Original article

Hepatitis G virus infection and genotypes in Iraqi thalassemia patients

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ABSTRACT

Background: HGV infects patients at risk for parenteral exposure and chronic blood transfusion, such as those with β- thalassemia major.

Objectives: This study was designed to investigate the prevalence of HGV infection in thalassemia patient and furthermore to sequence and analyze phylogentic of HGV.

Materials and methods: A prospective study conducted between Feb. to May, 2014. A total of 154 Beta thalassemia patients (87 male; 67 female), from Alkarama teaching hospital and Ibn AL-baladi hospital maternity &children's hospital; aged 18-50 years , who received regular blood transfusions were included in the study. All patients were screened for Antibody against E2 glycoprotein of HGV (Anti-E2 Ab) by using ELISA to detect the presence of HGV infection. While Detection of HGV genomes was done by RT-PCR.

Results: One hundred fifty four thalassemia patients (87 male, 67 female) of 18-50 years were involved in this study. Using the ELISA method, Anti-HGV was detected in 16 patients out of 154 (10.4%) with peak prevalence age group was between 20-24 years. Reverse transcription - polymerase chain reaction (RT-PCR) showed HGV-cDNA was detected in 28 (18.2%) only. The peak age prevalence of HGV infection was under 20 years; however, there were no significant differences in prevalence among two sexes or number of blood transfusion. The results of genotyping in 12 randomly selected patients showed presence of genotype 2 and genotype 5 with percentage of 91.7% and 8.3% respectively.

Conclusion: the prevalence rate of HGV RNA in β-thalassemia major patients is 18.2%, while the prevalence rate of anti-HGV (past infection) is 10.4%. No one of thalassemia patients had HGV RNA and anti-HGV simultaneously. The Gene sequence analysis of PCR products identified HGV genotypes 2 and 5 with percentage of 91.7% and 8.3% respectively

Key words: hepatitis G virus (HGV), prevalence, genotype, thalassemia

Introduction

Regular blood transfusions for patients of thalassemia have improved the overall survival although these transfusions carry a definite risk of transmission of certain viruses. Due to repeated blood transfusions hepatitis B, C, G, and HIV infection can occur. All these are transmitted by blood or blood products in 10% cases [1]. Hepatitis G virus (HGV, or GB virus C) is an enveloped positive-sense, single-stranded virus [2] GB agent hepatitis originally was described by Dienhardt and colleagues, who inoculated tamarins (a type of New World monkey (Saguinuslabiatus) with the serum of a 34-year-old surgeon G. Barker (GB) who fell ill with acute hepatitis of moderate enzymatic activity and three-week icteric period [3].In 1995, a group of investigators from Abbott Laboratories identified two viruses in the serum and liver of tamarins. These viruses were called GB virus A and B (GBV-A and GBV-B) due to the pedigree of the infectious serum .GBV-A and GBV-B belonging to closely-related viruses of the Flaviviridae family. Later on, A PCR product from a West African specimen discovered a novel virus-like nucleotide sequence that was named GBV-C, which is most likely derived from a human virus [4]. Only HCV and HGV, assigned within genus Hepacivirus (type species Hepatitis C virus). The GBV-C genome is similar to hepatitis C virus (HCV) RNA in its organization, i.e. the structural genes are situated at the genomic 5' region and non-structural genes are at the 3' end [5].The 5’ terminal of the HGV, and HCV virus genomes represent untranslated regions (UTRs), while the 3’ ends of GB viruses and HCV represent the U’I’Rs. GB viruses and HCV are similar in size and structure. Each possesses single, large, open reading frames encoding putative poly-proteins of about 3000 amino acid residues. Infection with HGV is common in the world. Frequencies of GBV-C/HGV infection are hard to determine, but prevalence studies suggest that 1–4 % of healthy blood donors in most developed countries are viraemic at the time of blood donation [6].Based on analysis of the 5’ noncoding region (NCR) or E2 sequence, there are 5 major genotypes [7], and a recently identified sixth genotype [8], all distributed distinctly in different geographical regions. The mechanism responsible for the development of GBV-C-induced hepatitis is not clear so far. In spite of the described cases of acute and chronic hepatitis G, its hepatotropicity remains controversial [9].Viral hepatotropicity is supported by the detection of GBV-C RNA in hepatocytes and by the development of acute and fulminant hepatitis following the transfusion of infected blood and its products [10]. The outcome of acute hepatitis may be: (1) recovery with the disappearance of serum

