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HBV Genotyping distribution among Iraqi patients in Wasit Province

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ABSTRACT: Hepatitis B viruses are one of the world's most important issues for public health. HBV production is driven by this high mutation rate which results in a high degree of genetic heterogeneity with a distinct geographic distribution of 10 resulting genotypes (A – J) .This study aimed to determination the HBV genotypes distribution among Iraqi hepatitis B virus patients.The study included 100 patientswith positive HBs Ag .Muliplex PCR results showed thatthe percentage of HBV genotypes distributed among patients was as follows: AE 1%, ACD 1% , ABCDEF 1% , ABCDFG 1% , B pure 30%, BC 9% , BD 1%, BE 3% , BF 1%, BCE 6% , BCDE 1%, BCDF 1%, BCEF 1%, C pure 16%, CD 13%, CE7%, CDE 2%, D pure 4%, E pure 1%, so the most common genotype is B pure genotype (30%) , and in the second rank C genotype (16%), however the C mixture genotype was (59%) was common followed by B genotype (55%) .Based on our knowledge this study is the first study in Iraq that documented genotype G in one sample.

KEYWORDS: HBV, Genotypes, Muliplex PCR, hepatitis, viral load, patients

INTRODUCTION

Hepatitis B virus (HBV) infection is globally a great issue for public health,About two billion people worldwide have been infected with HBV and more than 240 million suffer from chronic hepatitis B (CHB), while 600,000 patients annually suffer from acute hepatitis B (AHB) and CHB (*Brown et al* . , 2016), in addition to around 1 million deaths annually (WHO, 2013).

Characterization of HBV genotypes results from a nucleotide sequence variation of 8% or more, while subgenotypes are nucleotide sequences with variations between 4% and 8% (Kurbanov*et al*.,2010).A total of 10 genotypes of HBV (A- J) have been reported worldwide. In Asia genotypes B and C predominate as well as D. HBV production is driven by this high mutation rate which results in a high degree of genetic heterogeneity with a distinct geographic distribution of 10 resulting genotypes (A – J) (Sousa *et al*., 2018).In reality, in North America , Europe, South-East Africa and India, HBV genotype A is found; in Asia and Oceania, genotypes B and C are found, while in North America, North Africa, Europe , the Middle East

and Oceania, genotype D is most common. In West Africa, HBV genotype E is hyperendemic; genotype F is common in America; genotype G is common in Western countries; genotype H is found in Central and South America (Rajoriya*et al* ., 2017). The newest HBV genotype, genotype I was reported in Vietnam and Laos; while genotype J was reported in Japan (Ott*et al*., 2012; Lok*et al* ., 2017). HBV genotyping is an important tool that has been used to investigate the cause of outbreaks of hepatitis B (Arankalle*et al* , 2011) , modes of transmission (Ismail *et al* ,2015) andthe manner and pathogenesis of the virus should also be clarified (Baig et al., 2007). Sequencing, INNO-LiPA, RFLP, multiplex PCR, serotyping, oligonucleotide microarray chips, reverse dot blot, RFMP (Restriction Fragment Mass Polymorphism), invader assay, and real-time PCR have at least 10 different methods of HBV genotyping with variable sensitivity , specificity, turnaround time , and cost (Guirgis *et al* ., 2010) . Genotyping of HBV for the study samples was done by multiplex PCRassay method, easy to do and also appropriate for the identification of infections of mixed genotypes.This study aimed to determination the HBV genotypes distribution among Iraqi hepatitis B virus patients

MATERIALS AND METHODS

Samples collection

This study included 100 hepatitis B patients , 72 males and 28 females with median age 37 year, ranging from (10-70) years.The patients were outpatients attending Al-Karama Teaching Hospital and Central Health Laboratory in AL-Kut City/ Wasit Province / Iraqfrom December 2017 to June 2018. This work was carried out in the College of Science, Department of Biology, College of Medicine, PCR unit at AL-Karama Teaching Hospital and Central Health Laboratory in Wasit Province/Iraq.

Five milliliters of peripheral blood were collected from patients. Two milliliters of blood were used to the viral DNA and was frozen at a temperature of -70 Ċ for use in the molecular study and 3 milliliters for the rest of work. Patients co-infected with hepatitis C and HIV and cancer and undergo dialysis and other autoimmune diseases , as well as the pregnant women were excluded from our study.

