

Investigation of the Major HCV Genotype in Asymptomatic Patients in Iraq by the Use of Reverse- transcription PCR

Ghanima A. Al-mola*, Hashim R. Tarish**, Karar M. Abdulsada**, Rafah Hady Lateef

Abstract-

Globally, asymptomatic patients represent approximately 80% of total hepatitis C virus patients as indicated by Reverse-Transcription Polymerase Chain Reaction (RT-PCR) based viral genotyping. The present study was conducted to detect HCV genotypes and their prevalence among the asymptomatic populations from five Iraqi governorates (Najaf, Babylon, Qadisiya, Karbala and Baghdad governorates), as the asymptomatic patients globally represent about 80% from total HCV patients by using of viral genotyping through RT-PCR analysis. A875 samples were collected from different study groups. The total asymptomatic HCV positive cases by RT-PCR test were 103 out of 875, the genotype 4 was the predominant genotype that appeared in 89.4% of the patients followed by genotypes 1b, 2b, 3a and 6a which had been found in 6.79%, 2.91%, 2.91% and 1.94% of the asymptomatic patients, respectively. The current study provide the first information in Iraq at least in the study areas about the presence of HCV genotypes 2b and 6a

Key words: asymptomatic, Genotype, HCV, investigation, prevalence, predominant, RT-PCR

1 INTRODUCTION

Hepatitis C virus infection is one of the major public health problem in both developed and developing countries since discovering at 1989 (1,2). It is estimated that HCV infect 200 million peoples (3%) of the world's population and there are at least 21.3 million HCV carriers in the Middle East and Eastern Mediterranean countries (3). The infection is more often asymptomatic. About 85%-90% of acute cases and 70%-80% of chronic cases are asymptomatic and the jaundice develops only in one third of the symptomatic patients (4,5).

The virus exhibit substantial genetic variation so there are at least eleven distinct viral genotypes : 1 through 11 , and variable number of sub- types found usually in specific geographical regions and display significant difference in their aggressiveness and in response to antiviral therapy (6,7). Genotypes 1, 2 and 3 are widely distributed throughout USA, Europe, Australia and East Asia; genotype 4 is largely confined to the Middle East, Egypt and central Africa; genotype 5 and 6 are found predominantly in South Africa

and South East Asia, respectively (6,7,8); HCV genotypes 7, 8, and 9 have been identified mainly in Vietnamese patients; while genotypes 10 and 11 have been reported from Indonesia (9).

There are an increasing evidences that HCV genotypes possess different biological potentials, certain genotypes are more frequently associated with severe forms of liver disease and more amenable to interferon treatment make the genotyping of infecting virus is one of the prime predictors of the disease progression and the response to antiviral therapy. Consequently, typing of HCV isolates becomes an additional tool in diagnosis of the infection (10,11,12).

The distribution of HCV types in our country is still unclear because of the marked paucity in the studies about them, the purpose of this study was to estimate the prevalence of HCV genotypes among different asymptomatic study groups.

Methods:

Sampling

Serum samples were collected from a total of 875 clinically asymptomatic individuals were included in this study that

• Author name *Ghanima A. Al-Mola: *Department of Biology College of Science for Women, Babylon University, Babylon-Iraq
• E.Mil: almolaghanim@yahoo.com (corresponding author)
Co-Author name **Hashim R. Tarish: _ raheem2004@yahoo.com

**Karar M. Abdusada: kmabdulsada@yahoo.com ** Department of Microbiology College of Medicine-University of Kufa, Al-Najaf-Iraq

*Rafah Hady Lateef: Hadirafah@yahoo.com

performed from the beginning of June 2009 to the end of August 2010, 517 of these samples were randomly taken from asymptomatic individuals (general population) of all ages, both sexes, different residences and the occupations whom attended to hospitals from five Iraqi governorates; Najaf, Babylon, Qadisiya, Karbala and Baghdad governorates, 145 of samples were from thalassemic patients, 100 haemodialytic patients, 38 from the medical staff, 69 of blood donors (blood bank) whom positive for ELISA-II test and 6 were hepatocellular carcinoma patients from them also liver biopsies have taken. All samples were subjected to investigation of HCV genotypes by use of reverse transcription PCR (RT-PCR).

Asymptomatic HCV genotyping:

Genotyping through RT-PCR protocol consists of 4 main procedures:

Extraction, Reverse transcription, Amplification and Detection.

