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Frequency of Hepatitis C Virus Genotypes /Subtypes Association with Response to Therapy in a Sample of HCV Infected Iraqi Patients

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Abstract

Background Hepatitis C virus (HCV) is an important human pathogen affecting 120-170 million individuals in the world. Identification of the causative virus genotype is of a significance to both clinical practices and predict the likelihood to therapy response.

Objective To determine the distribution of HCV genotypes/subtypes and its association with response to therapy among newly diagnosed HCV patients.

Methods Fifty patients with confirmed anti-HCV antibodies were included in this study for HCV genotyping in association with response to therapy. Blood samples from patients were subjected to RNA extraction and reverse transcription step; viral load of HCV was measured by polymerase chain reaction (PCR) at time zero, 3 months and 6 months of dual therapy. Response to therapy was measured as a decrease in viral load (2 log or more) and was described as: good (median log is zero after 6 months of therapy), moderate (median log declines more than 2 log but not zero after 6 months of therapy), poor (median log does not decline or decline less than 2 log after 6 months of therapy).

Results Two genotypes of HCV were detected, genotype 4 was the predominant (27/50, 54%) followed by genotype 1 (23/50, 46%). For HCV subtypes, subtype 1a was of highest percentage (28%) followed by 4e (24%), 1b (18%), 4a (14%), 4b (12%), and 4e (4%).The results revealed a significant association between HCV subtypes, but not genotypes, with response to therapy. HCV subtype 1a followed by 4a showed the highest rate of response 85.7% and 71.4%, respectively, while interestingly HCV subtype 4d showed no response and 1b showed poor response 11.11%.

Conclusion HCV subtypes of great importance in predicting success to HCV therapy and it is believed this would affect the newly emerging directly acting drugs as well.

Keywords HCV, genotypes, response to therapy

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List of abbreviations: c DNA = Complementary DNA, CHC = Chronic hepatitis C, ELISA = Enzyme linked immunosorbant assay, HCC = Hepatocellular carcinoma, HCV = Hepatitis C virus, HIV = Human immunodeficiency virus, IC = Internal control, NR = Non-responder, R = Responder, SVR = Sustained virological response

Introduction

Hepatitis C virus (HCV) is an important public health problem worldwide that causes acute and chronic liver diseases like cancer. Approximately 80% of subjects with acute hepatitis C progress into a chronic disease [(1)]. Chronic hepatitis C virus (CHC) infection is an important cause for developing (10-20%) cirrhosis and hepatocellular carcinoma (HCC) that often results in liver failure and thus liver transplantation [(2)]. HCV is divided into six major genotypes and more than 80 subtypes [(3,4)].Identification of the causative virus genotype is of significance to

both clinical practices and epidemiological studies. Regarding the clinical practices, once the genotype is identified, the result of the treatment and determination of its duration are facilitated as the genotype is the strongest predictor of the sustained viral response (SVR), which is defined as undetectable HCV RNA after six months from completion of treatment [5]. Recent data strongly indicate that HCV genotype is the key determinant of response to interferon-alpha (IFN-α) based treatment regimens[6,7].Genotype should be determined in all HCV-infected persons prior to treatment in order to predict the likelihood of treatment response [8,9]. Patients with genotypes 1 and 4 generally exhibit a poorer response to IFN-based therapy than those with genotypes 2 and 3. HCV genotype 5 appears to be an easily treatable virus, with response rates compatible with those of genotypes 2 and 3 therapy [7,10].Treatment response in genotype 6 HCV patients may be at an intermediate level between that observed in genotype 1 and genotypes 2/3. The optimal duration of treatment for HCV genotype 6 is unclear and currently under investigation [7,10].

This study relates HCV genotypes/subtypes with response to therapy. This will provide basis for better predictability of treatment success in HCV patients in this area.

Methods

Patients and sampling:

This study was carried out from March 2015 to November 2015 on diagnosed HCV infected patients referred to Gastroenterology and Hepatology Center at Baghdad governorate; however, diagnosed cases of HCV infection included mostly chronic HCV infection. For each patient, 5 ml of blood were collected at three occasions, before treatment, after three months, and after six months of treatment.

