

Genotypic Study of Hepatitis Type B in Al- Diwaniya Province.

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Abstract:

Hepatitis B (HBV) with its possible serious sequels of liver cancer and cirrhosis is a well recognized worldwide health problem. These problems are particularly occurring as a complication of the chronic form of the disease. Relying on the sequence homogeneity, there are Ten HBV genotypes (A-J). In the current study nested PCR was utilized to spot HBV genotypes among hepatitis type B in Al- Diwaniya Province/ Iraq.

A total of 80 blood samples were collected from patients clinically suspected to have HBV hepatitis and diagnosed basically as HBs Ag positive. The study sample included 59 males and 21 females of ages (12-75 years). Recorded results for HBV DNA were as follow: 33(41.25%) samples were positive for HBV DNA while 47 (58.75%) samples were negative for HBV.

Nested PCR results done on the positive samples for HBV genotyping showed that genotype E was the only single genotype in a percentage of (3%), while most of the samples showed mixtures of more than one genotype as follows: A+E (3%) , C+D+E (12.12%) , B+C+D+E (15.15%) , A+D (3%) , A+B+C+D+E (18.18%) , A+B+D+ E (18.18) , B+D+E (3%) , A+C+D+E (3%) ,B+C+D (6%) , A+B+D (3%) , A+B+C+D (6%) , D+E (6%) . Genotype F was not found in any sample in this study. There was no significant difference in distribution of genotypes between males and females (P.Value = 0.369).

Introduction

Variable Hepatitis B genotypes have different geographical distribution, different way of disease progression and prognosis. These

factors justify the importance of genotyping of the virus (1). Several studies were conducted In Iraq that studied HBV prevalence and genotyping. In Wasit/ Iraq, all the tested samples were of mixed genotypes. The main mixture was “A +B+ C+ D+ E” in a percentage of (77.7%) and the other combinations were of less percentages ; “A+ B+ D+ E” , “A+ B+ C” , “A+ B+ E” , “A+ D+ E” of percentages of (16.6%), (2.7%), (1.3%) and (1.3%) respectively. No single genotype was found (2). In Duhok, North of Iraq a study stated that genotype D is the main genotype and the second genotype was B. High percentage of chronic hepatitis type B patients with genotype D were HBe Ag positive (3). A similar study in Basra/ South of Iraq found that the main single genotype was genotype D and the main mixture of genotypes was of D+E (4). The current study was conducted to establish the main genotypes of HBV clinical cases from Al-Diwaniya in the middle of Iraq.

Materials and Methods

The primer for the detection of the virus as well as the primers used for genotyping were supplied by “Bioneer. Korea” with the design described by Naito (5). (Table 1).

Table (1): The PCR and nested PCR primers sequences and size of amplicon for HBV:

	Primer	Sequence (5'-3') & Amplicon size
HBV -PCR	P1 (universal, sense)	“TCA CCA TAT TCT TGG GAA CAA GA” (1063bp)
	S1-2 (universal ,anti sense)	“CGA ACC ACT GAA CAA ATG GC”
Nested PCR- Mix A genotyping (A,B,C)	B2 (types A to E, sense)	“GGC TCM AGT TCM GGA ACA GT”
	BA1R (type A, antisense)	“CTC GCG GAG ATT GAC GAG ATG T” (68bp)
	BB1R (type B, antisense)	“CAG GTT GGT GAG TGA CTG GAG A” (281bp)
	BC1R (type C, antisense)	“GGT CCT AGG AAT CCT GAT GTT G” (122bp)

Nested PCR-Mix B genotyping (D,E,F)	BD1 (type D, sense)	“GCC AAC AAG GTA GGA GCT” (119bp)
	BE1 (type E, sense)	“CAC CAG AAA TCC AGA TTG GGA CCA” (167bp)
	BF1 bb (type F, sense)	“GYT ACG GTC CAG GGT TAC CA” (97bp)
	B2R (types D to F, antisense)	“GGA GGC GGA TYT GCT GGC AA”

Five millimeter of venous blood were collected from each of the 80 patients. The serum has been collected in Eppendorf tube then stored at -20c to be used for DNA extraction and nested PCR technique.