Screen of HBV infection by using ELISA kit for HBsAg

Serum samples were carried out according to the designation (Foresight, USA) on the ELISA worksheet.

DNA extraction

According to the manufacturer's instructions (Anatolia gene function, Turkey), DNA extraction was completed.

Multiplex PCR for detection of HBV Genotyping

Multiplex PCR was used to detection of HBV Genotyping . Two sequential reactions were performed on each sample, one targeting genotypes A, B, F and G and the other targeting genotypes C , D and E. By comparing the sample's band size with predicted band sizes, the genotype was established (table 1). The appearance of more than one band suggested co-infection with two or more genotypes,these samples were further processed.

Multiplex PCR was performed with the PCR System (Multigene , USA) thermocycler and was carried out in a total volume of 50µ l containing 2 µl each of sense and 0.5 antisense primers, (5 µl) template and (41 µ l) water ,both of these were added to the master mix tube, then the mixture was centrifuged for 2 seconds at 8000 rpm.

The thermocycler was first programmed to incubate the samples at 94 ° C for 5 minutes, followed by 40 cycles consisting of 94 ° C for 1 minute, 59.5 ° C for 1 minute and 72 ° C for 2 minutes. After PCR, the amplified products were visualized in a 2 % agarose gel, stained with ethidium bromide and examined under UV light by electrophoresis.

Table (1): Type-specific PCR primers for detection seven HBV genotyping (A-G)(Liu*et al* , 2008)

Amplicon	Sequence (5 -3)	Sense/ antisense	Product size bp
N1-ABFG set	**(core / polymerase)**		
N1 (forward primer)	**5 - GCCTCCAAGCTGTGCCTTG-3**	**S**	
AR1 (genotype A-specific primer)	**5 -TAGGGGACCTGCCTCGGTC-3**	**AS**	**500**
BR1 (genotype B-specific primer)	**5- GGGGCCCCACAAATTGCTT-3**	**As**	**190**
FR1 (genotype F-specific primer)	**5-AATTATTACCCACCCAGGAAGCCAA-3**	**As**	**209**
GR1 (genotype G-specific primer)	**5-TGTATGGTGAGGCGAACAATGATCAGAG-3**	**As**	**260**
N2-CDE set	**(Surface/polymerase)**		
N2 (forward primer)	**5 -CCTGCTGGTGGCTCCAGT-3**	**S**	
CR1-2 (genotype C-specific primer)	**5 -CGAATTTTGGCCAGGACAAGT-3**	**As**	**300**
DR1 (genotype D-specific primer)	**5 -TGTGATCTTGTGGCAATGACCCAT-3**	**As**	**906**

ER1 (genotype E-specific primer)	**5 -AGTGGGGGAAAGCCCGG-3**	**As**	**400**

Statistical Analysis

The data was analyzed statistically using SPSS 26 program. The comparisons between groups were performed with analysis of variance (ANOVA) test. Chi-square test was used to significant compare between percentage and T-Test was used to significant compare between means. P-Value less than 0.05 was considered significant.

RESULTS

In this study, (100) patients clinically suspected of having HBV infection were first screened for HBsAg by the ELISA screen test to diagnose their HBV infection.

Genotyping of HBV for the study samples was done by multiplex PCRassay method, easy to perform and is also suitable for detecting mixedgenotype infections. In this study the distribution percentage of HBV genotypes among thepatients were as follows: AE 1%, ACD 1% , ABCDEF 1% , ABCDFG1% , B pure 30%, BC 9% , BD 1%, BE 3% , BF 1%, BCE 6% ,BCDE 1%, BCDF 1%, BCEF 1%, C pure 16%, CD 13%, CE7%,CDE 2%, D pure 4%, E pure 1%, so the most common genotype is Bpure genotype (30%) , and in the second rank C genotype (16%),however the C mixture genotype was (59%) was common followed by B genotype (55%) , F genotype recorded in 5 samples (as a mixed)Table (2), (Figure 1). Based on our knowledge this study is the first study in Iraq that documented genotype G in one sample (ABCDFG) , Figure (2).