First of all, potential patients were screened by enzyme linked immunosorbant assay (ELISA) 3rdgeneration assay. Plasma samples from only those showed seropositive anti-HCV antibodies were included in this study. Accordingly, 50patients were included. The recruited cases were subjected for measuring HCV RNA load at time zero, 3 months and 6 months of therapy. In addition, cDNA of HCV RNA was subjected for genotyping. Ethical approval for interview, collecting information from records and taking specimens from patients was obtained.

Detection of serum anti-HCV antibodies

The initial screening for anti-HCV antibodies was carried out by a third generation ELISA. The procedure and result interpretations were done according to the manufacturer instructions HCV ELISA test kit 3rd generation (plasmatic, UK). The absorbance of the solution was read at 450 nm by the ELISA reader. The mean absorbance of the negative control (NCx) was calculated. The cut-off value = 0.120+NCx; specimens with absorbance values less than the cut-off value were considered to be negative, specimens with absorbance values greater than the cut-off value were considered to be positive.

Viral RNA extraction for viral load and viral genotyping

QIAamp viral RNA Minikit (Cat.No 52906. Qiagen, Germany) was used for HCV-RNA extraction from plasma samples according to the manufacturer guidelines. Briefly, the sample was first lyzed under denaturating condition to inactivate RNase and for isolation of intact viral RNA. Buffering solutions were used to provide optimum binding of RNA to the QIA amp membrane. Sample is loaded onto the QIA amp Mini spin column. The RNA binds to the membrane and contaminants are efficiently washed in two steps using two different wash buffers. High- quality RNA is eluted in a special RNase- free buffer ready for use.

Viral load detection

Twenty µl of extracted HCV-RNA were used for Real-time qPCR amplification step using HCV virus-RGRT-PCR (Ref No.4518233. Qiagen, Germany). The protocol of technique was according to the manufacturer guidelines. Briefly, the Hepatitis C virus RGRT-PCR kit consists of Master A and B containing reagents,

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enzymes for the reverse transcription and specific amplification of a 240 bp region of the HCV genome and for the direct detection of the specific amplicon in fluorescence channel cycling Green of the Rotor- Q-Gene. In addition, the protocol pursued included a second amplification target to identify possible PCR inhibition, namely internal control (IC) in fluorescence channel cycling orange of the Rotor-Q-Gene. External positive controls were allowing the determination of the amount of viral RNA.

Rotor-Q -gene thermo cycler (Qiagen, Germany) was used and run by the following program shown in table 1.

Table 1. Thermo cycling program of real-time qPCR for measurement of HCV RNA load

Temperature(°C)	Time	Number of cycle
50	30 min	1
95	15 min	2
95	30 sec	
50	60 sec	50
72	30 sec	

The standard curve was constructed by adding 20µl of serial dilutions of HCV standard RNA from 10 IU/µL to10000 IU/µL. Standard curve was also used for calculating PCR run efficiency which was above 96%. Internal control was added through extraction steps, PCR water grade used as negative control. After the run is finished, the data was analyzed via signal fluorescence. The HCV load in patients; blood was calculated as follows:

$$\text{HCV RNA (IU/ml)} = \frac{Result\ (IU/\mu l) \times Elution\ volume\ (\mu l)}{Sample\ volume\ (ml)}$$

Reverse transcription and HCV cDNA genotyping

The protocols used were according to the manufacturer guidelines. The amplification step was achieved by using HCV Real time-PCR 2.0 Kit AC 032/24 (NLM, Italy). Up to 10 µl of amplified PCR products were used to hybridize with universal specific probe immobilized on nitrocellulose strip through many steps using Gene GEN-C 2.0 AC004/24 (NLM, Italy).The reverse hybridization step is based on reverse-hybridization principle. Briefly, biotinylated amplicons generated by RT-PCR of the 5-UTR and Core regions of HCV RNA, were hybridized to specific probes that are bound to nitrocellulose strip, biotinylated hybrids were then detected using streptavidin bound to alkaline phosphatase; amplicons not complement were washed out. Then substrate reacted with the streptavidin-alkaline phosphatase complex forming a purple precipitate and coloring banding pattern on the strip. Genotype specific bands were developed on the strip and the resultant profile was analyzed using interpretation table came with the kit.

