that the prevalence of hepatitis C virus among Thalassemia patients is high in Iraq, but the numbers are somewhat conflicting. Despite the application of the screening program on donors and the low rates of spread of the virus through blood transfusion, the spread of hepatitis C virus infection was high in thalassemia patients. It was noted that the prevalence of infection among the young age groups was high. Various studies conducted in different parts of Iraq showed conflicting results: Al-Watifi and Hassan in Basra, Raham et al in Diyala Province, and Al-Doori in Anbar Province indicated that increasing age is an important factor for the risk of infection with hepatitis C virus, while Numan did not disclose there is no significant .association. It was noted that the seropositivity of HCV antibodies is decreasing in patients with hereditary anemia in Basra, and genotype 4 is the most prevalent among these patients. It was noted that patients in Basra Province had the lowest number of infections with hepatitis C virus compared with other parts of Iraq. However, survival still poses a great risk to these patients because of their high number there [5]. There is no doubt that transfusion of contaminated blood is an important means of spreading

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viral infection to blood recipients. It is well established that hepatitis C, B and human immunodeficiency virus (HIV) are mandatory screening tests by blood banks. In Iraq, the hepatitis C virus test was introduced in the blood bank in 1995. Premarital hepatitis C testing is a preventive health program in many countries for the purpose of detecting and treating unidentified disorders to prevent transmission of infection to spouses and children alike. The implementation of the pre-marriage screening program for infectious diseases is a huge and ambitious project in terms of cost and impact. Such a program was actually implemented in the Duhok Province in Kurdistan region of Iraq in 2008 [6]. This study was undertaken to study the correlation between some hematological parameters and liver function tests with viral hepatitis, to detect transfusion transmitted infections with hepatitis C virus in beta thalassemia patients and to evaluate the ELISA and RT- PCR techniques for the diagnosis of HCV.

2. MATERIALS AND METHODS

2.1 Subjects

This study has been conducted at Ibn-Al-Balady Hospital and Central Public Health Laboratories / Baghdad. Eighty patients with ß-thalassemia (major) were admitted to Thalassemia Center in Ibn-Al-Balady Hospital – Baghdad for blood transfusion. The diagnosis of thalassemia was done by specialized doctors, based on clinical examination and blood investigations (complete blood picture, blood group & Rh, PCV, Hb concentration and liver function tests). All age groups and both sexes of thalassemia patients were included in this study. According to the exclusion criteria, the following categories of thalassemia patients were excluded: Thalassemia minor patients who are not dependent on blood transfusions, patients with sickle cell anemia and alpha thalassemia, in addition to those with leukemia and any condition that contradicts the objectives of this study. Consent forms were obtained from all study participants, and complete information was collected for each patient regarding age, gender, comorbidities, treatment program and admission diagnosis.

2.2 Specimens and Procedures

Blood samples from all study patients were subjected to the following hematological and biochemical tests: PCV, Hb concentration, liver function tests (GPT, GOT, Alkaline phosphatase). Serum samples from all patients were tested for antibodies to hepatitis C virus (anti-HCV) using ELISA test and for HCV RNA by real time polymerase chain reaction (RT-PCR) test.

2.2.1 Hematological Tests

Measurement of hemoglobin concentration (Hb) and PCV was achieved by using the CELL-DYN Emerald (Abbott Diagnostic, USA) automated hematology analyzer. The CELL-DYN Emerald has easy to use software controlled by a color touchscreen with a built-in keyboard .

2.2.2 Biochemical Tests

Alkaline phosphate, GPT and GOT levels were tested by the Reflotron® Plus, which is an in vitro diagnostic device designed for the quantitative determination of clinical chemistry parameters using Reflotron® Test reagent strips. It works on the principle of reflectance photometry and ensures rapid and reliable results while being easy to use.The results are shown on a clear LC display and the profile can be printed out via the integral printer. Reflectance measurement is based on the color change in the test strips.

2.2.3ELISA Tests

Anti-HCV antibodies were detected by ELISA technique using IgM, IgG ELISA kite (Monobind, Inc. USA). The assay procedure is an immune-enzymatic method in which the wells of a microplate are coated with recombinant antigens representing epitopes of HCV: Core, NS3, NS4 and NS5. Serum or plasma samples are added to these wells. If antibodies specific for HCV are present in the sample, they will form stable complexes with the antigens on the well .