Table (2): Distribution of HBV genotype in patients

Genotyping	Number	Total	P value
A pure **AE** **ACD** **ABCDEF** **ABCDFG**	**0** **1** **1** **1** **1**	**0%** **1%** **1%** **1%** **1%**	
Total	**4**	**4%**	

B pure **BC** **BD** **BE** **BF** **BCE** **BCDE** **BCDF** **BCEF**	**30** **9** **1** **3** **1** **6** **1** **1** **1**	**30%** **9%** **1%** **3%** **1%** **6%** **1%** **1%** **1%**	**P=0.000**
Total	**53**	**53%**	
C pure **CD** **CE** **CDE**	**16** **13** **7** **2**	**16%** **13%** **7%** **2%**	
Total	**38**	**38%**	
D pure	**4**	**4%**	
E pure	**1**	**1%**	
G pure	**0**	**0%**	
F pure	**0**	**0%**	
Total	**100**	**100%**	

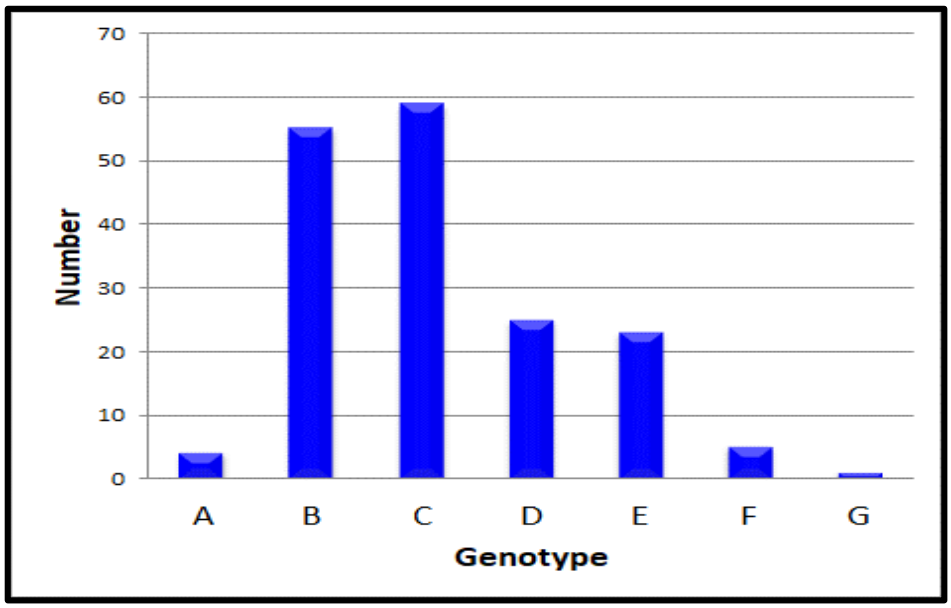

Figure (1): Distribution of HBV genotype in patients

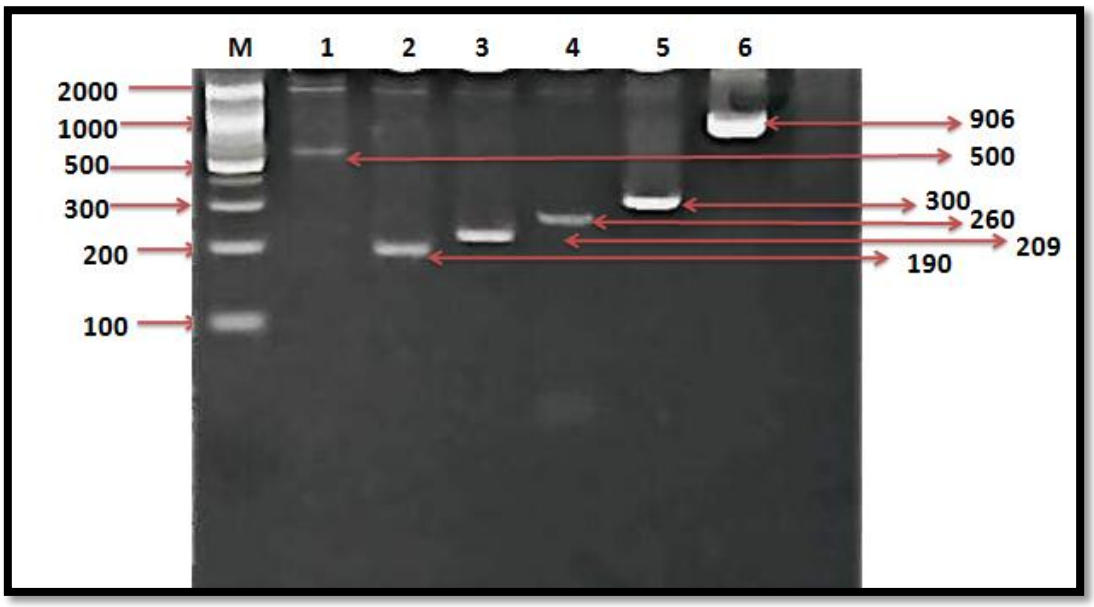

Figure (2): Agarose gel electrophoresis image that show Multiplex- PCR product analysis for Genotyping Hepatitis B virus (A,B,C,D,E,F) from serum sample (from one patient). M (Marker ladder 2000-100bp). Lane (1) Hepatitis B virus Genotyping A, at PCR product 500bp, and Lane (2) HBV Genotyping B, at PCR product 190bp , Lane (3) HBV Genotyping F at PCR product 209bp, Lane (4) HBV Genotyping G at PCR product 260bp, Lane (5) HBV Genotyping C at PCR product 300bp, Lane (6) HBV Genotyping D at PCR product 906bp