Statistical analysis

Data of this study were analyzed using SPSS software version 23. Descriptive statistics were done in terms of frequencies and percentages. Values of viral load of HCV were shown to be nonparametric; therefore, median ± confidence interval rather than mean was used. Qualitative assessment of response to therapy was grouped into responders (R) and non-responders (NR) patients based on two log reduction cutoff of viral load. Mann Whitney test was used for measuring P values for medians. P values less than 0.05 were considered significant.

Results

Population of the study

For age of patients, it was shown that age groups 41-50 and then31-40 years showed the highest percentages of HCV infection as 78% of hepatitis patients were of age older than 31 and younger than 50 years old, hepatitis C virus patients were shown to be of equal sex ratio (1:1).The control group was confirmed to be sex and age matched, moreover hepatitis patients were shown to be mainly non-smokers (smokers rate 18%) and non-alcoholics (alcoholic rate 8%) with no predilection for being diabetic (12%), thalassemic (6%), or had history of blood transfusion (14%).

Nineteen out of 50 HCV patients were presented with liver fibrosis. Stage 3 of fibrosis was of highest occurrence (36.8%) then stages 2 (26.3%), 4 (21.1%), and 1 (15.8%). This finding indicates that more than half (57.9%) of HCV patients with fibrosis were with advanced stage of liver fibrosis (stages 3 and 4).

Percentage of HCV genotypes and subtypes among Iraqi patients

The study was conducted on 50 HCV seropositive patients for both sexes; the result showed that two genotypes of HCV were detected: HCV genotype 4 in 27/50(54%) followed by HCV genotype 1 in 23/50(46%).The percentage of the most dominant HCV subtypes1a, 4e, and 1b, were 28%, 24%, and 18%, respectively as shown in figure 1 and 2, table 2.