Extraction of DNA

Genomic viral DNA was extracted from utilizing “gSYAN Genomic DNA Mini Kit” (Geneaid, USA).

Detection of HBV

PCR master mix was prepared for direct detection of HBV by using (AccuPower[®] PCR PreMix Kit). The resultant mixture was 20 µl. which is constituted of P1 gene primer(10pmol) 1µl, S1-2 gene primer(10pmol) 1µl , PCR water 13µl and DNA template (DNA extraction) 5µl .

After that, these PCR master mix components that mentioned in placed in standard AccuPower PCR PreMix Kit that contains all other components which needed to PCR reaction such as (Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, MgCl₂, stabilizer, and tracking dye). Then, all the PCR tubes transferred into Exispin vortex centrifuge at 3000rpm for 3 minutes. the Thermocycler program was as follow: Primary denaturation step by incubation at 94 °C for 5 minutes , proceeded for 40 cycles. Each cycle include the following: 1- denaturation at 94 °C for 30 seconds. 2- annealing at 55 °C for 1 minute. 3-elongation at 72 °C for 1.5 minute . 4- Extension at 72 °C for 5 minutes.

Genotyping Method

Direct detection of HBV genotypes was performed by means of two mix reactions: Mixed A reaction for the detection of the genotypes (A,B,C) and mixed B reaction for genotypes (D,E,F). Each positive serum sample was subjected for both reaction A and B. The master mix constituents were supplied by “(AccuPower[®] PCR PreMix Kit)”. The nested PCR master mix was composed of B2, BA1R, BB1R, B2, BA1R, BB1R (5 µl) or BD1, BE1, BF1 bb, B2R primers (10 pmol), PCR water (15 µl), DNA template (DNA template) with addition of 1 µl aliquot DNA product of the primary PCR step from each positive sample.

The thermocycler conditions of nested PCR were programmed for 40 cycles of; 20 cycles of 1- 94 °C for 30 seconds. 2- 58 °C for 30 seconds. 3- 72 °C for 40 seconds, and another 20 cycles of 1- 94 °C for 20 seconds. 2- 60 °C for 30 seconds. 3- 72 °C for 40 seconds, with the final extension at 72 °C for 5 minutes.

Electrophoresis of the products was performed using 1% agarose gel stained with ethidium bromide followed by visualization under UV trans illumination. A ladder of 100bp DNA was used for the prediction of the PCR products sizes and consequently determining the genotypes for each sample.

Results and Discussion:

HBV DNA detection by PCR

Out of the (80) serologically positive samples for HBV tested by PCR technique for HBV DNA, Thirty three samples (41.25%) were found to be positive, while forty seven (58.75%) were found to be negative (Table 2) (Figure (1)).

Table 2: HBV DNA by PCR distribution by gender among (33) positive samples

Gender	No. of sample	No. of positive sample	%
Male	59	24	40.67
Female	21	9	42.86
Total	80	33	41.25