1-Viral RNA extraction step have been done inside the biological cabinet

a- RNA Extraction from serum samples:

It was implemented by use of Ribo Virus Columns Extraction kit, Sacace Biotechnologies, Italy, the procedure was done according to the manufacturer's instructions; at which 150 µl of serum was loaded to each Column followed by consecutive steps of washing and finally the RNA that attached to the silica membrane in the Ribo Virus columns were dissolved in nuclease-free water and aggregated at bottom of Eppendorf tubes by centrifugation then plugged rapidly and stored at -70°C until used.

b- The extraction from liver biopsies was done through the Easy Express Viral Nucleic Acid Release Kit, (Biochain Company, USA), the procedure was done according to the manufacturer's instruction; 100 µl of thawed proteases solution was put in a sterile 0.5ml Eppendorf tubes, one mg of liver biopsies were added to each tube and mixed by vortex for 15 seconds followed by multiple rounds of heating and centrifugation, then the supernatant were aspirated and stored at -70 °C until use.

2- Reverse Transcription: This process was done by use of Reverse Transcription System Kit, (Promega Corporation, USA). The procedure have been done inside the biological cabinet and the steps conducted according to manufacturer's instructions, The frozen RNA samples directly thawed inside incubator at 70°C for 10 minutes then added to a mixture from kit components, mixed and underwent several steps of heating cycles. The mixture hence contain cDNA, stored at -20°C until use.

3- Amplification: Mixture sets of primers were used according to Ohnoet al. (13), the primer solutions were prepared by dissolving the lyophilized primers, Alpha DNA company, Canada in Tris-EDTA buffer as the instructions of the manufacturer. Two rounds of amplification had implemented, the products of first round were used in the second round.

4- Detection: Reaction products were analyzed by 2% agarose gel electrophoresis, 8 µl of amplification products added in each well. The products of the two mixture of primer were separately loaded. The DNA bands were observed through viewing under UV transilluminator, and pictures took by Bio-Documents Analyzer, (Biometra, Turkey).

Results:

The total asymptomatic HCV positive cases by RT-PCR test were 103 out of 875, The genotyping by use of RT-PCR revealed that 89 patients were infected with genotype 4 from total 103 HCV positive cases with percentages of 86.4% which indicates that genotype 4 is the predominant type.

Genotype 1b rank below genotype 4 and appeared in 7 cases (6.79%), followed by both genotypes 2b and 3a, each appeared in 3 cases and percentage of 2.91%, where as genotype 6a was found only in two cases and showed the lowest rate among others (1.94%); One thalassemic patient has experienced a mixed infection with HCV genotypes 4 and 1b. (Table-1), (Figure-1).

Discussion:

The new researches explained that the rate of development of persistent infection at the asymptomatic acute patients significantly higher than that of symptomatic patients, besides to about 80% of HCV patients were asymptomatic (14,15); therefore, it is important to know the present information of asymptomatic HCV infected subjects at our community. The variation in the geographical distribution among the viral genotypes in the world made it worthy to get a local information pertain viral genotypes as that considered as an epidemiological marker particularly in tracing the source of infection and elucidating the possible mode of transmission, assessment the duration and benefit of antiviral therapy and future development of vaccine (16).

In our study we used a genotyping method through RT-PCR analysis that has been originally employed by Ohnoet al. (13) which involved wide range of HCV genotypes: 1a, 1b, 2a, 2b, 3a,

3b, 4, 5a, and 6a. The current study was revealed that the genotype 4 is the predominant genotype which found in 86.4% of the infected cases followed by Genotypes 1b, 2b, 3a and 6a that came in percentages of 6.79%, 2.91%, 2.91% and 1.94% respectively. This study was the first at least in the study areas that involved the searching of HCV genotypes among asymptomatic patients by using of RT-PCR test and the first that recorded presence of genotypes 2b and 6a in Iraq.

Al-Kubaisy *et al.* (17) have studied the sero-prevalence of HCV genotypes by use of genotype specific ELISA-III test searching antibodies at thalassemic Iraqi children, they were reported that genotype 4 was the most frequent type that has been found it in 35.4% of the infected cases followed by 1a in 27.1% of cases and genotype 1b that occurred in 22.9% of cases whereas mixed infection with genotypes 1a and 4 was set in 14.6% of the HCV among infected thalassemic children. Other study, by Al-Kubaisy *et al.* (18) who used the same technique (genotype specific ELISA-III test searching antibodies) and observed differed result among haemophilic patients in Baghdad and co-infected with the human immunodeficiency virus (HIV), they referred that the genotype 1a was the dominant genotype followed by genotypes 1b and 4.

