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# Detection of Hepatitis B Virus (HBV) Genotypes by Nested Polymerase Chain Reaction (PCR) Technique in Diyala Province

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## Abstract

Hepatitis B virus (HBV) infection is a public health concern because it causes liver diseases such as hepatocellular carcinoma and liver cirrhosis. Depending on the virus sequence homogeneity, ten HBV genotypes (A-J) were identified. The aim of this study is the molecular and serological detection of HBV utilizing the nested polymerase chain reaction (PCR) technique, and biochemical test for liver function to estimate the levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and Alkaline phosphatase (ALP). A cross-sectional study was conducted in170 reviewers (103 males and 67 females) who attended the Baquba Teaching Hospital, Teaching laboratories, the Main Blood Bank, and Baladrooz General Hospital. Age ranging 13-75 years. The samples were collected from Diyala Province of Iraq, Enzyme- Linked Immune Sorbent Assay test (ELISA) was used to detect hepatitis B surface antigen (HBsAg) in serum. Automated machine (Mindray Bs-240 system) for quantitative determination of (ALT, AST, ALP) was used. Conventional Polymerase Chain Reaction (PCR) of HBV p gene was performed for HBV- DNA detection from serum samples, and Nested PCR technique with specific primers was used to detect HBV genotypes. Results of ELISA showed that 70 (41.20%) samples were positive and 100 (58.80%) samples were negative. PCR results showed that 14 (8.20%) samples were positive for HBV- DNA. Statistically, the positivity of HBV detected by ELISA was higher (41.2%) than positivity of

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HBV detected by PCR (8.20%). Liver function test found the levels of ALT (36.31±12.00), AST (47.92±21.00) ALP (100.24±45.92) was high in patients than controls. ALP and AST scored the highest sensitivity than ALT, while based on specificity, ALT and ALP scored the highest specificity than AST.

**Keywords**: Hepatitis B virus, ELISA, Liver function test (ALT, AST, ALP), Nested PCR.

# تحديد الانماط الوراثية لفيروس التهاب الكبد نوع بي بواسطة تفاعل البلمرة المتسلسل المتداخل في محافظة ديالى

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## الخلاصة

تعد عدوى فيروس التهاب الكبد نوع بي ( HBV)مشكلة صحية عامة لأنها تسبب أمراض الكبد مثل سرطان الخلايا الكبدية وتليف الكبد. اعتمادا على تجانس تسلسل الفيروس، تم تحديد عشرة أنماط وراثية من ( HBV (A-J) الهدف من هذه الدراسة هو الكشف المصلي والجزيئي عن فيروس التهاب الكبد B باستخدام تقنية تفاعل البلمرة المتسلسل (PCR) ، والاختبار البيوكيميائي لوظيفة الكبد لتقدير مستويات اللانين أمينوترانسفيراز (ALT) والأسبارتات أمينوترانسفيراز (AST) والفوسفاتيز القلوي (ALP). أجريت دراسة مقطعية على 170 مراجعًا (103 ذكور و 67 إناث) حضروا إلى مستشفى بعقوبة التعليمي والمختبرات التعليمية ، وبنك الدم الرئيسي ، ومستشفى بلدروز العام. تتراوح أعمارهم بين 13-75 سنة. تم جمع العينات من محافظة ديالى العراقية تم استخدام اختبار مقايسة الممتز المناعي المرتبط بالإنزيم (ELISA) للكشف عن مستضد التهاب الكبد B السطحي (HBs Ag) في مصل الدم. تم استخدام جهاز اوتوماتيكي (جهاز Bs-240 مندريه) للتحديد الكمي لـ (ALT و AST وALP ). تم إجراء تفاعل البلمرة المتسلسل التضخمي التقليدي (PCR) لجين HBV pللكشف عن DNA فيروس التهاب الكبد B في عينات المصل وتم استخدام تقنية تفاعل البلمرة المتسلسل المتداخلة مع بادئات محددة للكشف عن الأنماط الجينية لفيروس التهاب الكبدB . اظهرت نتائج ELISA كالتالي (41,20٪) عينه إيجابية و 100 (58,80.٪) عينة سلبية .كانت نتائج تفاعل البلمرة المتسلسل 14 (8,20٪) عينة موجبة لـHBV- DNA . إحصائياً كانت إيجابية HBV المكتشفة بواسطة ELISA عالية بنسبة (41,2٪), اما إيجابية HBV المكتشفة بواسطة PCRفكانت (8,2٪). وجد اختبار وظائف الكبد أن مستويات ALT (36,31 ±12,00) و AST( 47,92 ± 21,00)و ALP(100,24 ±45,92) حيث كانت مرتفعة في المرضى مقارنة بعينة السيطرة. وسجل ALP و AST أعلى حساسية من ALT بينما بناءً على النوعية ، سجل ALT و ALP أعلى خصوصية منAST .

