Genotypic Detection of Hepatitis C Infection Among Multi-transfused Thalassemic Patients In Erbil City by Using Real Time PCR.

Fattma A Ali (Ph.D)[1], **Sevan Hassen Bakir** (MS.c)[2] , **Ahmed Akil** (Ph.D)[3] **and Furat Tahseen Saber**(MS.c)[4]

Abstract

Background: Thalassemia is one of the most common genetic diseases in the world. It is a major health problem, causing much morbidity. The prevalence of hepatitis C virus (HCV) among multi-transfused patients varies from one area to another and depends on the endemicity of viral hepatitis in different regions.

Objective: To investigate the prevalence of hepatitis C virus among patients with thalassemia major and to determine the most prevalent genotype for this virus.

Patients and Methods: This cross sectional- descriptive study was carried on all patients registered for central laboratory in Erbil City-Iraq during the period from September 2015 till September 2016, thus a total of 176 thalassemia patients were included. Hepatitis C virus infection was tested by using serum for HCV RNA detection by real time- polymerase chain reaction.

Results: Out of (176) patients with thalassemia 61 patients had positive result for HCV genotypes by RT-PCR and the most common of genotypes was detected including, (gene-1a, gene-1b, gene-2, gene-3, gene-4, gene 1a,4, gene 1a,2,4, gene-1b,2 and gene 1b,4), the most prevalent gene in our study gene-3 was 20(32.8%) followed by gene-1a 16(26.2%) and then gene 1b,4 and gene-4 (6%, 6%, 9.8 %) respectively. There is difference between male and female patients according to HCV genotype in which the most common genotypes that appear among male patients was gene- 3 13(37.4%), gene-1a 7(20%) and gene 1b, 4 was 5(14.3%) respectively. While among female patients the most common type of gene was gene-1a 9(34.6%) and gene-3 7(26.9%) although there is no significant differences between sex (male and female) according to HCV genotype (P-value = 0.063).

Conclusion: Geographical distribution of various genotypes of HCV is useful for understanding the epidemiological status, detection of mode and source of infection. Preservation of screening and development and update of its laboratory methods seems to be the sole beneficial decision. PCR technique is essential to detect and treat active infection.

Key words: Hepatitis C virus, beta thalassemia, blood transfusions, blood, real time PCR.

Corresponding Author: fattmaabeer@yahoo.com.

Received: 20th March 2017

Accepted: 14th May 2017

[1,3] College of Health Sciences - Hawler Medical University - Erbil - Iraq.
[2] Medical technical institute - Erbil Polytechnic University - Erbil-Iraq.
[4]Ministry of Health Puplic - Health Laboratory of Erbil - Erbil - Iraq.

Introduction

Thalassemia is one of the most common genetic diseases in the world. It is a major health problem, causing much morbidity, early mortality and a great deal of misery for a family both financially and emotionally[1].

Hepatitis C virus infection is a major,

worldwide health problem. It is estimated that over (170- 200) million people are infected and the virus is distributed worldwide with a prevalence varying in different countries from 0.2% up to 40% [2].

The combination of chronic transfusion and iron chelating therapies has dramatically changed life expectancy and quality of life for patients with thalassemia major[3]. Among thalassemic patients transfused before the1990s, the prevalence of HCV infection was shown to be proportional to the number of units of blood received, and approached 80% in the adult patients[4]. Major essentially comprises of regular "safe blood transfusion" and a lifelong iron-chelating therapy. Unfortunately, the patients even those managed at relatively better management centers, are prone to develop both types of complication, i.e., those transmitted through blood transfusion (particularly hepatitis C) as well as sequelae of transfusion siderosis. Hepatitis B has a declining trend, probably as a result of regular pre-transfusion screening for HBs Ag, use of hepatitis B vaccination and improved public awareness about the disease. HIV infection, fortunately, is uncommon in our setup [5].