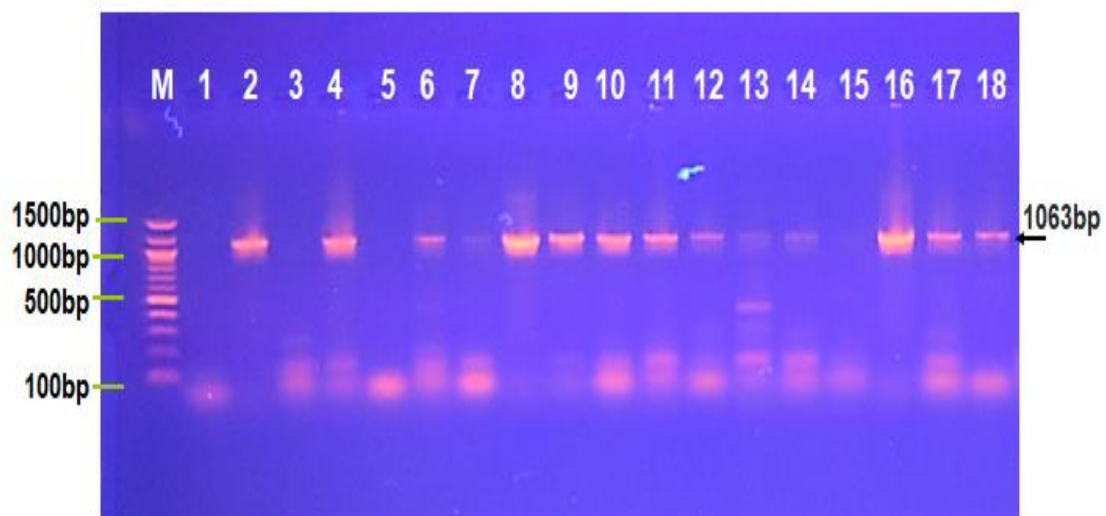
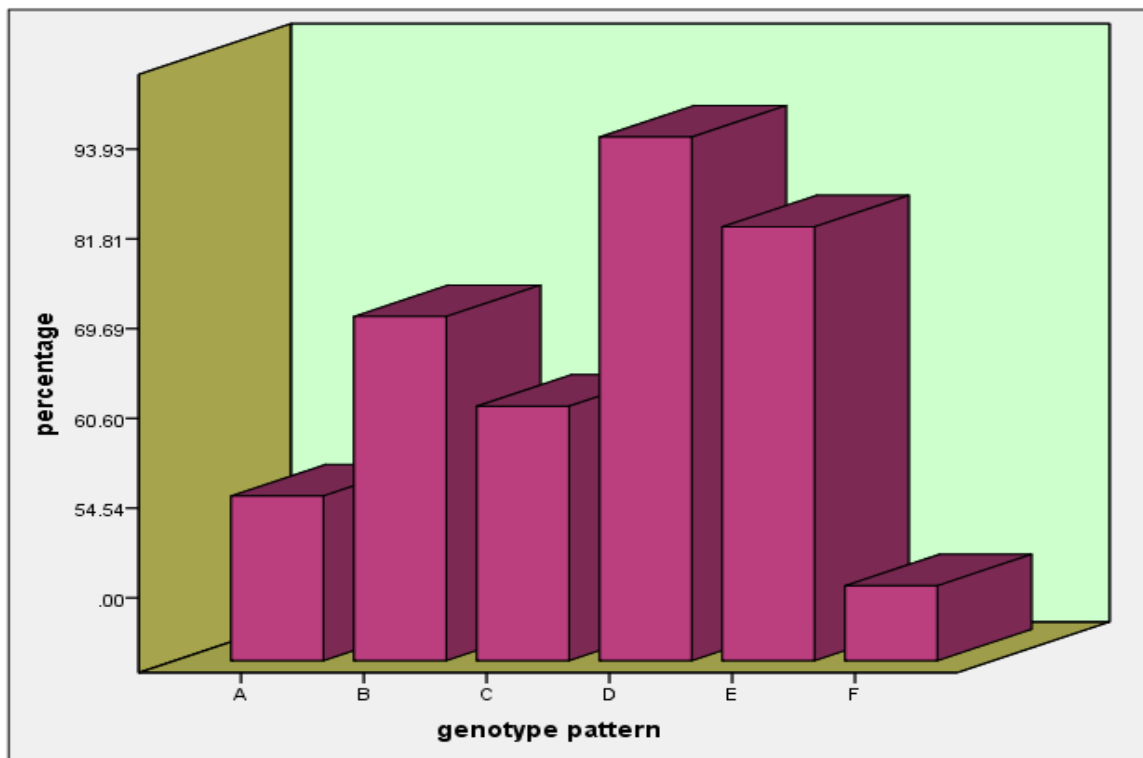


Figure (1): “Gel electrophoresis” of PCR assay product study for HBV from serum samples. M “Marker ladder 1500-100bp”. Lane (1-18) positive HBV at 1063bp size.