Table (3) shows the percentage of each type of genotype of HBV, regardless of whether it is single or mixed with another type.

Table (3): Distribution of each of HBV genotype in patients

Genotype	Number	Percent
A (Pure + Mixed)	4	4%
B (Pure + Mixed)	55	55%
C (Pure + Mixed)	59	59%
D (Pure + Mixed)	25	25%
E (Pure + Mixed)	23	23%
F (Pure + Mixed)	5	5%
G (Pure + Mixed)	1	1%

Relevant genotypes of primers (N1-ABFG set and N2-CDE set) were set separately (each primer forward and reverse) in the PCR mixture for the same sample to conduct simplex PCRin order to certify that these bands, due to the presence of several primers in the mixture, were not primer dimmer or unspecific bands. The findings showed bands of the same predicted size as in the multiplex primer for each hepatitis B virus genotype (figure 3), (figure 4)

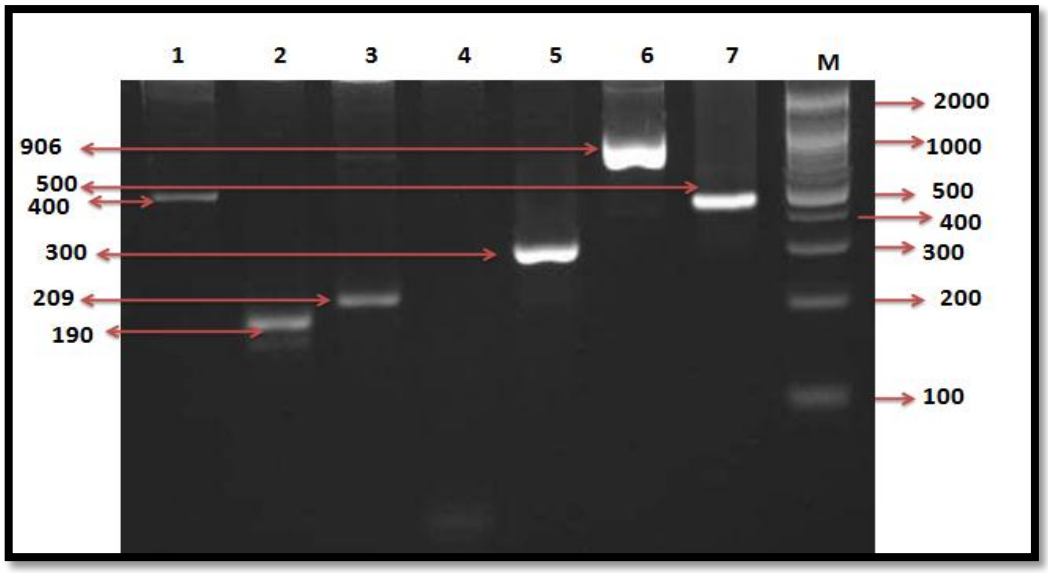

Figure (3): Agarose gel electrophoresis (2%) image that show Multiplex- PCR product analysis for Genotyping Hepatitis B virus (A,B,C,D,E,F) from serum sample (from one patient). M (Marker ladder 2000-100bp). Lane (1) Hepatitis B virus Genotyping A, at PCR product 500bp, and Lane (2) HBV Genotyping B, at PCR product 190bp , Lane (3) HBV Genotyping F at PCR product 209bp, Lane (4) (not present band), Lane (5) HBV Genotyping C at PCR product 300bp, Lane (6) HBV Genotyping D at PCR product 906bp