GBV-C RNA and the emergence of anti-E2; (2) development of chronic hepatitis (CH) with serum GBV-C RNA being persistently detectable; (3) presence of GBV-C RNA without biochemical or histological signs of liver disease [11] Following clearance of GBV-C/HGV viraemia, most individuals develop conformation-dependent antibodies to the envelope glycoprotein E2, and thus E2antibody serves as a marker of past infection[11,12].

Patients, materials and methods

A prospective study including 154 Beta thalassemia patients (87 male; 67 female), from Alkarama teaching hospital and Ibn AL-baladi hospital maternity &children's hospital; their aged between 18-50 years , all patients received regular blood transfusions, the study had been conducted between February to May, 2014.

HGV serological analysis: All patients were screened for Antibody against E2 glycoprotein of HGV (Anti-E2 Ab) by using ELISA to detect the presence of HGV infection.

Detection of HGV genomes: Steps of RT-PCR for the detection of HGV infections

1. Extraction of viral RNA from specimens.
2. Estimation of RNA concentration
3. Reverse transcription of the RNA (cDNA synthesis)
4. Amplification of the cDNA: and this includes: Primers preparation:

Viral RNA in plasma sample was detected by RT-PCR using oligonucleotide primers deduced from highly conserved 5'UTR region of GBV-C genome/HGV. These primers are shown in table (1):

Table (1): Primers for PCR amplification of HGV

Primer	Primer sequence	Size of product (bp)
Forward - G1	**5′CGGCCAAAAGGTGGTGGATG -3′**	**185**
Reverse –G2	**5′- CGAGGAGCCTGAGGTGGGG- 3′**	

B. PCR Amplification Mixture and Condition.

Each 25 µl PCR mix composed of the ingredients listed in table (2). DNA amplification was carried in a thermal cycler according to caudai *et al.*, (1998).A negative control without template DNA was used in each reaction to reveal the hazardous cross contamination of the PCR components

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Table(2): Master Mix components of HGV gene PCR

Component	Final Concentration	Amount
GoTaq Master Mix 2X (5X GoTaq flexi reaction buffer, Mgcl2, PCR nucleotide mix, and GoTaq Flexi DNA polymerase.	1X	12.5 µl
Primer Forward G1	40 Pmol	1µl
Primer Reverse G2	40 Pmol	1µl
cDNA	<250ng	5 µl
Nuclease free water	-	5.5µl
Total volume	-	25 µl

C. PCR program:

To detect the 5'UTR HGVgene amplification, cDNA amplification was carried in a thermal cycler, following the cycles listed in table (3).

Table (3): The PCR program for the 5'UTR HGV gene amplification

No.	Step	Temperature(C°)	Time	No.Of Cycles
1	Denaturation	94	1 min.	40
2	Annealing	55	45 sec.	
3	Extension	72	1 min.	
4	Final Extension	72	4 min.	1

The PCR products (10µg) with 100bp Ladder (promega) were subjected to electrophoresis on 2% agarose gel stained with ethidium bromide electrophoresis Then the gel was visualized on UV transilluminator at 320 nm. Gel was was performed at 80 V for 1 hr.30 min. photographed by Polaroid system (figure 1).

