

Detection of Hepatitis -B virus Genotypes among Chronic Carriers in Duhok - Iraq

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Abstract:

Background: Hepatitis B virus (HBV) is one of the major etiological agents causing acute and chronic liver disease worldwide with significant morbidity and mortality. The high genetic variability of HBV is reflected by eight genotypes (A to H), each with a particular geographical prevalence.

Objectives: The study was conducted to find out HBV genotypes in chronic hepatitis B- (CHB) carriers in association with serological markers of HBV.

Methods: This work was carried on from March to, December 2012 in Duhok/Iraq and enrolled 134 HBsAg positive carrier cases. recruited to Central Public Health Lab. Specific primers PCR technique was used to detect HBV genotypes. The carrier cases were screened for markers of HBV infection by Enzyme Linked Immunosorbent Assay (ELISA). Automated machine for quantitative determination of ALT was used.

Results: The carrier cases were 91 males (67.9 %), 43 females (32.1 %), and their age range was 10-87 year old (mean=31.4 SD± 13.3). Among the studied patients 133(99.2%) were found to have genotypes D including 91(67.9%) males and 42(31.3%) females and only one female patient carried genotype B (0.8%). Anti-HBc(total), IgM anti-HBc, HBeAg and Anti HBeAb were detected in rates of 100%,0%,50%and 46.9 % respectively. The patient with genotype B had positive HBe Ag, negative HBeAb and normal ALT level.

Conclusion: This study revealed that hepatitis B virus genotype D is the main genotype in Duhok/ Iraq followed by genotype B. High percentage of CHB patients with genotype D are HBeAg positive. Most of CHB patients with high ALT level had positive HBeAg rather than negative HBeAg.

Key words: HBV genotype; HBeAg; Nested PCR; ALT; ELISA;

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Introduction:

Hepatitis B virus (HBV) continues to cause viral hepatitis in developed and developing countries. Currently over two billion people have evidence of previous HBV seroconversion infection and 350 million individuals who become chronic carriers of the virus [1]. Genotypes of HBV are considered as natural strains of the virus that appeared during last years and correlate with their distribution in different geographical areas. [2].

The HBV genotypes show 8% difference in the whole nucleotide sequence of the virus and in the surface gene sequence leading to different serotypes [3]. The prevalent genotype in Northwestern Europe, North America and Africa is A [4] while in South-Eastern Asian populations it's genotypes B and C [5]. In the New World and East Africa Genotype E and F are prevalent, on the other side genotype D is present in Europe, Middle East, and India. A new genotype G was discovered in patients from France, America, and Germany and lastly genotype H is detected in patients from Central America [6]. Several genotyping studies of HBV have been done in Middle

East including Iran, Jordan, Syria and Turkey which revealed that the most common genotype is D [7-12] Many laboratory methods have been approved for detection of HBV genotypes as colorimetric point mutation assay [13], enzyme linked immunosorbent assay [14], analysis by restriction fragment length polymorphism [15], line probe assay [16], type specific primers PCR [17], ligase chain reaction assay [18] and direct sequencing [19].

Different studies indicate that the clinical course, development of complications and appearance of AntiHBe antibodies are affected by different genotypes. Other studies found that response of patients with chronic hepatitis B to treatment with interferon depends on the type of genotype B [20].

Several studies reported a correlation between HBV genotype and HBeAg clearance. These studies, all of Asian patients, found that the prevalence of HBeAg was higher in patients with genotype C compared to those with genotype B, this suggest that chronic carriers with genotype B can clear HBeAg more efficiently [21,22]. Similarly, Sumi et al (2003)[23] conducted a large cross-sectional study involving patients with hepatitis B and showed that HBeAg seroconversion rate in patients with chronic liver disease was significantly higher in genotype B patients compared to genotype C. Correlation between HBV

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genotypes and clinical outcome of patients with HBV has also been reported.

Therefore the aims of this study are to detect the prevalent genotypes of HBV in Duhok- Iraq and to reveal the influence of genotype on the clinical course of the chronic carriers.

Materials & methods:

This study was carried out on HBsAg positive carriers recruited from Central Public Health Lab, in Duhok City, Iraq from March 2012 to December 2012. One hundred and thirty- four patients were enrolled in this study, they visited the central lab for regular serological assessment and were selected conveniently: Hepatitis C virus (HCV) and (HIV) infections were excluded through appropriate serological tests. "Informed consent was obtained from every patient prior to sample collection which was performed according to standard protocols approved by the local health authority in Duhok city."The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee.»