The above difference in genotype patterns in Iraq may be attributed to the test that has been used in the diagnosis (ELISA test searching antibodies), number of samples, type of the patients (haemophilic and thalassemic and not asymptomatic) and the co-infection with HIV.

In Saudi Arabia (Arabic neighboring country), similar finding was showed by Shobokshi *et al.* (19) who found that the majority (62%) of the HCV infections in the Saudis were with genotype 4, while the other genotypes found with a lesser extent as follows: 1 (24.1%); 2 (7.4%); 3 (5.9%); and 5 (0.3%). In Syria, the frequency of HCV genotypes was similar to that of the current study, Antakiet *et al.* (20) had pointed out that genotype 4 was the most prevalent type at the Syrian HCV patients it was present in 59% of the patients followed by genotype 1 (in 28.5% of patients) and genotype 5 that found in 10% of the infected cases.

In Kuwait, Pasaca *et al.* (21) also observed similar result, they were recorded that genotype 4 was the predominant genotype followed by genotype 1. Where as In Jordan, Bdour (22) had drew absolutely varied picture from that in above Arabic countries, she studied HCV genotypes at the Jordanian haemodialysis patients and found that

genotype 1a was the most prevalent type which found in 40% of the patients whereas genotypes 1b and 4 were found in 33.3% and 26.6% of the HCV patients, respectively.

In Iran (non-Arabic neighboring country), the genotypic pattern was differed, for example: Zarkesh-Esfahani *et al.* (23) had observed that the frequency of HCV genotypes was determined as follows: genotype 3a (61.2%), genotype 1a (29.5%), genotype 1b (5.1%), genotype 2 (2%) and mixed genotypes of 1a plus 3a (2%).

The difference in the frequency of HCV genotype from that in Jordan and Iran was in congruence with observations of Ramia and Eid-Fares (24) whom referred that the genotype 4 was the prevalent type at the Arabic Middle Eastern countries (except for Jordan) and genotype 1 was dominant at non-Arabic Middle East countries.

In Turkey (non-Arabic neighboring country), the genotype 1 was the predominant genotype which has been observed in 92% of the patients, followed by genotype 4 in 5% and genotype 2 in 2% of the patients (25).

The interpretation of the presence of genotypes 1b, 2b and 3a in some Iraqi patients in the current study is that these types might had been introduced to our country from Iran, Jordan and Turkey where the prevalence of these genotypes was relatively high as a result of traveling, immigration and the matrimonies.

Genotype 4 was found to be the predominant genotype among the HCV-infected Lebanese patients (53.3%), genotype 1a was ranking below in 43.3% of infected cases and genotype 1b found in 8% of the patients (26). This result was in agreement with our study result concerning the dominance of genotype 4 amongst other types at HCV patients. Similar finding was also reported by Ohno *et al.* (27) at the HCV Yemeni patients where genotype 4 was the most prevalent type followed by genotype 1.

In Egypt, similar result was obtained by El-Kady *et al.* (28), they observed that the genotype 4 was detected in 89.6% followed by genotype 1 in 7.7% of the patients. The prevalence of genotype 4 in the Egyptian HCV patients was quite similar to that of our study and the majority of other Arabic middle Eastern countries too, that may attributed to similarity in the peoples traditions, their ethnicity and the geographical vicinity.

In Libya, which is an Arabic North-African country, the genotype 4 was the commonest and appeared in 35.7% of the HCV patients, genotype 1 was came in a lesser frequency (32.6%) followed by

genotypes 2 and 3 which has been found at 16.7% and 13.2% of the patients, respectively (29). The dominance of genotype 4 was not only in consistence with the our result but also with Egypt and most other middle-Eastern Arabic countries that may attributed to the traveling, working and the mating.