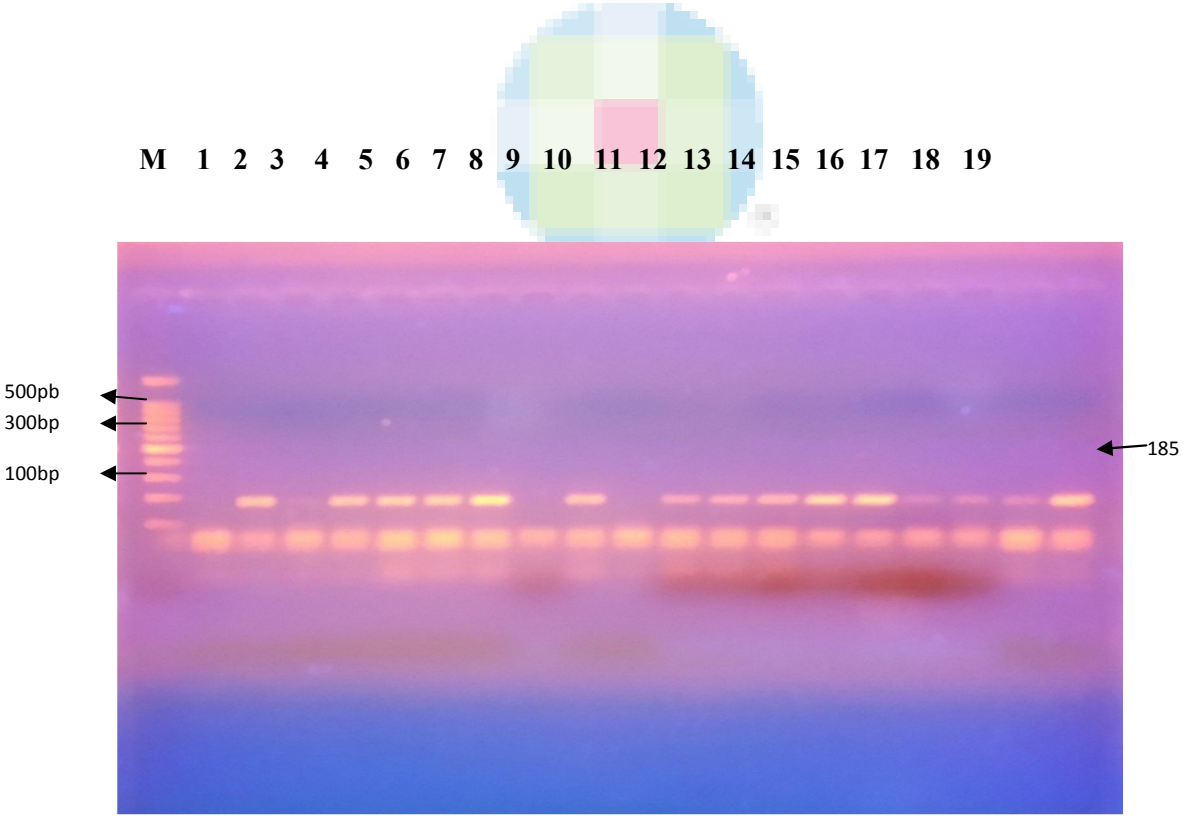


Figure 1. Agarose gel electrophoresis of 5'UTRPCR products of HGV-cDNA (M: 100 bp molecular weight marker, 2 % agarose, TAE buffer (1X), 80V, 1 hr.30 min; lane 1: Negative control, lane 2,4-7,9,11-19: amplicon of 185 bpPosetive HGV-cDNA , lane 3,8,10 Negative HGV-cDNA.

Gene sequencing:

Sequencing of PCR product was carried out by NICEM Company (South Korea) in reverse direction, and primer (G1&G2) was used in each sequencing reactions. Homology search was conducted between the sequence of standard gene BLAST program which is available at the National Center Biotechnology Information (NCBI) online at (http://www.ncbi.nlm.nih.gov)

and using BioEdit program. Evolutionary analysis was conducted in MEGA 5 [15]

Statistical analysis: Analysis of data was carried out using the available statistical package of SPSS-22 (Statistical Packages for Social Sciences- version 22). Data were presented in simple measures of frequency and percentage .The significance of difference of different percentages (qualitative data) was tested using Pearson Chi-square test.

Results

ELISA was used for identification of hepatitis G virus antibody (IgG). Anti-HGV was detected in 16 patients (10.4%).Table (4) shows that the peak prevalence age group of thalassemia patients with anti-HGV was between 20-24 years, so There were a significant ($p<0.05$) decrease in prevalence of anti-HGV with increasing age, in addition male to female percentage was 37.5% to 62.5% respectively, however there were no significant differences in prevalence of anti-HGV in frequency of blood transfusion.