Hepatitis C virus genome encodes a single poly protein of 3000 amino acids; it is cleaved post translation to yield at least 10 structural and nonstructural proteins[6].

Sequence comparisons of the virus led to the identification of at least six major genotypes [7]. These differ in nucleotide sequence by more than 30% over the complete virus genome. Additionally, there are more than 50 subtypes, which also differ in nucleotides sequence by more than 20% [8]. Genotype 4 is found most commonly in the Middle East [9]. Genotyping and sub-typing for HCV are not only required for therapy initiation and monitoring, but they also assist in vaccine development [10].

Studies have shown that HCV genotypes 1 and 4 are more resistant to treatment with paginated interferon and ribavirin than genotypes 2 and 3 [11]. A study conducted in Jordan among patients on regular hemodialysis showed that the prevalence of HCV infection was correlated with the history of blood transfusion before the introduction of anti-HCV screening in Jordanian blood banks in 1993[12]. HCV genotype 1a was found to be the predominant sub-type among blood donors and Jordanian hemodialysis patients as well as other Middle Eastern countries including Lebanon, Turkey, Cyprus, and Syria [13][14]. HCV genotype 4 is the most prevalent in Saudi Arabia, Egypt, Yemen, and Bahrain [15].

The prevalence of HCV among multi-transfused patients varies from one area to another and depends on the endemicity of viral hepatitis in different regions. The highest reported prevalence was in Egypt, where 75% of patients with homozygous β-thalassemia are infected with HCV, while the prevalence ranges from 33-67.3% in the neighboring countries of Kuwait [16]. Chronic hepatitis C viral infections are a co morbidity associated with regular blood transfusions; up to 80% of multi-transfused thalassemic patients around the globe are infected with transfusion related hepatitis infections are therefore a major concern for those with β-thalassaemia [17]. Because of the importance of hepatitis C infection and its adverse effect on the society, Thalassemia is an important health problem throughout Iraq.

The main objectives were to investigate the prevalence of HCV among patients with β-thalassemia, and to determine the most prevalent genotype.

Materials and Methods

This cross sectional- descriptive study was carried on all patients registered for central Laboratory in Erbil City - Iraq during the period from September 2015 till September 2016, thus a total of 176 thalassemia patients

were recruited hepatitis C virus infection was tested by real time- polymerase chain reaction CRT-PCR (Artus and Amplisens kit, Qiagen Company) was done. their age range from 15 to 78 years with mean age (44.39 ± 15.06 S.D.) years. The clinical diagnosis was obtained from patient records and interview and ethical approval for use of all specimens was obtained.10 ml of blood was obtained by vein puncture using disposable latex gloves and syringes . Sera were separated from whole blood under optimal conditions for RNA extraction. For this purpose, The blood samples were allowed to clot in the room temperature for 20 minutes and then centrifuged at 2,000 rpm for 10 minutes (-4ºC). All samples were divided into three aliquots then immediately frozen and stored at (-20ºC) and (-80ºC), for serological and molecular assays respectively to minimize degradation of viral nucleic acid, prevent cross contamination and unnecessary.