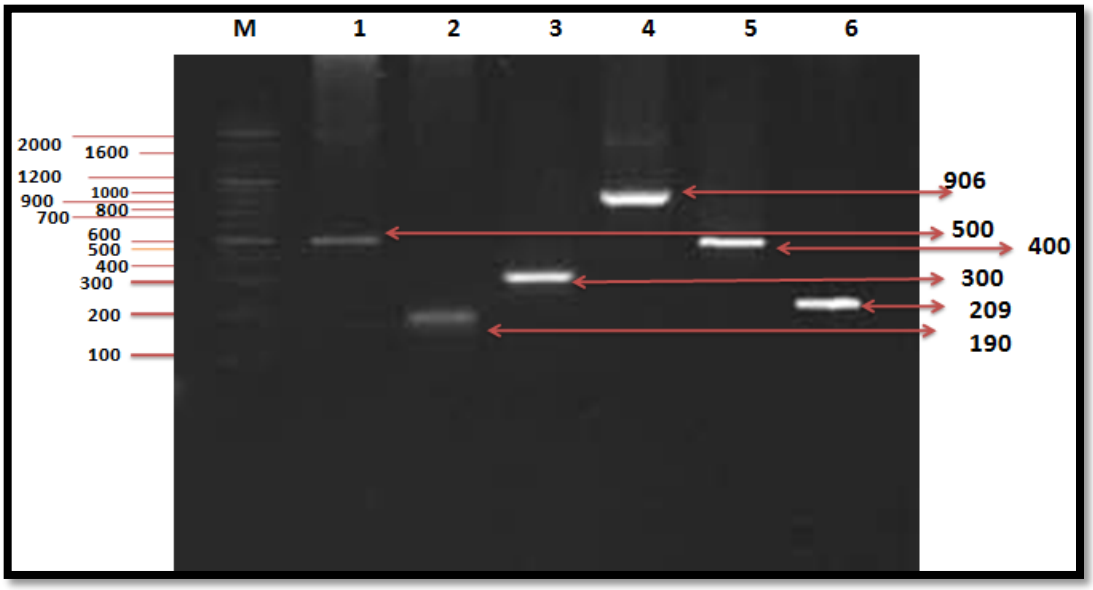

Figure (4): Agarose gel electrophoresis image that show Multiplex- PCR product analysis for Genotyping Hepatitis B virus (A,B,C,D,F,G) from serum sample (from one patient). M (Marker ladder 2000-100bp). Lane (1) Hepatitis B virus Genotyping A, at PCR product 500bp, and Lane (2) HBV Genotyping B, at PCR product 190bp , Lane (3) HBV Genotyping C at PCR product 280bp, Lane (4) HBV Genotyping D at PCR product 906bp , Lane (5) HBV Genotyping E at PCR product 400bp, Lane (6) HBV Genotyping F at PCR product 209bp

The correlation between Genotype and gender, age groups, activity

In this study there was no significant correlation between genotype and gender ,table (4).

Table:(4): The correlation between Genotype and gender.

Genotype	Chi-Squair	P-Value
A (Pure + Mixed)	1	0.317
B (Pure + Mixed)	2.317	0.128
C (Pure + Mixed)	0.449	0.503
D (Pure + Mixed)	1.058	0.304
E (Pure + Mixed)	0.088	0.767
F (Pure + Mixed)	0.167	0.683
G (Pure + Mixed)	2.597	0.107

Table (5) showed no correlation between Genotype (A,B,D,E,F,G) and age groups (p= 0.086, p= 0.974, p= 0.401, p= 0.514, p= 0.823, p= 0.070), however, only C genotype has a correlation with age groups (p=0.031).

The correlation between viral load and genotype of HBV

Figure (5) showed no correlation between viral load with genotype (p= 0.589). Tran *et al* ., 2015 also found there was no obvious relationship between genotype and viral load.Yu *et al*., 2005 found that genotype C HBV was associated with increased viral load, and associations of HBV genotype and viral load with HCC risk were additive.