However the pattern of genotypes' frequencies at the other three Arabic north African counties (Tunisia, Morocco and Algeria) was extremely differed; the genotype 1 and 2 were the dominant genotypes while genotype 4 has a trivial frequencies (6,30,31). Idress *et al.* (32) had reported that the genotype 3a was the dominant genotype (34.1%) at the Pakistani HCV patients followed by 2a (8.1%) , 3b (7%) and 1a (5.4%). That similar to the result of Verma *et al.* (33) in India who were reported that genotype 3 is the prevalent type (40.81%) while the other genotypes; 1a, 1b and mixed of 1 + 3 have been found in prevalence of 6.12%, 8.16% and 17.3% in HCV patients, respectively.

The presence of genotype 3a (which is the prevalent type in the Indian subcontinent) in our study at about 2.91% of HCV Iraqi patients may be that this genotype has been introduced to our country by traveling.(33)

In Indonesia which is one of southeast Asian countries, Utama *et al.* (34) were registered that genotype 1b was the most frequent type which was found in 47.3% of HCV patients. Genotype 1 also prevalent in Japan Whereas the viral genotypes 6, 7, 8, 9, 10 and 11 were observed in Vietnam, Thailand, Myanmar and Philippines (6,24).

In the Gabon which is located at the middle of Africa , Ndong-Atome *et al.* (35) were reported that genotype 4 was the most dominant type that had been recorded in 90.9% of HCV patients, this frequency was quite similar to that of our study whilst in South Africa, Vardas *et al.* (36) had registered that genotype 5 was dominant followed by genotype 1a.

In the current study the genotype 5 was not observed at any of the HCV patients, that may be due to the limitation in the geographical distribution of this genotype which was found mainly at the south African countries(24).

In Spain, Torres-Puente *et al.* (37) had recorded that genotype 1 was the predominant followed by types 2 and 3. In United States, Eyster *et al.* (38) had showed that the genotype 3a was prominent type which found in 41% of the patients followed by genotypes 1a and 1b that appeared in 31% and 13% of the patients, respectively.

). In Brazil which is a south American country, Sawada *et al.* (39) had registered that genotype 1 was the predominant type which came in percentage 94% of HCV patients while genotype 3 has been recorded at 6% of the patients.

It is of interest to note that the recording of genotypes 1b and 2b in our study at frequency of 2.91% for each of them may be attributed to the transmission of these genotypes from other regions such as European or American countries where the prevalence of these types was high, might as a result of immigration, traveling, outside treated patients, importing of contaminated blood or the blood products, this finding was in congruence with the observations of Sy and Jamal (3) who were mentioned that these genotypes have a cosmopolitan distribution but the highest frequencies were at Europe and America.

Conclusions

In the present study, we concluded that the HCV genotype four was the predominant genotype among asymptomatic HCV patients in the study areas(Najaf,Babylon,Qadisiya,Karbala and Baghdad Iraqi governorates). This study also recorded that the HCV genotypes 2b and 6a circulating among asymptomatic patients in Iraq.

Author's contributions

Ghanim A.A. and Hashim R.T.conceived the study. Karar M.A and Ghanim A.A. collected the samples and perform the molecular analysis. Hashim R.T. and Karar M.A.and Rafah H.L. search literature and drafted the manuscript. Ghanim A.A. reviewed the manuscript. All authors read and approved the final manuscript.