For HCV RNA detection by RT-PCR, all seropositive samples were tested individually for the presence of HCV RNA by qualitative RT-PCR (Sacace Biotechnologies, REF V-1-100R, Italy). To permit the molecular analysis of the large number of seronegative samples, a pooling strategy was developed, similar to the method described by [11]. This involved the pooling of four seronegative serum samples and the analysis of the mixture for the presence of HCV RNA. Twenty-five µl of each of the four samples were mixed together, and then 100µl pool was used for the assay. The RT-PCR procedure based on four major processes: isolation of HCV RNA from specimens using RNA/DNA extraction kit (Ribo-Sorb, Sacace Biotechnologies, REF K-2-1, Italy), reverse transcription of the RNA using reverse primer and M-MLV reverse transcriptase provided with the kit and incubated in thermal cycler at 37oC for 30 minutes, and then the cDNA was amplified by PCR with primers specific for the 5' untranslated region of the viral genome. The amplification were carried as follows: 95ºC for 5 min, then 42 cycles of 95ºC for 30 sec, 67ºC for 30 sec, and 72ºC for 30 sec, followed by a final extension at 72ºC for 1 min. After that detection of the amplified products was done on agarose gel. The kit contains the internal control which may be used in the isolation procedure and serves as an amplification control for each individually processed specimen and to identify possible reaction inhibition. Negative and positive controls were extracted, reverse transcribed, and amplified in each batch of samples tested by PCR. The serum samples proved to contain HCV RNA by RT-PCR were further subjected for HCV genotype/subtype analysis by Re-extraction of viral RNA from HCV RNA-positive clinical samples utilizing RNA/DNA Extraction Kit, Ribo Virus (HCV Real-TM Genotype) (Sacace Biotechnologies, REF K-2/C, Italy), which is designed for the rapid preparation of highly pure viral nucleic acids. The PCR method used for this part of the study utilizing one step reverse transcriptase nested PCR using HCV Genotyping Kit (Genekam Biotechnology, Germany, Ref.MK757).

This ready to use PCR kit contains primers, buffers, PCR master mix, positive control, negative control, molecular marker and loading dye. The procedure was done according to manufacturers' instructions.

Statistical analysis

Finally, we analyzed the data by Statistical Package for Social Sciences SPSS version (23) software and descriptive tests including frequency mean and standard deviation. The research was approved by ethical committee at Hawler Medical University - College of Health Science.

Result

Out of (176) patients with thalassemia HCV detection was done by HCV RNA-PCR to all patients and only (61) samples were showing positive results. Genotypes was detected including: (gene-1a, gene-1b, gene-2, gene-3, gene-4, gene 1a,4, gene 1a,2,4, gene-1b,2 and gene 1b,4). the most prevalent gene in our study gene-3 (20/61,32.8%) followed by gene-1a (16/61, 26.2%) and then gene 1b, 4 and gene-4 (6/61, 9.8%) respectively.

Table (1): The types and total number of detected HCV Genotype in thalasemmia patients.

Type of gene	Number of samples	Percentage%
gene- 1a	16	26.2
gene- 1b	5	8.1
gene-1b,2	1	1.6
gene- 1b,4	6	9.8
gene- 2	4	6.6
gene- 3	20	32.8
gene- 4	6	9.8
gene- 1,2 and 4	1	1.6
gene-1,4	2	3.3
Total	61	100%

Table (2) show that HCV more prevalent among male thalassemia patient 35(57.4) than female thalassemia patient 26 (42.6), difference between type of gene in male and female patients in which the most common genotypes that appear among male patients was gene-3 (13/61, 371%), gene-1a (7/61, 20%) and gene 1b,4 (5/61, 14.3%) respectively. While among female patients the most common type of gene was gene-1a(9/61, 34.6%) and gene-3 (7(/61, 6.9%) although there is a difference in the number of genotypes between male and female patients but there is no significant differences between gender (male and female) according to type of gene (P-value = 0.063) as shown in table (3).

Table (2): The relation between sex and genotype.

Sex	gene-1a	gene-1b	gene-2	gene-3	gene-4	gene-1,4	gene-1,2,4	gene-1b,2	gene1b,4	Total
Male No. (%)	7 (20%)	2 (5.7%)	3 (8.6%)	13 (37.1%)	3 (8.6%)	1 (2.9%)	1 (2.9%)	0	5 (14.3%)	35 (57.4%)
Female No. (%)	9 (34.6%)	3 (11.5%)	1 (3.8%)	7 (26.9%)	3 (11.5%)	1 (3.8%)	0	1 (3.8%)	1 (3.8%)	26 (42.6%)
Total	16 (26.2%)	5 (8.2%)	4 (6.6%)	20 (32.8%)	6 (9.8%)	2 (3.3%)	1 (1.6%)	1 (1.6%)	6 (9.8%)	61 (100%)

In total of (61) patients with thalassemia, showing positive hepatitis C virus detection test (35) of them male and (26) were female. As appear in table (3) the differences between the mean of the male and female of the patient's samples, although there is a difference in the number of between male and female patients but there is no significant differences between gender (male and female) according to number of samples (P-value = 0.063).