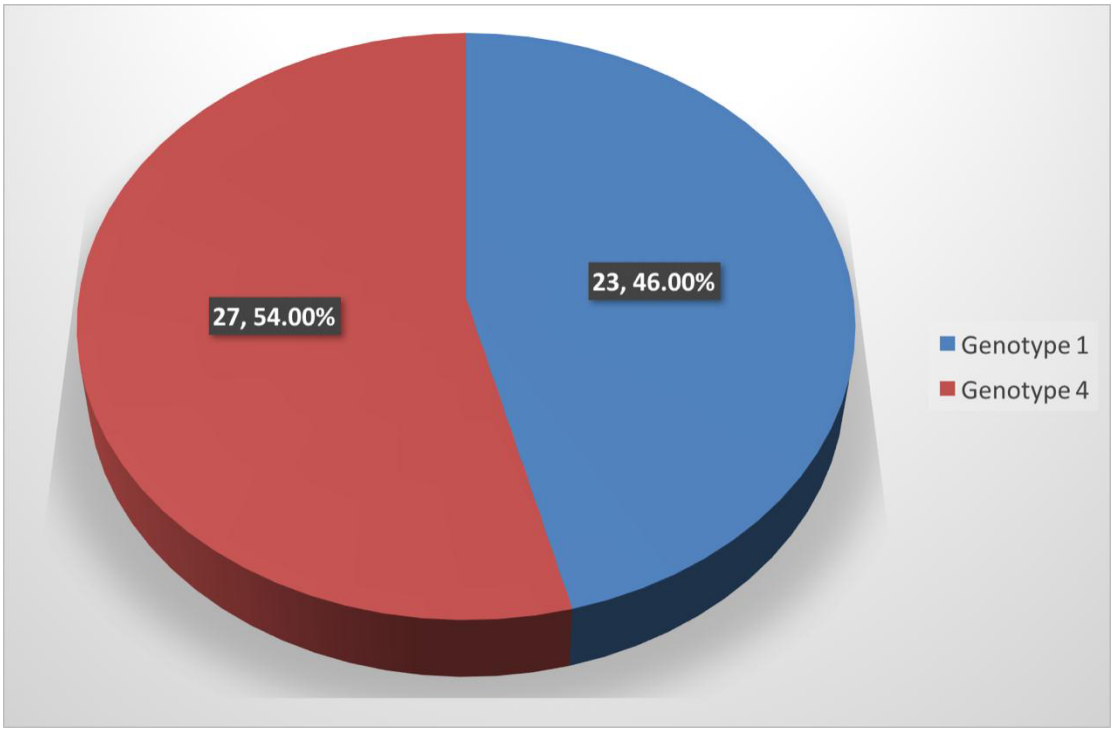

Figure 1. The percentage of HCV genotypes in a sample of Iraqi HCV patients

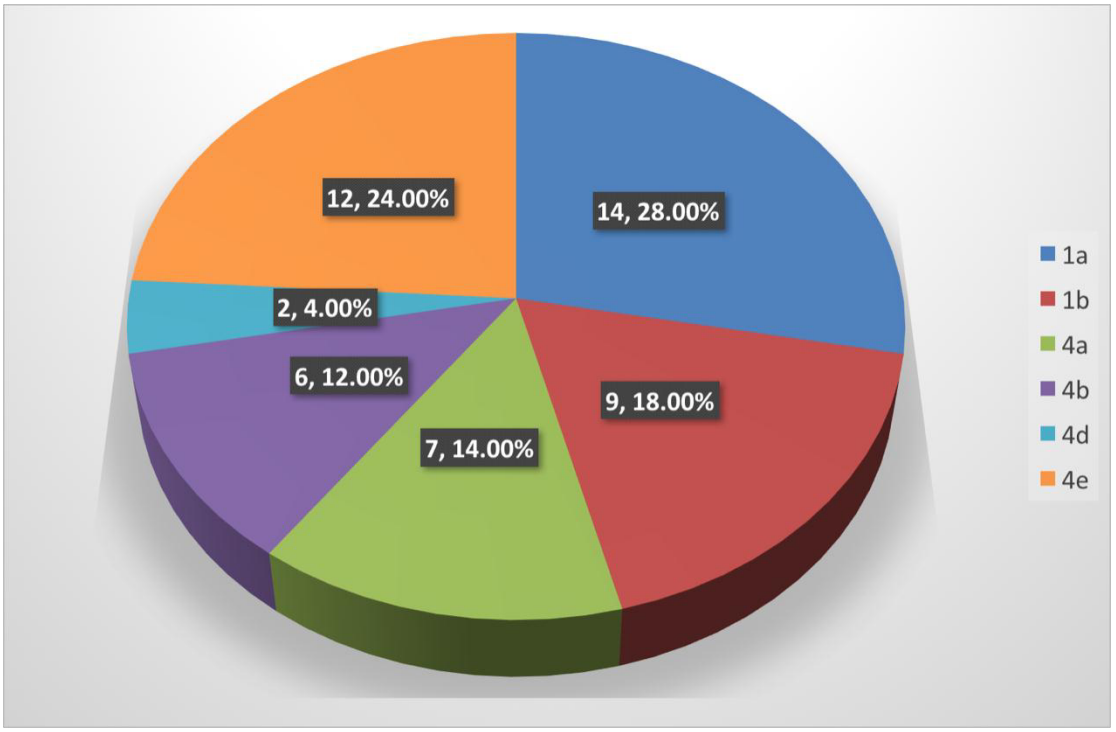

Figure 2. The percentage of HCV subtypes in a sample of Iraqi HCV patients

Table 2. (A) Frequency of HCV genotypes and (B) subtypes in a sample of Iraqi patients.

		Frequency	%
A. Genotype	1	23	46.0
	4	27	54.0
	Total	50	100
B. Subtype	1a	14	28.0
	1b	9	18.0
	4a	7	14.0
	4b	6	12.0
	4d	2	4.0
	4e	12	24.0
	Total	50	100

Association between Hepatitis C virus genotypes and the demographic characteristics of hepatitis C virus patients

This study revealed significant association between HCV genotypes and sex of patients ($P<0.05$), while no significant association was seen between HCV genotypes and age, body mass index, smoking, and alcohol intake ($P>0.05$). Female patients were shown to be infected with HCV genotype 1 (56%) more than genotype 4 (44%) while male patients were infected more with genotype 4 (64%) than genotype 1 (36%) as shown in table 3.