Blood samples were collected from (134) patients. Each sample consisting of (5) milliliters was obtained from antecubital vein by a sterile disposable syringe. The blood sample was poured into a clean plain tube without anticoagulant and centrifuged at 3000 rpm for 5 minutes. The serum was separated and stored in multiple marked clean tubes at (-20 °C) for both ELISA and biochemical test, and for PCR assay

The blood samples were tested for the following serological tests to confirm the chronic carriers by ELISA (Bioprobes -Italy):

HBsAg detection by ELISA: All 134 patients were tested for HBsAg

All the HBsAg positive cases were tested for total Anti-HBc antibodies

All the HBsAg, total Anti-HBc positive patients, were tested for Anti HBc-IgM to detect the acute HBV infection. HBsAg positive serum samples that showed positive anti-HBc (IgM) were excluded. Then all chronic carriers were examined for HBeAg, Anti- HBeAb by ELISA. Biochemical test was used to detect the liver enzyme ALT Alanine transferase (GPT) to measure the hepatic cellular damage.

DNA extraction: The QIAamp DNA extraction kit from (QIAGEN GmbH) was used to extract DNA from blood samples following the manufacturer's instructions. DNA extracts were stored at -20°C to be used later for amplification of HBV DNA by nested PCR polymerase chain reaction (Genekam Biotechnology AG-Germany).

HBV genotyping: The nested PCR is based on the use of two types of primers (outer primer pairs) for the first round and (inner primer pairs) for the second round PCR. They were selected from nucleotide sequence of the conserved regions in Pre S1-S gene regardless the HBV genotype. The universal

outer primers included P1 (sense) and S1-2 (antisense). The inner primers were: 1. B2 (sense) combined with mix A which contains antisense primers for genotypes A,B,C. 2. B2R (antisense) in combination with mix B which contains sense primers for genotypes D, E, F.

The second round PCR primers were selected depending upon the conserved nature of the nucleotide sequence in each genotype. Then correlation of genotyping results with the serological markers and ALT levels to set out the effect of genotypes on chronic carriers' status and to find out the probable role of different genotypes in determining the clinical outcome of chronic infection.

Statistical analysis: The differences between different parameters in this study were evaluated by using the chi-square test (X^2) and T test which was used to test the null hypothesis for significant relation between HBV genotypes and clinical outcome. Any value that is below 0.05 was considered significant [24].

Primer	Sequence* (position, specificity, and polarity)
First PCR	
P1**	5'-TCA CCA TAT TCT TGG GAA CAA GA-3' (nt 2823–2845, universal, sense)
S1-2	5'-CGA ACC ACT GAA CAA ATG GC-3' (nt 685–704, universal, antisense)
Second PCR Mix A	
B2	5'-GGC TCM AGT TCM GGA ACA GT-3' (nt 67–86, types A to E specific, sense)
BA1R	5'-CTC GCG GAG ATT GAC GAG ATG T-3' (nt 113–134, type A specific, antisense)
BB1R	5'-CAG GTT GGT GAG TGA CTG GAG A-3' (nt 324–345, type B specific, antisense)
BC1R	5'-GGT CCT AGG AAT CCT GAT GTT G-3' (nt 165–186, type C specific, antisense)
Mix B	
BD1	5'-GCC AAC AAG GTA GGA GCT-3' (nt 2979–2996, type D specific, sense)
BE1	5'-CAC CAG AAA TCC AGA TTG GGA CCA-3' (nt 2955–2978, type E specific, sense)
BF1	5'-GYT ACG GTC CAG GGT TAC CA-3' (nt 3032–3051, type F specific, sense)
B2R	5'-GGA GGC GGA TYT GCT GGC AA-3' (nt 3078–3097, types D to F specific, antisense)

Step	Temp	Duration	Repeat
Initial polymerase activation	95°C	600 sec	1
Denaturation	94°C	20 sec	40
Annealing	55°C	20 sec	
Elongation	72°C	60 sec	

Results:

The studied patients were 91 males (67.9 %), 43 females (32.1 %), and their age ranges were 10-87 year old, (mean=31.4 SD± 13.3). Among the studied patients 133(99.2%) were found to carry genotype D including 91(67.9%) male and 42(31.3%) female and only one female patient carried genotype B (0.8%).