Table4: Prevalence of anti- HGV in thalassemia patients according to age, gender and frequency of blood transfusion

Parameter		Anti-HGV				P value
		Positive		Negative		
		No	%	No	%	
Age (years)	<20	1	6.3	56	40.6	0.041*
	20---24	10	62.5	45	32.6	
	25---29	3	18.8	23	16.7	
	=>30years	2	12.5	14	10.1	
Gender	Male	6	37.5	81	58.7	0.105
	Female	10	62.5	57	41.3	
No. of Blood transfusions (pints)	<30	1	6.3	17	12.3	0.120
	30---	3	18.8	58	42.0	
	60---	5	31.3	34	24.6	
	=>90	7	43.8	29	21.0	

*Significant difference between proportions using Pearson Chi-square test at 0.05 level.

All samples were subjected to PCR testing for detection of HGV nucleic acid**.** HGV-cDNA was detected in 28of 154 (18.2%) of patients with thalassemia. Statistical analysis of the data which was shown in the table (5) reveals that there were no significant differences in prevalence of HGV RNA among different age groups, gender and frequency of blood transfusion. The peak age prevalence of HGV infection was under 20 years; however, there were no significant differences in prevalence among two sexes.Nevertheless, no HGV one had RNA and anti-HGV simultaneously (table 6).

Table 5: Prevalence of HGV RNA in thalassemia patients according to age, gender and frequency of blood transfusion.

Parameter		HGV RNA				P value
		Positive		Negative		
		No	%	No	%	
Age (years)	<20	11	39.3	46	36.5	0.848
	20---24	9	32.1	46	36.5	
	25---29	4	14.3	22	17.5	
	=>30years	4	14.3	12	9.5	
Gender	Male	17	60.7	70	55.6	0.618
	Female	11	39.3	56	44.4	
No. of Blood transfusions(points)	<30	-	-	18	14.3	0.190
	30---	12	42.9	49	38.9	
	60---	9	32.1	30	23.8	
	=>90	7	25.0	29	23.0	

*Significant difference between proportions using Pearson Chi-square test at 0.05 level.

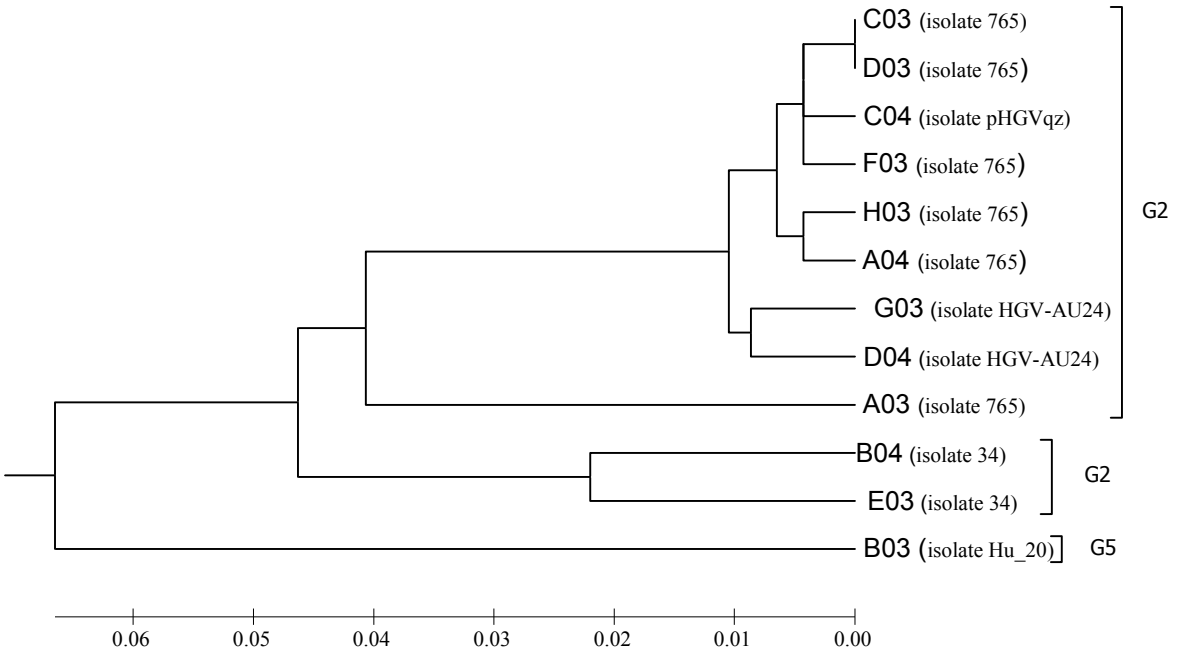
Table 6: Prevalence of HGV RNA in thalassemia patients with or without anti- HGV.