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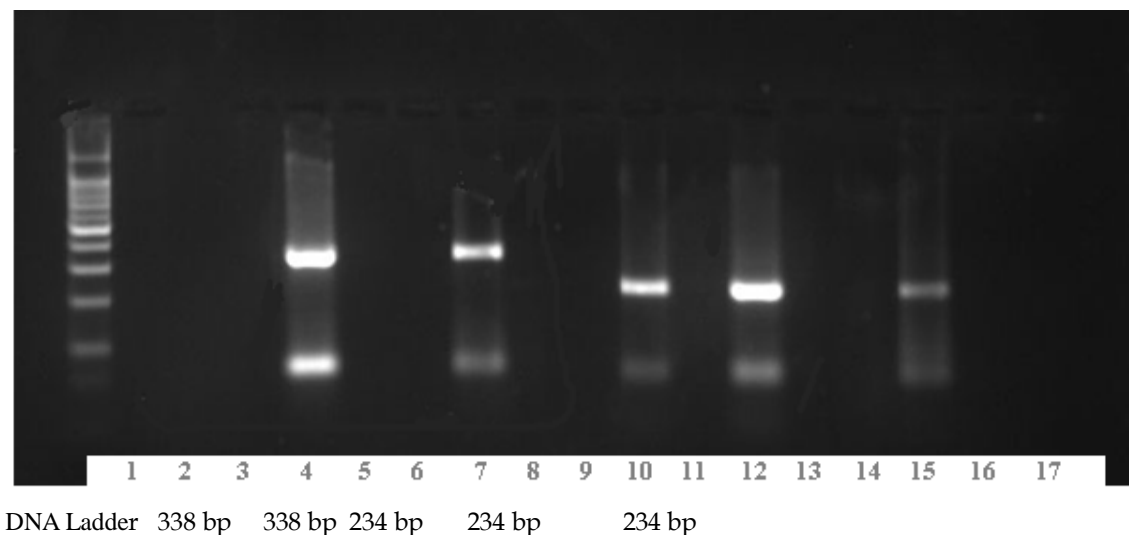
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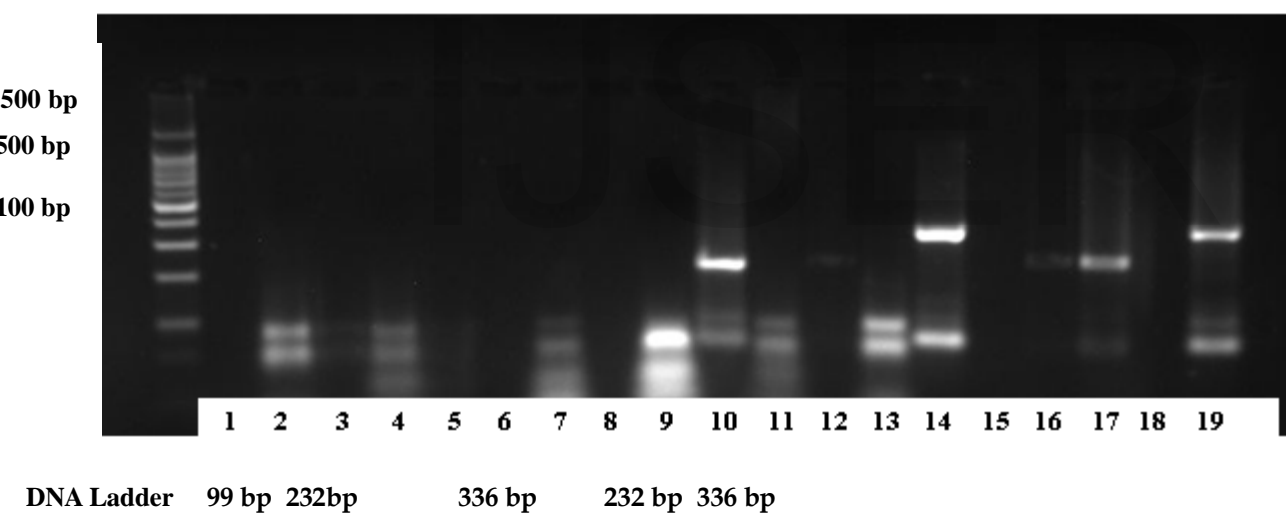
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Mixture 1



Mixture 2



Transcription Polymerase Chain Reaction by Using of Two Mix- tures of Primers

Mixture 1:	Lanes 4 and 7	genotype 2b
	Lanes 10, 12, and 15	genotype 1b
Mixture 2:	Lane 9	genotype 4
	Lanes 10 and 17	genotype 3a
	Lanes 14 and 19	genotype 6a

Table (1): The Results of genotyping of asymptomatic HCV by Using RT-PCR Analysis in the Study Groups

Genotypes (G)	Normal asymptomatic individuals					Thalas-se-mic patients	Medical staff	HCC. patients	Renal dialysis	Blood donors	Total	percentages
	Najaf	Baby-lon	Qa-disyia	Kar-bala	Bagh-dad							
G1b	1	1	0	0	0	2	0	0	0	3	7	6.79%
G2b	0	0	0	0	0	2	0	0	0	1	3	2.91%
G3a	1	0	0	0	1	1	0	0	0	0	3	2.91%
G4	5	3	2	3	1	21*	2	4	7	41	89	86.4%
G6a	0	0	0	0	0	2	0	0	0	0	2	1.94%

* Genotypes mixed infection (G1b + G4)