The discrepancy between the results of the screening ELISA test and PCR does not deny the occurrence of infection with HBV in these 47 patients. HBs Ag is still regarded the main HBV serological indicator in the confirmation of the occurrence of the HBV infection (6 and 7). This

may be explained by the presence of the what is termed “non-replicative phase” in the chronic HBV disease course since its natural course is known to pass through four phases which are HBs Ag positive but some are non-replicative (8 and 9). Furthermore, it is recognized that seroconversion of HBe Ag positive into HBe Ab positive is common in patients at the non-replicative phase. HBV DNA during this phase is merely undetectable in serum HBV DNA levels referring to an immunological response to the infection (9 and 10). This conclusion is agreed by other studies in other areas in Iraq where similar results were obtained (12 and 2).

The present study established no significant difference between



female and male patients.

HBV Genotypes

Although different genotypes were all presented in different mixed forms, but there were different percentages for each genotype. According to this study, the different percentages of the different genotypes were as follows: genotype D in a percentage of (93.94%), followed by genotype E in a percentage of (81.82%). Genotype B percentage was (69.70%), and C was (60.61%) while A percentage was (54.55%) as shown (Figure2).

Figure.2: Number and percentage of each Genotype found in the genotype mixture of HBV documented by” nested PCR” among (33) patients.

In this study, only one single genotype E (3%) was found among other genotypes which were all present in mixed forms (Figure.3) (Figure.4) (Figure.5).

The distribution of different mixtures of the different HBV genotypes in this study were as the following percentages: A+E (3%) , C+D+E (12.12%) , B+C+D+E (15.15%) , A+D (3%) , A+B+C+D+E (18.18%) , A+B+D+ E (18.18%) , B+D+E (3%) , A+C+D+E (3%) ,B+C+D (6%) , A+B+D (3%) , A+B+C+D (6%) , D+E (6%) , Genotype F was the only genotypes not discovered in any of the patients samples. (Figure 3.).