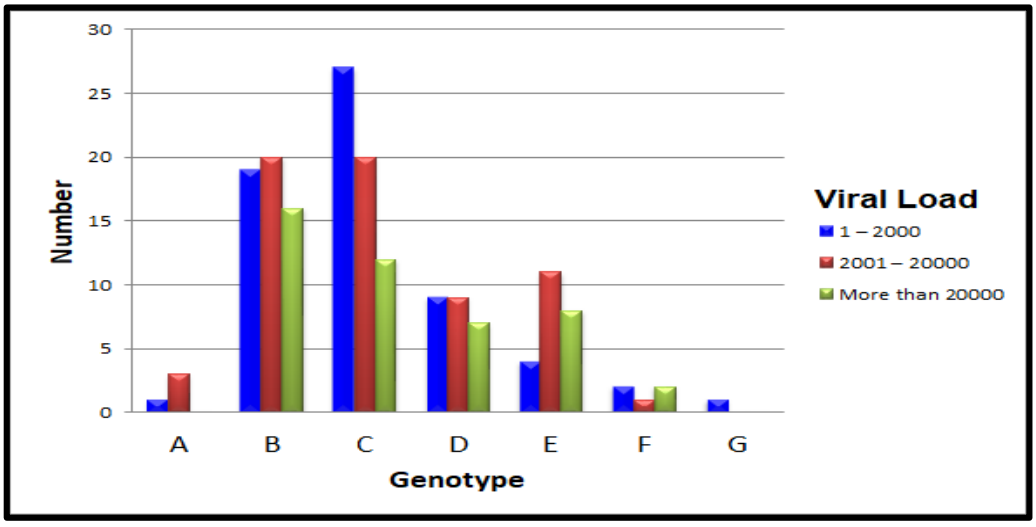

Figure (5): The correlation between viral load with genotype of HBV

Table (5): The correlation between Genotype and age groups

Genotype	Chi-Squair	P-Value
A (Pure + Mixed)	9.649	0.086
B (Pure + Mixed)	0.852	0.974
C (Pure + Mixed)	12.308	0.031
D (Pure + Mixed)	5.121	0.401
E (Pure + Mixed)	4.252	0.514
F (Pure + Mixed)	2.184	0.823

G (Pure + Mixed)	10.213	0.070

Table (6): The correlation between Genotype and activity

Genotype	Chi-Squair	P-Value
A (Pure + Mixed)	1.316	0.251
B (Pure + Mixed)	0.718	0.397
C (Pure + Mixed)	0.006	0.939
D (Pure + Mixed)	0.292	0.589
E (Pure + Mixed)	0.715	0.398
F (Pure + Mixed)	0.046	0.830
G (Pure + Mixed)	0.319	0.572

Table (6) showed no correlation between Genotype and activity ($p \geq 0.05$).

The correlation between genotyping and concentration of anti HBV core IgM and concentration of anti HBV core IgG

Table (7) showed that all genotype has no a significant different with the concentration of anti HBV core IgM ,(except B and C genotype) which has a highly significant different, (p= 0.000 with B genotype, and p= 0.008).

Table (7): The correlation between genotyping and concentration of anti HBV core IgM

Genotype	Coefficient	Standard Error	t	P-Value
A (Pure + Mixed)	-0.060	0.816	-0.074	0.941
B (Pure + Mixed)	0.740	0.198	3.741	0.000
C (Pure + Mixed)	0.629	0.232	2.709	0.008
D (Pure + Mixed)	-0.034	0.320	-0.106	0.916
E (Pure + Mixed)	-0.450	0.329	-1.368	0.175
F (Pure + Mixed)	-0.568	0.712	-0.798	0.427
G (Pure + Mixed)	-0.614	1.597	-0.384	0.702

Table (8) showed there was no a significant relationship between all of the genotyping of HBV patients with the concentration of anti HBV core IgG ($p > 0.05$).

Table (8): The correlation between genotyping and concentration of anti HBV core IgG

Genotype	Coefficient	Standard Error	t	P-Value
A (Pure + Mixed)	8.591	127.400	0.067	0.946
B (Pure + Mixed)	47.003	30.896	1.521	0.132
C (Pure + Mixed)	-10.460	36.249	-0.289	0.774
D (Pure + Mixed)	6.584	50.015	0.132	0.896
E (Pure + Mixed)	-12.978	51.361	-0.253	0.801
F (Pure + Mixed)	-35.729	111.140	-0.321	0.749
G (Pure + Mixed)	-12.580	249.471	-0.050	0.960

DISCUSSION

Hepatitis B virus genotyping system depended on Multiplex PCR using specific primers for the detection of 7 genotypes from A to G of HBV based on the method that was described by (Liu *et al* , 2008).