2.2.4 Polymerase Chain Reaction (PCR) Test

AmpliSens® HCV-FRT PCR kit is intended for analysis of RNA extracted with an RNA/DNA isolation kits from peripheral blood plasma. Blood samples were taken after overnight fasting into the tube with EDTA solution as anticoagulant. Closed tubes with blood were turned several times upside down and back again. The following nucleic acid extraction kit was used: RIBO-sorb, REF K2-1-Et-100-CE. RNA/DNA was extracted according to the manual provided by the manufacturer. The purified RNA was stored at 2–8 °C for at most 4 h, at temperatures not higher than minus 16 °C. Amplification of extracted RNA was done in Thermal cycler (Stratagene, USA). To prepare the reaction mixture, the reagents (10 µl of RT-PCR-mix-1-FL HCV, 5 µl of RTPCR-mix-2-FEP/FRT, 0.25 µl of RT-G-mix-2, 0.5 µl of Polymerase (TaqF), and 0.25 µl of TMRevertase (MMlv) per one reaction) were mixed in a new sterile tube. A quantity of 15 µl of the prepared reaction mixture was added to each PCR tube. A quantity of 10 µl of RNA samples isolated from the clinical samples was added to each PCR tube . The Rotor-Gene instrument was programmed according to manufacturer's manual and the Guidelines. A temperature profile was created on Rotor-Gene instrument as follows:

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Table 1. *AmpliSens-2 RG program for rotor-type instruments*

Step	Temperature °C	Time	Fluorescence detection	Cycle repeats
1 (Hold)	50	15 min	-	1
2 (Hold)	95	15 min	-	1
3 (Cycling 1)	95	5 Sec	-	5
	60	20 Sec	-	
	72	15 Sec	-	
4 (Cycling 2)	95	5 Sec	-	40
	60	20 Sec	FAM/Green, JOE/Yellow, ROX/Orange,Cy5/ Red	
	72	15	-	

The fluorescence channel sensitivity was adjusted as described in the Guidelines. The iCycler iQ or iQ5 instrument was programmed according to manufacturer's manual and the Guidelines. A temperature profile was created on iQ5 instrument as follows:

Table 2*. The fluorescence channel sensitivity was adjusted as described in the Guidelines.*

Step	Temperature °C	Time	Fluorescence detection	Cycle repeats
1	50	15 min	-	1
2	95	15 min	-	1
3	95	5 Sec	-	5
	60	20 Sec	-	
	72	15 Sec	-	
4	95	5 Sec	-	40
	60	20 Sec	FAM/HEX ROX/ Cy5	
	72	15	-	

Data analysis

The signal from the Internal Control cDNA amplification product was detected in the FAM channel, the signal from the HCV cDNA amplification product was detected in the JOE/HEX channel.

Results interpretation

The results were interpreted by the real-time PCR instrument software by the crossing or not crossing of the threshold line by the fluorescence curve.

Table 3*. The boundary Ct values are summarized in the Important Product Information Bulletin.*

Control	Stage for control	Ct in channel		Interpretation
		Green/FAM	JOE/HEX/ Yellow	
C–	RNA isolation	Pos (≤ Ct)*	Neg.	OK
PCE	RNA isolation	Pos (≤ Ct) *	Pos (≤ Ct) *	OK
NCA	Amplification	Neg.	Neg.	OK
C+	Amplification	Pos (≤ Ct) *	Pos (≤ Ct) *	OK

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1. The sample was considered positive for HCV RNA if the Ct value detected in the JOE/HEX/Yellow channel did not exceed the boundary value specified in the important product information bulletin.
2. The sample was considered negative for HCV RNA if the Ct value in the JOE/HEX/Yellow channel was absent or if the Ct value detected in the JOE/HEX/Yellow was greater than the specified boundary value and the Ct value in the FAM channel did not exceed the boundary value specified in the important Product Information Bulletin.
3. The sample was considered equivocal if an equivocal result was obtained in any of the channels. In this case, PCR analysis of this sample should be repeated once again. Results were accepted as relevant if both positive and negative controls of amplification as well as negative and positive controls of extraction were passed properly.

2.3 Statistical Analysis

Variables were presented as counts and percentages, mean ± SD. The Chi-square test and correlation analysis for calculating the least significant difference (L.S.D.) test were applied to determine the statistical significance of the data. *P* value ≤ 0.05 was considered statistically significant. Data analysis was performed by using SPSS (Statistical Package for Social Sciences) version 19.