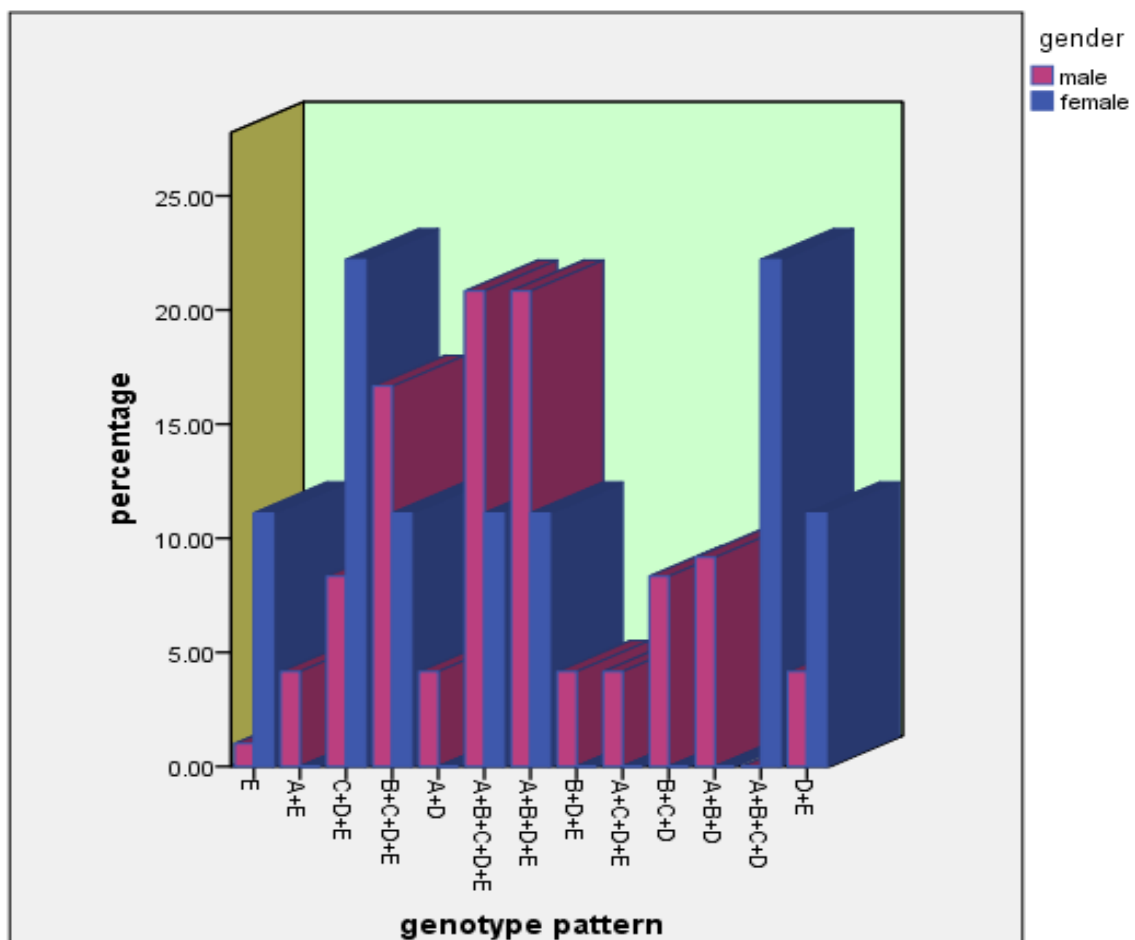


Figure 3. Genotypic mixtures of HBV in males and females

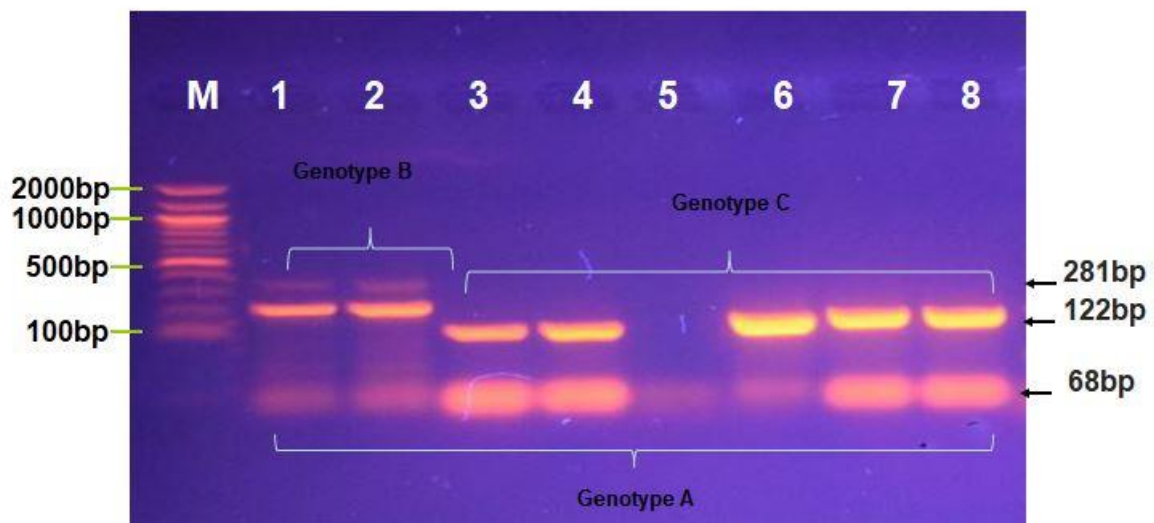


Figure (4): Electrophoresis of product of PCR assay for HBV genotypes A,B, and C from positive samples. M “Marker ladder 1500-100bp”. Lane 1 and 2 at 281 bp (genotype B). Lane 2, 4, 6, 7 and 8 at 122bp (genotype C). Lane 1-4 and 6-8 at 86 bp (genotype A)