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**الكلمات المفتاحية**: فيروس التهاب الكبد نوع B، ELISA الاليزا ، اختبار وظائف الكبد ( ALT، AST، ALP ), PCR المتداخل.

## Introduction

Hepatitis B virus (HBV) infection is considered a global public health problem because it causes significant morbidity and mortality in the liver. Globally, around two billion people are infected with HBV [1]. Hepatitis B virus (HBV) is a partly double-stranded DNA virus that belongs to the genus *Orthohepadnavirus* and the family Hepadnaviridae. It is one of the leading causes of acute or chronic HBV infection worldwide [2]. Although extensive vaccination programs and developments in the treatment of hepatitis B (HBV) in many countries have resulted in a reduction in the disease's spread, inadequate vaccination coverage and the disease's status as a major global health concern have resulted in a high HBV global burden [3]. Geographical variation in HBV genotype distribution may be an indication of how the virus spreads. North America and North West Europe are the main locations for genotype A[4]. B and C genotypes are extremely common in Australia and Asia [5]. Genotype D is found throughout the world, with the highest proportion in the Middle East and Southern Europe while, genotype E is nearly restricted to West Africa however, genotype F is found in Central and South America[6]. Several researches on HBV prevalence and genotyping were conducted in Iraq. In Wasit Al-Suraifi *et al*. [7] discovered that all samples had mixed genotypes, no single genotype was determined, and no patient possessed genotype F. In Duhok, Kurdistan region of Iraq, Abdulla and Goreal,[8] discovered that genotype D is the dominant genotype, followed by genotype B while in Basra Al-Aboudi and Al-Hmudi,[9] were suggested (92.3 %) had genotype D and (7.69 %) had mixed genotypes D+ E. This study aimed to determine the genotypic distribution of HBV in patients of Diyala / Iraq.

## Materials and Methods

### Samples collection

Blood samples were collected by venipuncture from 170 reviewers (103 males and 67 females), who attended the Baquba Teaching Hospital, Teaching Laboratories, the main blood bank and Baladrooz General Hospital. Age ranging 13-75 years. The samples were collected from Diyala Province of Iraq, during the period November 2021 to January 2022. Blood samples (five millimeter of venous blood) were drawing by disposable syringe under a septic technique. Each blood sample was collected directly in a sterile tube without anticoagulant and allowed to clot then serum was separated by centrifugation at 4000 rpm for 4 minutes. The serum was separated and stored in multiple marked clean tubes then stored at -20$^{o}$c to be used for ELISA, biochemical test for liver function, DNA extraction, and PCR technique.

### Hepatitis B virus screening test by ELISA

One hundred and seventy samples were tested for the detection of Hepatitis B surface antigen (HBs Ag) using ELISA kit (fortress - U.K). This kit employ monoclonal antibodies (IgG antibody to HBsAg), in the sandwich type method. The procedure was done following manufacturer's instructions. Blank, three negative control and two positive controls were included when the Kit was run. The optical densities (O.D.) of each well were measured at wave length 450 nm. The cut off value equals the mean absorbance value for three negative controls×2.1.The calculated absorptions of the samples were compared with the cut-off value. Positive results samples giving an absorbance greater than or equal to the Cut-off value .Negative results samples giving an absorbance less than the Cut-off value.

$$\text{Cut- Off- value (C.O.)} = \text{NC} \times 2.1$$

NC: The Mean Absorbance Value for 3/ Negative- Controls

**Biochemical Test**

The liver function test was used to measure the levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) to assess cellular damage in the liver.

**DNA Extraction**

Viral genomic DNA was extracted from serum samples using extraction kits each of which contains sufficient reagents for 48 purifications according to the manufacturer guidelines (Maxwell® 16 Viral Total Nucleic Acid Purification Kit, CAT.# AS1150, Promega, USA).