3. RESULTS

Table (3.1) *Distribution of study patients according to age*

Age groups in years	Frequency	Percent	Valid Percent	Cumulative Percent
1-10	6	7.5	7.5	7.5
11-20	18	22.5	22.5	30.0
21-30	40	50.0	50.0	80.0
31-40	16	20.0	20.0	100.0
Total	80	100.0	100.0	

3.1. Distribution Of Study Patients

according to age is shown on Table (3.1). It was shown that 50% of study patents was within the age group 21-30 years.

Table (3.2) *Distribution of study patients according to gender*

Gender	Frequency	Percent	Valid Percent	Cumulative Percent
Male	48	60.0	60.0	60.0
Female	32	40.0	40.0	100.0
Total	80	100.0	100.0	

3.2. Distribution Of Study Patients According To Gender Is Shown On Table (3.2).

It was shown that the male patients were higher than female ones (60% versus 40%).

Table (3.3) *Distribution of study patients according to PCV*

PCV	Frequency	Percent	Valid Percent	Cumulative Percent
15-20%	54	67.5	67.5	67.5
21-30%	26	32.5	32.5	100.0
Total	80	100.0	100.0	

3.3. According To PCV Measurement,

67.5% of study patients were detected with a range of PCV15-20%, whereas hemoglobin concentration (Hb), as shown in table (3.3).

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Table (3.4) *Distribution of study patients according to Hb*

Hb	Frequency	Percent	Valid Percent	Cumulative Percent
Mild anemia (10-12 g/dL)	0	0	0	0
Moderate anemia (5-9 g/dL)	80	100.0	100.0	100.0
Severe anemia (<5 g/dL)	0	0	0	0

3.4. According To PCV Measurement

100% of study patients were detected with a range of (5-9 g/dL).These parameters indicate moderate and severe anemia that accompanied their thalassemia as shown in table (4).

Table (3.5) *Distribution of study patients according to GPT*

GPT	Frequency	Percent	Valid Percent	Cumulative Percent
<22 U/I 25C	1	1.3	1.3	1.3
>22 U/I 25C	79	98.8	98.8	100.0
Total	80	100.0	100.0	

3.5. The Results Of Liver Enzyme Tests (GPT) Are Shown On Table (3.5).

It was shown that the vast majority of study patients (98.8%) were detected with elevated liver enzymes.

Table (3.6) *Distribution of study patients according to GOT*

GOT	Frequency	Percent	Valid Percent	Cumulative Percent
<18 U/I 25C	1	1.3	1.3	1.3
>18 U/I 25C	79	98.8	98.8	100.0
Total	80	100.0	100.0	

3.6. The Results Of Liver Enzyme Tests (GOT) Are Shown On Table (3.6).

It was shown that the vast majority of study patients (98.8%) were detected with elevated liver enzymes.

Table (3.7) *Distribution of study patients according to alkaline phosphatase*

Alkaline phosphatase	Frequency	Percent	Valid Percent	Cumulative Percent
<129 U/I 25C	1	1.3	1.3	1.3
>129 U/I 25C	79	98.8	98.8	100.0
Total	80	100.0	100.0	

3.7. The Results Of Liver Enzyme Tests (Alkaline Phosphatase) Are Shown On Table (3.7).

It was shown that the vast majority of study patients (98.8%) were detected with elevated liver enzymes.

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Volume 5, No:1. 2022

Table (3.8) *Frequency of Anti HCV Ab in serum specimens from study patients according to ELISA.*

Anti HCV	Frequency	Percent	Valid Percent	Cumulative Percent
Positive	80	100.0	100.0	100.0
Negative	0	0	0	0

3.8. Frequency of Anti HCV.

antibodies in serum specimens from study patients according to ELISA is shown on table (3.8).

Table (3.9) *Frequency of HCV in serum specimens from study patients according to RT-PCR assay*

PCR	Frequency	Percent	Valid Percent	Cumulative Percent
Positive	69	86.3	86.3	86.3
Negative	11	13.8	13.8	100.0
Total	80	100.0	100.0	

3.9. Frequency of HCV in serum specimens from study patients according to PCR assay is shown on table (9). It was clearly demonstrated that 86% of study patients were detected with positive RT-PCR assay. It was shown from a previous study that the overall prevalence rate of anti-HCV was 28.1%. Forty six of anti-HCV positive patients (46/58, 79.3%) were also HCV RNA positive. HCV–positive patients were significantly older from HCV-negative ones (p<0.001).