In this study the distribution percentage of HBV genotypes among thepatients were as follows: AE 1%, ACD 1% , ABCDEF 1% , ABCDFG 1% , B pure 30%, BC 9% , BD 1%, BE 3% , BF 1%, BCE 6% , BCDE 1%, BCDF 1%, BCEF 1%, C pure 16%, CD 13%, CE7%, CDE 2%, D pure 4%, E pure 1%, so the most common genotype is B pure genotype (30%) , and in the second rank C genotype (16%), however the C mixture genotype was (59%) was common followed by B genotype (55%) , F genotype recorded in 5 samples (as a mixed). Based on our knowledge this study is the first study in Iraq that documented genotype G in one sample (ABCDFG). The results of this study could be explained in the basis that HBV genotypes have distinct geographical distribution and because of the HBV genotype B predominantly distributed in the Asia and also C genotype and as Iraq is a part of this region, so the results of this study regarding the HBV genotypes distribution could be acceptable (Kao , 2011) .

A results of mixed genotype coincide with result of Abbood and Al-Mhanah, (2019) who found the most of patients with mixed genotype of HBV, Although Al-Suraifi *et al* ., (2016) found that no single genotype was determined and all of these samples displayed mixed genotype infection and their findings showed that the HBV genotypes were distributed as follows among patients: A+B+C+D+E (77.77%), A+B+D+E (16.66%), A+B+C (2.77%), A+B+E (1.38%), A+D+E (1.38%), while genotype F was not detected. Other study conducted in Sulaimania in Iraq (Rashid and Salih, 2015) using the same method, where they found 100% of samples had mixed genotypes , 25% of them had mixed genotypes B+C+D while 75% had mixed genotypes A+B+C+D . As well in Duhok region of Iraq , Abdulla and Goreal(2016) found that the main genotype in Duhok / Iraq is genotype D, followed by genotype B. HBe Ag is positive in a high percentage of CHB patients with genotype D. Whereas 92.3 percent of genotype D was found in Basra Al-Aboudi and Al-Hmudi (2015) and 7.69 percent had mixed genotypes D+ E. Also, genotype D (80 percent) was the predominant among CHB patients and mixed genotypes D+F (20 percent) in Baghdad by Ahmed (2013). Abdulrazaq and AL-Azaawie, (2017) were found that A, B, C, and F genotypes are reported for the first time in Iraq and may be Arab countries and Mediterranean Region.

Reports on HBV genotypes in neighboring countries demonstrated that; in Iran, Yoosefi *et al*., (2016) found that genotype D is most common which had a mixture of C / D genotype in 154/163 specimens (94.5 percent) and 9/163 (5.5 percent). Asaad*et al*.(2015) in Saudi Arabia

reported that among 160 HBVpatients, Genotype D was found in 135 (84.4%) patients, followed by A (18; 11.3%) and E (7; 4.3%). In Oman ,HBVgenotypes D was (130/170; 76.47%) and A (32/170; 18.28%) are predominant in Oman , while the HBVgenotypes C and E were less frequent (each 1.18%) (Al Baqlani *et al*., (2014) . In United Arab Emirates , Alfaresi *et al*., (2010) found all 88 patients (100%) have HBVDNA , and the genotype D was the most prevalent in patients (79.5%) followed by genotype A (18.2%) and genotype C (2.3%). As well as, In Egypt Khaled *et al*.,(2011) showed that genotype D constituted 87% of the total infections HBV DNA positive , the other 13% showed mixed infections of D/F. In Jordan Genotype D represented the predominant genotype in all Jordanian patients infected with *HBV* (Hamoudi *et al*., 2016).In Pakistan ten separate studies performed in different regions of Pakistan found that genotype D is the most common HBV genotype in Pakistan, with a total prevalence rate of 63.71 percent , followed by genotype A (10.036 percent), genotype C (7.55 percent) and genotype B (5.335 percent), while mixed genotypes were 2.377 percent and 9.931 percent , respectively (Ali *et al*., 2011). In Syria, Antaki *et al*., (2010) found that 97% of patients had genotype D.