Table (3): Show the mean and Std. Deviation between male and female.

Sex		N	%	Mean	Std. Deviation	Std. Error Mean
Gene type	**Male**	35	57.4	5.14	2.510	.424
	Female	26	42.6	3.96	2.323	.456

Discussion

The results of this study showed that in Erbil city of Iraqi Kurdistan 61 out of 176 (34.65%) of thalassemia patients were infected with HCV as shown in table 3. Transfusion transmitted infection have always been a major problem in multi transfused patients in past. This study is the most comprehensive to data on ascertaining related viral infections amongst a group of thalassemia in Erbil City of Iraqi Kurdistan. Studies from other countries where shown that the thalassemia is common health problem and showed varying rates of HCV for example Ninavha City - Iraq [17]. The result of this study lower than result reported by Al-Kubaisy *et al* (2004) who found a prevalnce of 61.5% in large study for Iraqi thalassemic patients in Baghdad [18]. while higher than Muhsin *et al* (2013) found a prevalence of 25% in Bbylon [19]. On the other hand lower than results recorded in Egypt 75% this is a relatively high percentage, keeping in mind that all the donated blood is regularly screened for HCV at all thalassemia centers in Egypt [20]. Lower than some reports from Saudi Arabia infection was 63%, and 40% in Bahrain [21]. Higer than Italy 44% [22] and lower than India 16.7% [23]. However, this figure was lowered upon confirmation of anti HCV by recombinant immunoblotting assay to 19.5%. In different parts of the world the prevalence of HCV infection in thalassemic patients differs in Malaysia 22.4% [24] and Lebanon 14% [25].

There are many reasons for lower prevalence in our country, including awareness about hepatitis C and other blood-borne diseases indicating more advanced blood safety. Thalassemic patients may acquire HCV through the administration of HCV infected blood collected during the donor window period.

There is a great variation of the prevalence rate of HCV in thalassemic patients among different parts of the world, although difference in laboratory technique can play a role in this variability also.

The types of HCV genotypes was detected including: (gene-1a, gene-1b, gene-2, gene-3, gene-4, gene 1a, 4, gene 1a, 2, 4, gene-1b,2 and gene 1b,4),the most prevalent gene in our study gene-3 20(32.8) followed by gene-1a 16(26.2) and then gene 1b,4 and gene-4 6(9.8) respectively. While HCV genotypic distribution among Al-Yarmouk Teaching Hospital, Al-Kadhimiya Teaching Hospital, and Al-Karama hospital in Baghdad recorded that the predominant HCV genotype was 4, detected in 12/29 (41.38%), whilst genotypes 1a and 1b were found in 10/29 (34.48%) and 4/29 (13.79%), respectively. One of 29 (3.45%) had genotype 3a and two of 29 (6.90%) had mixed infection with 1b & 4 [26]. According to Ludwig *et al*., (1994) staging system HCV RNA was performed by RT-PCR to detect 65 anti-HCV-positive patients [27]. HCV RNA was detected in 33 patients (51%); 18 (55%) had genotype 1,

three (9%) had genotype 2, three (9%) had genotype 3 and nine (27%) had genotype4 .

Although there is a difference in our study between male and female regarding HCV. HCV more prevalent among male thalassemia patient 35(57.4) than female thalassemia patient 26(57.4) and there were differences in number of HCV genotypes between male and female patients but there is no significant differences between male and female (P <0.05), in which the most common genotypes that appear among male patients was gene-3 13(37.1), gene-1a 7(20) and gene 1b,4 5(14.3) respectively. While among female patients the most common type of gene was gene-1a 9(34.6) and gene-3 7(26.9). Shakeri *et al*., [28] from Iran mention that the association between the HCV antibody positivity and sex was not statistically significant (P <0.05). Overall, the prevalence of antibodies against HCV in women and men was 0.14% and 0.29% respectively.