4. DISCUSSION

It was shown that 50% of study patents was within the age group 21-30 years. This observation was in accordance with previous studies **[7].** Thalassemia affects men and women equally and occurs in approximately 4.4 of every 10,000 live births. Studies have shown that alpha thalassemia is more common in individuals of African and Southeast Asian descent, while beta thalassemia is more common in individuals of Mediterranean, African and Southeast Asian descent. It was also noted that thalassemia affects 5-30% of individuals in these ethnic groups. **[8].** It was found from a previous study that there was a significant decrease in the values of hemoglobin and PCV in beta thalassemia patients compared to controls. **[9].** A previous study also indicated that the vast majority of study patients (98.8%) were found to have elevated liver enzymes. A significant association of elevated alanine aminotransferase (ALT) or (GPT) with iron overload, transfusion index, age, and anti-HCV positivity in thalassemia patients was further observed in the same study **[10].** The present study demonstrated that 100% of study patients revealed with positive Anti HCV antibodies in their serum specimens. From a previous study, hepatitis C virus (HCV) seroprevalence and risk factors in north Iran were investigated in 105 thalassemia sufferers, 93 haemodialysis patients and 5976 blood donors by second generation ELISA. The previous study showed that haemodialysis patients and thalassemia sufferers were at higher risk of having HCV infection; the prevalence being 55.9% and 63.8% respectively in comparison to the prevalence of blood donors (0.5%). A confirmatory immunoblotting was employed using HCV-positive cases (54 thalassemia sufferers and 19 blood donors). The result showed that 92.6% of samples of the first group and 10.5% of the latter were positive. Thus, it can be suggested that ELISA in low-risk cases may produce considerable false positives. In HCV-positive patients with thalassemia, the incidence of HCV among different age groups and genders was similar but a strong correlation in respect to the number of blood transfusion (P=0.008) was observed. In HCV-positive haemodialysis patients, it was found that there was no correlation with liver function tests (alanine aminotransferase and aspartate aminotransferase: ALT and AST), but a significant correlation was observed in respect to the duration of dialysis (P=0.000) and the number of units transfused (P=0.000). Consequently, it still seems blood transfusion is the main factor for increasing the incidence of HCV in thalassemia sufferers and haemodialysis patients **[11].** The current study revealed that 86% of study patients were detected with positive RT-PCR assay. It was shown from a previous study that the overall prevalence rate of anti-HCV was 28.1%. Forty six of anti-HCV positive patients (46/58, 79.3%) were also HCV RNA positive. HCV–positive patients were significantly older from HCV-negative ones (p<0.001). In addition, those results indicate that higher

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Journal port Science Research
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prevalence of anti-HCV or HCV RNA were significantly associated with longer duration of transfusion ($p<0.003$ and $p<0.001$, respectively. This previous study concluded that more accurate technology should be used to diagnose viral infection and treat thalassemia patients with HCV infection more accurately, even though the blood donor screening project has reduced HCV infection **[12].** In one of the previous Iraqi studies on the same subject, the seroprevalence of HCV antibodies among pregnant women was recorded as 3.2% **[13].** 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 (as cited by W.A. Al-Kubaisy *et al*) **[14].** It was clearly demonstrated that 100% of study patients revealed with positive ELISA Anti HCV antibodies in their serum specimens, whereas 86% of study patients were detected with positive RT-PCR assay. Taking into account that RT-PCR is a gold standard technique, it is concluded that serum specimens subjected to ELISA technique was revealed with 14% false positive results. High correlation rate was observed between some hematological parameters and liver function tests, in addition to the ELISA and RT- PCR results in thalassaemic patients.

5. CONCLUSIONS

Thalassemia patients should be aware of the risks of viral infection during their blood transfusions, and those with chronic infections should beware of transmitting the infection to uninfected individuals. This study confirms that thalassemia patients living in countries that have not adopted a child immunization program against hepatitis C should strongly undergo hepatitis C virus screening and vaccination before commencing transfusion therapy **[15].** In addition, infants born to mothers infected with the hepatitis C virus should be tested for anti-HCV at 18 months of age. Accurate detection and appropriate treatment of viral infection in thalassemia patients with hepatitis C virus requires a more accurate diagnostic procedures. Genotyping procedures for HCV is recommended to be applied in chronic case prior to antiviral treatment programs. Further studies are needed for the evaluation of the sophisticated tests which can be used for accurate detection of HCV in thalassaemic patients.

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