Table 3. Association between Hepatitis C virus genotypes and the demographic characteristics of hepatitis C virus patients

Parameter		HCV genotype		Total Frequency (%)	p value
		Genotype 1 Frequency (%)	Genotype 4 Frequency (%)		
Gender	Female	14 (56.0)	11 (44.0)	25 (100)	0.035
	Male	9 (36.0)	16 (64.0)	25 (100)	
Age groups (years)	31-40	11 (61.1)	7 (38.9)	18 (100)	0.156
	41-50	8 (38.1)	13 (61.9)	21 (100)	
	51-60	3 (37.5)	5 (62.5)	8 (100)	
	>60	1 (33.3)	2 (66.7)	3 (100)	
BMI (kg/m^2)	<20	1 (50.0)	1 (50.0)	2 (100)	0.517
	20-25	12 (40.0)	18 (60.0)	30 (100)	
	25-30	9 (56.2)	7 (43.8)	16 (100)	
	>30	1 (50.0)	1 (50.0)	2 (100)	
Smoking	No	19 (46.3)	22 (53.7)	41 (100)	0.547
	yes	4 (44.4)	5 (55.6)	9 (100)	
Alcohol intake	No	20 (43.5)	26 (56.5)	46 (100)	0.089
	yes	3 (75.0)	1 (25.0)	4 (100)	
Others	No	19 (46.3)	22 (53.7)	41 (100)	0.231
	DM	1 (16.7)	5 (83.3)	6 (100)	
	Thalassemia	3(100)	0 (0)	3 (100)	
Employment status	Employee	11 (47.8)	12 (52.2)	23 (100)	0.976
	Unemployed	12 (44.4)	15 (55.6)	27 (100)	
Total		23 (46.0)	27 (54.0)	50 (100)	

Association between Hepatitis C virus genotypes/subtypes and stage of liver fibrosis

It was shown no significant association between stage of liver fibrosis and HCV genotypes (P> 0.05). However, when taking HCV subtypes into account, it is found that liver fibrosis was associated significantly with the HCV subtypes (P<0.05) in that HCV subtypes 1b, 4d, and 4e showed predilection to advanced (3 and 4) stages of liver fibrosis while the percentage of advanced stages of liver fibrosis in subtypes 1a, 4a, and 4b showed either mild predilection to early stages or equal preference to early and advanced stages of fibrosis as shown in table 4.

Response to therapy in respect to HCV genotypes/subtypes

No significant difference in response to therapy, in terms of logarithmic reduction of viral load, was found between viral genotype 1 and 4 (P>0.05). Both genotypes showed good response to dual therapy and the response was seen within the first 3 months of therapy. However, both genotypes 1 and 4 reached median log 1 after 6 months of therapy as shown in table 5.

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Table 4. Association between stage of fibrosis and HCV subtypes

Subtype		Fibrosis				Total
		1	2	3	4	
1a	Count	2	1	2	0	5
	% within subtype	40.0	20.0	40.0	0.0	100
	% within Fibrosis	50.0	33.3	50.0	0.0	26.3
1b	Count	0	0	1	1	2
	% within subtype	0.0	0.0	50.0	50.0	100
	% within Fibrosis	0.0	0.0	20.0	20.0	10.5
4a	Count	0	2	2	0	4
	% within subtype	0.0	50.0	50.0	0.0	100
	% within Fibrosis	0.0	40.0	28.6	0.0	21.1
4b	Count	0	1	0	0	1
	% within subtype	0.00	100	0.0	0.0	100
	% within Fibrosis	0.00	25.0	0.0	0.0	5.3
4d	Count	0	0	0	1	1
	% within subtype	0.0	0.0	0.0	100	100
	% within Fibrosis	0.0	0.0	0.0	14.3	5.3
4e	Count	1	1	2	2	6
	% within subtype	16.7	16.7	33.3	33.3	100
	% within Fibrosis	20.0	20.0	28.6	66.7	31.6
Total	Count	3	5	7	4	19
	% within subtype	15.8	26.3	36.8	21.1	100
	% within Fibrosis	100	100	100	100	100
p value		0.003				

Table 5. Quantitative Association between patients' response totherapy and hepatitis C virus genotypes