Table (1): Correlation between Genotypes of HBV chronic carriers and Gender

Gender	Genotype B No. %	Genotype D No.%	Total
Male	0	91(100%)	91(67.9)
Female	1(2.3%)	42(97.7%)	43(32.1)
Total	1	133	134

Serological Markers:

Anti-HBc (total): Anti-HBc (total) antibodies were positive for all 134 out of 134 HBsAg positive carrier cases (100%) while anti-HBc IgM antibodies were negative for all 134 carriers (100%).

HBeAg by (ELISA test) was positive in 66 out of 131 studied HBsAg positive carrier cases (50.4%) while anti-HBeAb (by ELISA test) was positive for 61 out of 131 studied HBsAg positive carrier cases (46.5%)

Table (2): Serological Markers of HBV among HBsAg Positive patients

Markers	Positive No.(%)	Negative No.(%)	Total No.(%)
HBc total	134(100%)	0(0%)	134(100%)
HBc-IgM	0(0%)	134(100%)	134(100%)
HBe Ag	66(50.4%)*	65(49.6)	131(100%)
Anti-HBe Ab	61(46.5%)	70(53.5%)	131(100%)

*P>0.05 no significant difference between HBeAg+ve and HBeAg -ve carriers

ALT Levels among the Studied Patients: Abnormal ALT levels were set up to be > 2 times of the upper limit of normal (ULN). (Normal range 0-40 U/L for males, 0-30 U/L for females).

Among 130 HBsAg carriers with detectable HBV genotype D; 35(26.7%) showed raised ALT levels with a mean value of 111.2 U/L and 95(72.5 %) had normal ALT levels with a mean value of 23.9 U/L, while only one patient with HBV genotype B had normal ALT level of 19 U/L (0.8 %).

Table (3): Correlation between HBV Genotype and ALT levels among HBV chronic carriers

ALT	Genotype D	Genotype B	Total
*Raised ALT No. (%)	35(26.7%)	0(0%)	35(26.7%)
Mean (U/L)±SD	111.2 (23.27)	0	0
Normal ALT No. (%)	95(72.5 %)	1(0.8 %)	96(73.3%)
Mean (U/L)±SD	25.5 (6.029)	19	131

Table (4): Correlation between HBe Ag and ALT levels among HBV-chronic carriers

	Raised ALT *			Normal ALT			Total
	No.%	Mean U/L±SD	Mean Age	No.%	Mean U/L±SD	Mean Age	
+ve HBeAg	22(62.8%)	124.8** (20.39)	26.2 year	44(45.87%)	26.4 (4.85)	28.7 year	66
-ve HBeAg	13(37.2%)	91.9 (10.49)	36.0 year	52(54.2%)	21.8 (3.35)	35.0 year	65
Total	35			96			131

*> 2 ULN (Upper limit of normal), normal range 0-40 U/L male, 0-32 U/L in female.

**Pvalue<0.05significant correlation was found

A significant correlation was found between mean ALT level of HBeAg positive carriers and HBeAg negative carriers with high ALT level (P value<0.05).

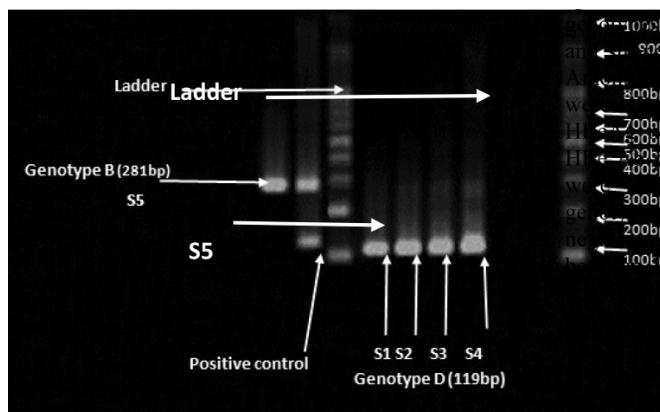


Figure1. Results of HBV genotypes revealed by 2% gel electrophoresis showing S1,S2,S3 and S4 for genotype D (119bp) and S5 for genotype B (281bp)

Discussion:

This is the first study performed in Duhok city and probably in Kurdistan Region/ Iraq to detect HBV genotypes. The main result of this study was the detection of genotype D in almost all HBV carriers (99.2%) followed by genotype B that constituted only 0.8% while genotype A, C, E and F were not

*>2 ULN (Upper limit of normal), normal range 0-40 U/L male, 0-30 U/L in female.