In Taiwan, Yu *et al* ., (2005) showed that that the most common genotype are B and C genotype ,B genotype (81.8%), and C genotype (15.4%). Tran *et al*., (2015) found that HBV genotypes B and C was predominance in Asian patients and our results are coming in agreement with this results . Sunbul, (2014) found that genotype A, B, and C are common in the Asian continent, and viral mutations were frequently associated with genotype C . Our results differ from all previous studies , and genotype C (single and mixture 59%) is the most common .

In infected hepatocytes, HBV will persist and elaborate strategies to evade the immune system have developed, replicating via reverse transcription (Glebe and König, 2014). Drugs approved for HIV reverse transcriptase inhibition lower the viral load of patients with chronic HBV but do not cure the infection. However, safe and reliable vaccines currently available that cause protective antibody levels in > 95 percent of babies, children and young adults can be avoided (Ott *et al* ., 2012).

The effect of HBV genotypes on clinical results has been investigated in various studies. Compared to genotype B, genotype C is associated with rapid development of fibrosis, elevated HCC development rate, recurrence, and metastasis. Compared to A, genotype D may be associated with more serious disease, and genotype F is associated with elevated mortality rates (Rajoriya *et al* .,2017). Although HBV genotypes may not have a major impact on nucleoside / nucleotide antiviral therapy response, genotype responsiveness to IFN is clearly affected.

Compared to those with genotype C, HBeAg-positive patients infected with genotype B have a better response and genotype A responds better than genotype D, especially during short term therapy. Genotype A reacts well to PEG-IFN as well (Raimondi*et al* .,2010) .

Notably, the intergenotype recombination has also been explained previously, and plays an essential role in the history of evolution of HBV infection. Recombination is favored in particular geographical areas (Sugauchi *et al*., 2002 ; Yang *et al*., 2006). Moreover, a recent study showed that recombination between HBV genotypes B and C is also frequent in Asia, including Vietnam (Sugauchi*et al* .,2002; Shi *et al*., 2012). Other recombinants like as A+D, A+E, C+D and G+C have also been observed in various geographical areas (Shi *et al*., 2012). Also, the frequent of mixed genotypes were higher in patients with chronic HBV infection than those with acute hepatitis B infection, cirrhosis and hepatocellular carcinoma, also suggesting that co infection with diverse hepatitis B virus genotypes is associated with alteration in clinical outcomes and pathogenesis (Toan*et al*., 2006).

In this study there was no significantly correlation between genotype and gender .Abbood and Al-Mhanah, (2019), stated there was no significantly different in distribution of genotypes among males and females . Al-Suraifi *et al* ., (2016) found that there was non-significant difference between the genotypes among males and females. Finally, Abdo *et al* .,(2006) found no difference between different genotypes according to gender, all these studies ensure that sex don't impact on genotyping of hepatitis B virus. These results showed no a significant different between viral load with genotype (p= 0.589). Tran *et al* ., 2015 also found there was no obvious relationship between genotype and viral load.Yu *et al*., 2005 found that genotype C HBV was associated with increased viral load, and associations of HBV genotype and viral load with HCC risk were additive.

There was no correlation between Genotype (A,B,D,E,F,G) and age groups (p= 0.086, p= 0.974, p= 0.401, p= 0.514, p= 0.823, p= 0.070), however, only C genotype has a correlation with age groups (p=0.031). Kao *et al* ., (2000) found the prevalence of genotype C tended to decrease by age (41% in carriers aged ≤ 35 years, 13% in those aged > 65 years, $P = 0.03$).

There was no correlation between Genotype and activity ($p \geq 0.05$). The experiments are limited to comparing patients with these two genotypes because of the preponderance of genotypes B and C in Asian countries. About genotypes, however, such comparisons provide very useful evidence on the relationship between genotypes B and C of HBV and the rate of progression of liver disease**.** These studies have clearly shown that HBV genotype B is

associated with spontaneous seroconversion of HBeAg at a younger age, less active liver disease and slower progression to cirrhosis relative to genotype C (Sumi *et al.*, 2003).

Our results reported that all genotype has no a significant different with the concentration of anti HBV core IgM ,(except B and C genotype) which has a highly significant different , this result coincide with result of Jia *et al* ., (2014) .

CONCLUSION

This study concluded that the most common genotype is B pure genotype (30%) , and in the second rank C genotype (16%), however the C mixture genotype (59%) was common followed by B genotype (55%) , F genotype recorded in 5 samples (as a mixed), and also The study recorded the first case infected with genotype G in our province and Iraq according to our knowledge.

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