Viral load		Genotype 1	Genotype 4
HCV viral load before treatment (IU /ml blood)	Median	3.00E+05	6.00E+05
	25-75 CI	(4.E+04-1.E+06)	(1.E+05-2.E+06)
HCV viral load 3 months after treatment (IU /ml blood)	Median	7.00E+02	1.00E+03
	25-75 CI	(0.E+00-3.E+05)	(0.E+00-1.E+05)
HCV viral load 6 months after treatment (IU /ml blood)	Median	0.00E+01	0.00E+01
	25-75 CI	(0.E+00-3.E+02)	(0.E+00-4.E+04)
Baseline-after 3 months		<0.001	<0.001
Baseline-after 6 months		<0.001	<0.001
3 - 6 months		1	0.943

As an attempt to illustrate deeply the association of dual therapy response by patients with different HCV viral subtypes, it was shown that all HCV subtypes in this study, except subtype 4d, responded in some way to dual therapy and the response was excellent in subtypes 1a, 4a, and 4b while the response to dual therapy was less remarkable in 1b and 4e, as summarized in table 6.

Table 6. Quantitative association between patients' response to therapy and hepatitis C virus subtypes

Viral load		HCV subtypes					
		1a	1b	4a	4b	4d	4e
HCV viral load before treatment (IU /ml blood)	Median	**3.00E+05**	**9.00E+05**	**1.00E+06**	**2.00E+05**	**2.00E+06**	**1.00E+06**
	25-75 CI	(3.E+04-4.E+05)	(2.E+05-1.E+06)	(2.E+05-2.E+06)	(3.E+04-2.E+05)	(6.E+05-3.E+06)	(3.E+05-2.E+06)
HCV viral load 3 months after treatment (IU /ml blood)	Median	**2.00E+01**	**7.00E+03**	**1.00E+03**	**2.00E+02**	**1.00E+06**	**6.00E+03**
	25-75 CI	(0.E+00-3.E+04)	(0.E+00-8.E+05)	(0.E+00-2.E+05	(0.E+00-4.E+03)	(1.E+05-2.E+06)	(0.E+00-7.E+04)
HCV viral load 6 months after treatment (IU /ml blood)	Median	**0.00E+00**	**4.00E+02**	**0.00E+00**	**0.00E+00**	**9.00E+05**	**0.00E+01**
	25-75 CI	(0.E+00-3.E+02)	(0.E+00-0.E+00)	(0.E+00-0.E+00)	(0.E+00-4.E+04)	(9.E+04-2.E+06)	(0.E+00-2.E+04)
Baseline-after 3 months		0.084	<0.001	0.032	0.017	0.945	<0.001
Baseline-after 6 months		<0.001	<0.001	<0.001	<0.001	0.899	<0.001
3 - 6 months		1	1	0.439	0.129	0.932	0.324
Description of response*		Good	moderate	good	Good	poor	moderate

*Description of response: good (median log is zero after 6 months of therapy), moderate (median log declines more than 2 log but not zero after 6 months of therapy), poor (median log does not decline or decline less than 2 log after 6 months of therapy

In the qualitative assessment of association, patients were classified into responders (R) and non-responders (NR). Subtype 1a showed the highest percentage of responders to dual therapy, 85.71%, followed by 4a 71.43%, and 4b 66.67%. On the other hand, HCV subtype 4d followed by 1b showed the poorest percentage of response,0% and11.11% respectively, while 4e subtype of good response but much less evident than 1a, 4a, and 4b as shown in table 7.

Discussion

Viral genotypes and subtypes of HCV are considered as markers for morbidity and mortality [(11)], clinical status, pathogenesis and outcome of disease [(12)]. Moreover, HCV genotypes/subtypes can be used for elucidating the possible mode of transmission, assessing the duration and benefit of antiviral therapy and future development of vaccine [(13)]. The present study showed that only HCV genotype 4 and 1 were detected in 54%, 46%, respectively of HCV-RNA positive patients. The rate of HCV genotypes found in this study is similar to these reported from different Middle East countries like Saudi Arabia and Lebanon where genotype 4 is the most common [(14)]. However, the results of this study are close, but not similar to that shown by previous studies conducted in Iraq; A study conducted in 2012 [(15)], found that genotype 1 is slightly more common than genotype 4 in that genotype 4 was 41.38%while genotype 1 was 48% of cases. Another study in Iraq, Al-Kubaisy et al, [(16)] reported that genotype 1 was 49% and genotype 4 was 35.4% of thalassemic Iraqi children. The above difference in genotype/subtype patterns in Iraq may be

attributed to that HCV genotype 4 might become more resistant to therapy and became under selective pressure of therapy especially the most resistant subtypes to therapy found in this study were 4d and 4e which might explain the increasing percentage of HCV genotype 4 over 1 over time in Iraq.