ALT Levels among HBeAg+ve and HBeAg-ve Carriers: Among 66 carriers with positive HBeAg 22 (33.3%) showed raised ALT levels with mean value of 124.8 U/L while 44 (66.7%) had normal ALT levels with mean value of 26.4 U/L. while among 65 carriers with negative HBeAg 13 (20%) had raised ALT levels with mean value of 91.9 U/L, and 52(80%) had normal ALT levels with mean value of 21.8 U/L. Table (4.). So 35 carriers had raised ALT, 22 of them (62.8%) with positive HBeAg and 13 (37.2%) were HBeAg negative.

found in any case. These findings conform to a study done in Egypt by Saady et al (2003)[7] who studied the genotypes of HBV isolated from 105 serum samples of Egyptian carriers by sequencing and found genotype D in all 105 carriers. The results of this study goes with other studies revealing that prevailing genotype in Middle East is D [25, 26]. A study done in Syria showed that 97% of the enrolled patients were of genotype D, and 72% were HBeAg negative [12]. Moreover, a study in Turkey revealed that all patients included in the study had genotype D[8] . In addition, two studies in Iran revealed that genotype D was found in all patients [10,27].

Nazish et al [28] from Pakistan investigated in 2012 (214) samples and only genotypes C and D were identified in local population with (9.8%) of genotype C and (90.2 %) of genotype D. So in conclusion, results of HBV genotyping in Duhok/Iraq are in agreement with all other studies done in the Middle East mentioned above. Many studies concluded that HBV genotypes and serotypes are good markers for clinical and epidemiological assessment [29,30], knowing that genotypes have distinct geographical distribution and strongly associated with ethnic groups [31,32]. Among our 130 patients with genotype D, 65(50%) were HBe Ag positive and the other 65(50%) were HBeAg negative, while 61 (46.9 %) patients had HBe Ab positive and the other 69 (53.1 %) patient were HBe Ab negative. Only one patient with genotype B was HBeAg positive and HBeAb negative. No significant

association was detected between HBeAg+ve and HBeAg –ve carriers with genotype D in response to different phases. Regarding chronic active hepatitis (CHB) patients, 37.1% of them were HBeAg –ve while the remaining 62.9 % with HBeAg +ve, but in other studies from Syria and India it was found that genotype D was more and often associated with HBeAg negative chronic active hepatitis B (CHB), and more severe course [33,34,12]. In another study, it was observed that HBeAg negative state predominated in patients with genotype D (presumably indicating pre-core or core promoter mutation) [35]. So the rate of HBeAg –ve (CHB) in our study was less than that detected in the above mentioned studies. This indicates that the rate of core/precore mutation is less in the studied patients in Duhok in contrast to the other studies. The mean age of patients was significantly higher in the anti-HBe-positive group as compared with the HBe- Ag-positive group. This result was similar to other studies from Iran and Turkey [36, 37]. The mean value of ALT in CHB patients with +ve HBeAg was higher than in CHB with –ve HBe (124.8 verses 91.9 IU/L) P value<0.05. In a study done by Muhammad et al (2010)[38] in Duhok found that HBeAg-positive patients had elevated ALT level with mean value of 96.2 IU/L while among HBeAg-negative patients with elevated ALT level the mean value was 74.2 IU/L. Another study by Abbas (2006) in Iraq showed that 61.2% of HBeAg-positive patients had elevated ALT level and 38.8 % had normal ALT level[39]. In contrast to our results (Sevgi et al., 2012) from Turkey found that no significant differences observed between the HBeAg status and ALT levels [37].

The mean ALT level in HBeAg-positive patients was 1.35 times higher compared to HBeAg negative patients, which could be due to a higher degree of inflammation. This is close to result obtained by another study from China which found that about 60% of HBe Ag +ve patients had ALT up to 1.5 times the normal level of HBe Ag –ve [40]. So in CONCLUSION: Hepatitis B virus genotype D was the main and almost the only one genotype in Duhok (99.2%) followed by genotype B (0.8%). High percentages of active CHB patients with genotype D were HBeAg positive (62.9%), which may indicate low rate of pre core /core mutant ratio in the studied patients. Most patients with high ALT level were HBeAg positive.

Author's contribution:

Study design and conception: Ibrahim 40%, Amer 60%

Acquisition of data: Ibrahim 60%, Amer 40%

Analysis and interpretatin of data: Ibrahim 40%, Amer 60%

Drafting a amnscript: Ibrahim 50%, Amer 50%

Critical revision: Ibrahim 40%, Amer60%

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