Parameter		Anti-HGV				P value
		Positive		Negative		
		No	%	No	%	
HGV RNA	Positive	-	-	28	20.3	-
	Negative	16	100.0	110	79.7	

*Significant difference between proportions using Pearson Chi-square test at 0.05 level.

Molecular typing and phylogenetic analysis of Hepatitis G Virus (HGV):

The amplified products from 12 / 28 HGV positive were subjected to sequencing procedure for the tree for each sample was obtained as seen in figure (2). region (5'UTR). Sequencing of PCR product was carried out by NICEM Company/ (South Korea) using an ABI 3730XL DNA Analyzer in reverse direction by primer G1&G2. Nucleotide sequence and phylogenetic analysis of 12/28 (%) HGV positive isolates belong to G2 (91.7%), G5(8.3%).This allowed us to construct a phylogenetic tree based on a partial polymerase 185bp fragment of 5'UTR in ORF1.Phylogenetic



G2 = **genotype 2** , G5 = **genotype 5**

Figure 2: Phylogenetic tree on the basis of the HG virus partial open reading frame 1 (ORF1) sequence as constructed by the neighbor-joining (N-J) method. Note that there are two major clusters, tentatively named G2, G5.

Genotyping results for the amplified region of each sample are shown in table (6). Nucleotide sequence analysis showed that the HGV genotypes in Iraqi thalassemia patients were closely related to the novel that isolated from the United States , Europe, Egypt, Thailand, , India, Turkey, Nepal, Saudi Arabia, UAE, Pakistan, Myanmar (genotype 2) and south Africa (genotype 5) that deposited in the Gene Bank with accession number as shows in table(6)

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Table 6: The characteristics of genotyped HGV-positive clinical samples

	Name of gene	Accession N.	Homology	Genotype	Organism
1	Hepatitis G virus isolate 765	AY196904.1 GI:28543032	92%	2	GB virus C
2	GB virus C isolate Hu_20	KC618400.1 GI:558518560	96%	5	Uganda
3	Hepatitis G virus isolate 765	AY196904.1 GI:28543032	99%	2	GB virus C
4	Hepatitis G virus isolate 765	AY196904.1 GI:28543032	99%	2	GB virus C
5	Hepatitis G virus isolate 765	AY196904.1 GI:28543032	99%	2	GB virus C
6	Hepatitis G virus isolate HGV-AU24	AF177614.1 GI:9836767	98%	2	GB virus C
7	Hepatitis G virus isolate 765	AY196904.1 GI:28543032	99%	2	GB virus C
8	Hepatitis G virus isolate 765	AY196904.1 GI:28543032	98%	2	GB virus C
9	Hepatitis G virus isolate 34	AF489995.1 GI:20271200	94%	2	GB virus C
10	Hepatitis G virus isolate 34	AF489995.1 GI:20271200	94%	2	GB virus C
11	Hepatitis G virus strain HGV-Iw isolate pHGVqz	AF081782.1 GI:4093140	100%	2	GB virus C
12	Hepatitis G virus isolate HGV-AU24	AF177614.1 GI:9836767	99%	2	GB virus C

Discussion

Viral hepatitis infections, including HGV and HCV and their related hepatic and extra hepatic clinical manifestations are at the higher risk of interfering in the pathophysiology of thalassemia [16].Diagnosis of HGV depends on two methods; acute infection can be diagnosed by detection of HGV RNA by PCR, while past infection can be diagnosed by detection of antibody to E2 protein of the virus by ELISA. However, anti E2 antibodies to HGV and HGV RNA are almost mutually exclusive [17]. It had been reported that 60%-70% patients develop antibodies after infection [18]. As such, the detection of HGV RNA and anti-E2 is necessary to accurately define the prevalence of HGV infection in a population [19].