Table 7. Qualitative Association between patients' response totherapy and hepatitis C virus subtypes

		Subtype						Total
		1a	1b	4a	4b	4d	4e	
Responsiveness	R	12	1	5	4	0	7	29
		85.7%	11.1%	71.4%	66.7%	0.0%	58.3%	58.0%
	NR	2	8	2	2	2	5	21
		14.3%	88.9%	28.6%	33.3%	100%	41.7%	42.0%
Total		14	9	7	6	2	12	50
Description of response*		good	poor	good	good	No response	good	
p value		<0.001						

* Description of response. Good (R>NR), poor (NR>R), no response (all patients are NR).

Concerning HCV subtypes, in general, the current study findings indicated that 1a subtype was of the highest percentage, 28%, followed by 4e (24%), 1b (18%), 4a (14%), 4b (12%), and 4d (4%). This is the contrary to the previous study conducted on 492 histologically proven chronic HCV cases recruited from all region of Saudi Arabia between 1999 and 2002; they found that HCV subtype 4c/d are the major subtypes observed in the genotype 4 and as follows: 4a (0.5%), 4b (2.2%), 4c/d (19.5%), 4e (3.7%), and 4h (5.7%), while they found the rate of subtypes within HCV genotype 1 to be 1a (5.1%) and 1b (16.1 %)[17].

The results of the current study exhibited a remarkable lowering in the median of HCV viral load in blood of the studied patients in response to therapy for 3 and 6 months in both HCV genotypes 1 and 4 without any significant difference between HCV genotype 1 and 4. These findings are consistent with a recent study performed at Gastroenterology and Hepatology teaching hospital in Baghdad from 2011-2012 on 90 HCV antibody positive patients which found that the patients infected with HCV genotypes 1 and 4 exhibited similar early virological response in 93.3%of patients underwent dual therapy [18]. In another study conducted in Baghdad 2011; investigators found similar results by interpreting end treatment virological response to HCV genotypes 1 and 4 [19]. Regarding studies abroad, several studies found the same [20-22]. On the other hand, there are some other reports showed inverse outcome where response to therapy was different with different genotypes of HCV, some revealed better response to therapy in HCV genotype 4 [23,24].

Concerning HCV-1 subtypes, the findings of this study coincide with that of another study [25] which revealed dual antiviral therapy is more effective against HCV subtype 1a than subtype 1b (55% vs 43%), respectively. Another study confirmed this finding, showing that HCV subtype 1b is associated with more severe liver disease, not because it is a more aggressive form of HCV but because it reflects a longer duration of infection [26].

Regarding HCV-4 subtypes, the results of this study are pioneering in Iraq; this study showed that rate of response to therapy was lowest in subtypes 4d, then 1b and 4e while subtypes 1a, 4a and 4b showed best response to therapy.

This finding is in agreement with another international study [27] which found that patients infected with HCV -4a subtype respond significantly better to combination therapy than HCV-4d subtype, 4a achieved 77% while 4d achieved 52% of successful therapy in term of SVR. Another study in France [28] reported a poor response to 4d group of 10 HIV-positive patients, who were acutely infected with HCV. Also, another study on French patients infected with HCV-4, where subtype -4a had significantly higher rate of SVR (58%) than subtype 4d (43%) [29].

Taken together, HCV genotypes and subtypes showed remarkable association with response to HCV dual therapy, sex of patients, and stage of liver fibrosis. This highlights the importance of extending the genotyping tests of HCV infections to the level of subtypes within HCV genotypes1 and 4 in Iraqi population to help predict success of treatment and likelihood of development of advanced stages of liver fibrosis.

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Authors Contribution:

Dr. Abdulamir made the drafting of the article and revising it critically for important intellectual content; Dr. Al-Khalidi did clinical examination and diagnosis; Dr. Alwaysi helped in provided the samples and Abdulhassan collected blood samples, did the laboratory analyses and preparation of the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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