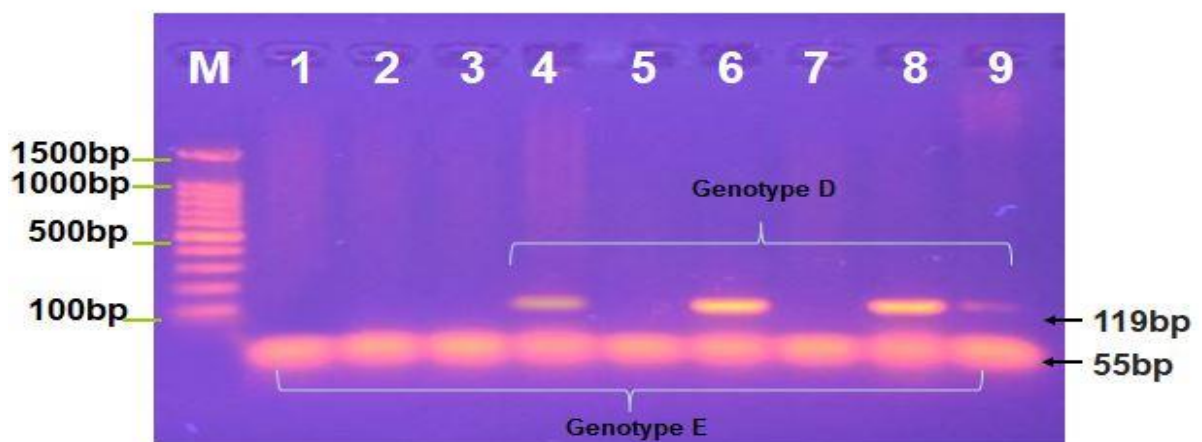


Figure (5): Gel electrophoresis of PCR assay product study for HBV genotypes D,E, and F from positive samples. M “Marker ladder 1500-100bp”. Lane 1 -9 at 55 bp (genotype E). Lane 4, 6, 8 and 9 at 119bp (genotype D).

HBV is known to be differentiated into 8 genotypes (A to H) (13). These genotypes have some variation in pathogenesis, virulence, and other factors affecting the outcome of the disease and response of the immune system to the infection as well as treatment (1 and 5). Genotypes “A, C and D” are the most common at Asia (14). The 6 major

genotypes (A-F) were chosen to search for in this study using nested PCR technique described by Naito (5). This method is approved to be highly sensitive for recognition of HBV genotypes (15).

In the current study, the majority of the patients having HBV were confirmed to have a mixture of more than one genotype (96.97%), while it is found that only small proportion showed a single genotype infection (3.03%) for genotype E. These results are consistent with the conclusion of a study in Sulaimania in Iraq used the same technique. This study reported that all the examined samples were of a mixed genotypic viruses (16). In Wasit province, a similar study were conducted and reported that that 100% of the samples were of mixed genotypes. The combination of the genotypes “(A, B, C, D and E)” was the major cause (77.7%), while it is found that the mixture of (A, D and E) was the least (1.3%). Furthermore, no single genotype were recognized and no sample were found to contain genotype F neither single nor mixed (2). In Duhok, North Iraq a study reported that the predominant genotype causing HB infection was genotype D and genotype B is coming next to it (3). Another study in Baghdad stated similar results (17).

In Asia, the main well documented genotypes to be predominant are (A-G), mainly “A, C and D”. “A, C, D and G” genotypes are the predominant genotypes in Europe. Mixed genotypes “A+C and C+D” are the main mixtures in Asia, while “C+D” in Europe and “A+D” are recognized in Africa (14)..

The opportunity of the occurrence of mixture of multiple genotypes may be explained by the assortment between multiple digenotypes (18). In Wasit/ Mid Iraq, a researcher found a high incidence of mixed genotypic infections (2). Patterns of genotypic mixtures were documented in Sulaimaniya, North of Iraq where they attributed this high percentages to the emigration movements of refugees to Europe and longstanding periods in refugee camps, at which many opportunities to get infection from other nationalities in the same camps (16). In addition, low hygienic levels may enhance the disease transmission particularly in dental clinics, hairdressing and tattoo salons are other important risk factors to HBV transmission.

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