Although there is a difference in our study between male and female regarding HCV. HCV more prevalent among male thalassemia patient 35(57.4) than female thalassemia patient 26(57.4) this was in agreement with studies recovered by Raham in Diayla [29]. Whereas females have a higher prevalence in Iran [30]. Males and females are similarly affected according to Ansar and Kooloobandi study in north Iran [30].

There is no statistically significant relation between sex and thalassemic patients with HCV dissimilarity was statistically not significant when compared to negative patients because male gender was already more recruited in the study.

The reason for this wide range between our study and others can be due to differences in the total prevalence of selected population. this is a relatively high percentage keeping in mind that all the donated blood is regularly screened for HCV at all thalassemic Centers in Iraq.

In conclusion, geographical distribution of various genotypes of HCV is useful for understanding the epidemiological status, detection of mode and source of infection. Preservation of screening and development and update of its laboratory methods seems to be the sole beneficial decision. PCR technique is essential to detect and treat active infection.

References

[1] El-Beshlawy A, Ilham Y. Prevention of Hemoglobinopathies in Egypt. Hemoglobin. 2009; 33 Suppl 1:S14-20.

[2] Alavian SM. Hepatitis C infection inIran; A review article. Iran J Clin Infect Dis 2009; 4:47-59.

[3] Angelucci E. Antibodies to hepatitis C virus in thalassemia. Haematologica. 1994; 79:353-5.

[4] Rehman M, Akhtar GN, Qadeer M, Shams T, Usman A, Lodhi Y. Safe blood begins with safe donors. Pak J Med Sci. 2003; 19(3):161-168.

[5] Lauer GM, Walker BD. Hepatitis C virus infection. N Engl J Med 2001;345:41-51.

[6] Simmonds P, Holmes EC, Cha TA, Chan SW, McOmish F, Irvine B, *et al*. Classification of hepatitis C virus into six major genotypes and a series of subtypes by phylogenetic analysis of the NS-5 region. J Gen Virol 1993; 74: 2391-9.

[7] Simmonds P, Alberti A, Alter HJ, Bonino F, Bradley DW, Brechot CE *et al*. A proposed system for the nomenclature of hepatitis C viral geno types. Hepatol 1994;19:1321-4.

[8] Kolberg J. Evaluation of a novel serotyping system for hepatitis C virus: strong correlation with standard genotyping methodologies. J Clin Microbiol 1995; 33: 2978-83.