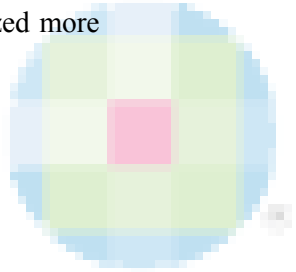
In our study the prevalence of anti-HGV was 10.4% in thalassemia patients. In Iraq Al-karkhy in his study demonstrate that 14.63 % of healthy volunteer blood donors have positive HGV-IgG [20], while the prevalence of HGV/GBV-C was reported in Iraq by Al-Obeidy et al. who revealed that HGV-IgG were detected in 25% of healthy blood donors [21]. The ongoing HGV infection can be diagnosed by demonstration of viremia in patient blood by reverse transcriptase-PCR [22]. The present study shows that 68.8 (62.5+6.3) of anti-HGV was found below age 24 years, so the prevalence was more in young age. In our result HGV RNA was detected by RT-PCR in 18.2% of thalassemia patients. In earlier studies, the significantly higher prevalence of HGV viremia was found in blood donors versus patients with multi-transfused anemia (1.7 vs. 18%) in Taiwanese children (23). However, HGV RNA was also detected in 12.9% of Iranian patients with thalassemia [24], which is similar to Italian patients (11%) with thalassemia [25].Moreover, a higher prevalence (19%) of serum HGV-RNA positive has been reported in Greek thalassemic patients [26]. Also Moatter et al. was detected the HGV RNA in 21% of thalassemia patients examined in Pakistan [27]. This may be explained by a greater number of transfused blood units per year in those thalassemia patients. Although a positive correlation between the prevalence of HGV infection and the history and/or numbers of blood transfusion have been reported in some studies from Japan [28] and [29] from France. Other reports indicated that infectiousness of HGV through blood products was low [22] or the clearance of the virus was rapid. Various factors have been present in patients who developed HGV infection with renal failure and those who were on dialysis despite they had multiple blood transfusions [30] or they had a history of surgeries and multiple injections.

In our study, we found that the HGV RNA was prevalent among individuals who were younger (39.3% in <20 year). In contrary to our findings, Shev et al. found no age relation in GBV-C/HGV positive patients with chronic hepatitis [31]. However, in many studies, HGV infection appeared to be acquired at younger ages than other known types of viral hepatitis, [32] which is similar to our finding. By contrast, the mean ages of anti-HGV were from 20-24 years. These findings might be explained by the fact that clearance of HGV may only occur after a long period of viremia [33] Hence, patients who were exposed to HGV may not yet have completely recovered. This theory is supported by the findings of Dille et al. who found that in patients with a post-transfusion hepatitis, the development of E2-specific HGV antibodies was associated with the loss of HGV viremia [34]. Gene sequencing, based on the nucleotide sequence of residue 134-395 of

the 5'-UTR region of HGV samples were studied and the results showed that genotype 2 was the most prevalent genotype and genotype 5 was only detected in one sample out of twelve. Reports from United States and Europe [35] and from other Middle Eastern countries (Iran, United Arab Emirates, Turkey, and Saudi Arabia) [36-39] showed that genotype 2 was the most prevalent genotype in these countries. In one case, genotype 5 was detected in thalassemia patient; this may reflect the African origin of this patient or donated blood. Finally, diagnosis of significant higher serological and molecular prevalence of HGV in patients with thalassemia in Iraq emphasized more on the importance of these viral hepatitis infections in introducing and complicating the clinical outcome of thalassemia patients.

Conclusion

In Iraq, the prevalence rate of HGV RNA in β-thalassemia major patients is 18.2%, while the prevalence rate of anti-HGV (past infection) is 10.4%. No one of thalassemia patients had HGV RNA and anti-HGV simultaneously. The Gene sequence analysis of PCR products identified HGV genotypes 2 and 5 with percentage of 91.7% and 8.3% respectively



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