[9] Maertens G, Stuyver L. Typing of hepatitis C virus isolates. In: Rizzetto M, Purcell RH, Gerin JL, Verme G, editors. Viral Hepatitis and Liver Disease. Turin: Minerva Medica, 1997:181-6.
[10] Mahaney K, Tedeschi V, Maertens G, Bisceglie A, Vergalla J, Hoofnagle J, *et al.* Genotypic analysis of hepatitis C virus in American patients. Hepatology1994;20:1405-14.
[11] Salwa B. Hepatitis C virus infection in Jordanian haemodialysis units: serological diagnosis and genotyping. J Med Microbiol 2002; 51:700-4.
[12] Dusheiko G, Schmilovitz H, Brown D. Hepatitis C virus genotypes: an investigation of type-specific differences in geographic origin and disease. Hepatol 1994;19:13-8.
[13] Abdulkarim AS, Zein NN, Germer JJ. Hepatitis C virus genotypes and hepatitis G virus in hemodialysis patients from Syria: identification of two novel hepatitis C virus subtypes. Am J Trop Med Hyg 1998;59:571-6.
[14] Davidson F, Simmonds P, Ferguson JC. Survey of majorgeno types and subtypes of hepatitis C virus using RFL Pof sequences amplified from the 59 non-coding region.J Gen Virol 1995; 76:1197-204.
[15] Al-Fuzae L, Abool backer KC, Al-Saleh Q. Beta-Thalassaemia major in Kuwait. J Trop Pediatr 1998; 44:311–12.
[16] Angelucci E. Antibodies to hepatitis C virus in thalassemia.Haematologica 1994; 79:353–5.
[17] Khaled MD. Prevalence of Hepatitis B, Hepatitis C and human immunodeficiency virus infection among Thalassemia patients in Ninavha Governorate/ Iraq. J. Of Biotechnology Research Center (Special edition). 2014; 8(2).
[18] Al-Kubaisy WA, Al-Naib KT, Habib M. Seroprevalence of hepatitis C virus specific antibodies among Iraqi children with thalassemia. Eeastern Mediterranean Health J. 2006; 12(1/2):2004-10.
[19] Muhsin MA, Abdul-Husin IF. Seroprevalence of hepatitis B and C among thalassemic, hemophilic patients in Babylon governorate-Iraq. MJB 2013; 10(2): 445-54.
[20] Nanawy AA, El- Azzoun OF, Soliman AT. Prevalence of hepatitis C antibodies sero positivity in healthy Egyptian Children and four high risk groups. J Trop pediato. 2005; 41: 341-3.
[21] Alavian SM, Tabatabaei SV, Lankarani KB. Epidemiology 0f HCV infection among Thalassemia patients in eastern Mediterranean countries: a quantitative review of literature. IR CMJ 2010; 12:365-76.
[22] Rebulla P, Mozzi F, Contino G, Locatelli G, Sirchia G. Antibody to hepatits C virus in 1305 Italian multiply transfused thalassemics: Comparison of first and third generation tests. Transfuse Med. 2008;2:69-70.
[23] Agarwal MB, Malkan GH, Bhave AA. Anti-body to hepatitis-C virus in multi-transfused thalassemics- India experience. J Assoc phys India. 2009; 41:195-197.
[24] Jamal R, Fadzillah G, Zulkifli SZ, Yasmin M. Seroprevalence of hepatitis B, hepatitis C, CMV and HIV in multiply transfused thalassaemia patients: results from a thalassaemia day care center in Malaysia. Eur J Clin Microbiol Infect Dis. 1999; 18: 709-715.
[25] Ramia S, Koussa S, Taher A, *et al.* Hepatitis- C virus genotypes and hepatitis-G-virus infection in Lebanese thalassemics. Ann Trop Med Parasitol 2002; 96(2):197-202.
[26] Abdullah AM, Hardan AR, Latif II. Genotyping of hepatitis C Virus isolates from Iraqi hemodialysis patients by Reverse Transcription-PCR and One Step Nested RT-PCR. Diyala Journal of Medicine. 2012; 3(1).

[27] Ludwig J, Batts KP, Moyer TP, Poterucha JJ. Advances in liver biopsy diagnosis. Mayo Clin Proc. 1994; 6(9):677–8.
[28] Shakeri MT, Nomani H, Ghayour Mobarhan M, Sima HR, Gerayli S, Shahbazi S, *et al.* The Prevalence of Hepatitis C Virus in Mashhad, Iran: A Population-Based Study. Hepat Mon. 2013;13(3).
[29] Raham TF, Abdul Wahed SS, Al Haddad HN. Prevalence of hepatitis C among patients with B- thalassaemia in Diyala-Iraq.Al-Taqani. 2011; 24(4): 113-120.
[30] Tamaddoni A, Mohammadzadeh I, Ziaei I. Seroprevalence of HCV Antibody among Patients with β-Thalassemia Major in Amirkola Thalassemia Center, Iran. Iran J Allergy Asthma Immunol. 2007; 6(1): 41.
[31] Ansar MM, Kooloobandi A. Prevalence of hepatitis C virus infection in thalassemia and haemodialysis patients in north Iran-Rasht. J Viral Hepat. 2002; 9(5):390-2.