**HBV genotyping**

All of DNA that has been extracted from HBV samples in the current study underwent PCR procedure in order to target p gene that is under study. Reactions mix was prepared according to the manufacturer's instructions (Promega, USA) using the in previous studies [10] and provided by (Macrogen, Korea), as shown in table (1). PCR thermocycler condition included initial denaturation step for 5 minutes at 95°C, then cycling step including 30 cycles of 95°C for 30 second , 55°C for 30 second, and 72º C for 30 second followed by final extension for 7 minutes at 72º C, and hold for 10 minutes at 10º C. Nested PCR master mix was prepared for direct detection of HBV genotypes (A,B,C)in Mixed A reaction and genotypes (D,E,F) in mixed B reaction by using PCR kit (GoTag Green Master Mix) and this master mix done according to company instructions. Nested PCR thermocycler system condition included initial denaturation step for 5 minutes at 95°C, then cycling step including 30 cycles of 95°C for 30 second , 60°C for 30 second, and 72º C for 30 second followed by final extension for 7 minutes at 72º C, and hold for 10 minutes at 10º C.

**Table1:** The PCR primers with their sequence and product size.

| Primer | Sequence (5'-3') | | Product Size (bp) |
|---|---|---|---|
| First PCR –HBV- universal sense Primer | P1 | TCA CCA TAT TCT TGG GAA CAA GA | 1063bp |
| First PCR –HBV- universal antisense Primer | S1-2 | CGA ACC ACT GAA CAA ATG GC | |
| Nested PCR-Mix A sense primers | B2 | GGC TCA AGT TCA GGA ACA GT | 68bp |
| Genotype A antisense primer | BA1 R | CTC GCG GAG ATT GAC GAG ATG T | |
| Genotype B antisense primer | BB1 R | CAG GTT GGT GAG TGA CTG GAG A | 281bp |
| Genotype C antisense primer | BC1 R | GGT CCT AGG AAT CCT GAT GTT G | 122bp |
| Nested PCR-Mix B antisense Primers | B2R | GGA GGC GGA TCT GCT GGC AA | 119bp |
| Genotype D sense Primer | BD1 | GCC AAC AAG GTA GGA GCT | |
| Genotype E sense Primer | BE1 | CAC CAG AAA TCC AGA TTG GGA CCA | 167bp |
| Genotype F sense Primer | BF1 | GTT ACG GTC CAG GGT TCA CA | 97bp |

**Statistical analysis**

Data of current study were analyzed by using statistical package for social studies SPSS version 25.0 and Graph pad prism version 6. Categorical variables were compared by Pearson-Chi-square test or two-tailed Fisher exact probability (p). A level of P values ≤0.05 was considered statistically significant.

## Results

The present study included 103(60.60%) male and 67(39.40%) female reviewers. Age ranging 13-75 years. The positivity of HBV detected by ELISA of reviewers was high (41.20%) than healthy (0.00%), as shown in table (2). Hepatitis B surface antigen (HBs Ag) is regarded as the most important HBV serological marker for infection diagnosis and detection. The results in the current study were reported 41.20% positivity rate of HBs Ag. This result does not match with other research in Iraq, numerous researches reported a positivity rate of Hepatitis B surface antigen (HBs Ag) of 0.4 %, 3 %, 1.3 %, 3 %, and 0.7 %, respectively [11-15]. Research round

the world have revealed varying HBs Ag positive rates, in Iran Hosnie [16] reported a HBs Ag rate of (3.8%), while in Saudi Arabia Faisal *et al*. [17] reported (3.24%) HBs Ag positivity rate and in Turkey Ayla *et al*. [18] reported HBs Ag positivity rate of (0.3%).The disagreement between these results could be attributable to the study's design, as the current study was cross-sectional. This could be attributable to viral serum load, the sensitivity and specificity of the ELISA test used, sample size, or geographical location.

**Table2:** Comparative of ELISA (HBs Ag) test between study groups

| Variables | | | Groups | | Total | P value |
|---|---|---|---|---|---|---|
| | | | Reviewers (n=170) | Controls (n=30) | | |
| ELISA (HBsAg) | Positive | N | 70 | 0 | 70 | P<0.001* RR=1.30 (1.11-2.11) OR=42.72 (23.19-100.12) Sn= 41% Sp.=100% |
| | | % | 41.20% | 0.00% | 35.00% | |
| | Negative | N | 100 | 30 | 130 | |
| | | % | 58.80% | 100.00% | 65.00% | |

Abbreviations: relative risk (RR), odd ratio (OR), sensitivity (Sn), specificity (Sp).

*Significant difference between proportions using Pearson Chi-square test at 0.05 level

The results of liver function tests revealed that the levels of ALT, AST, and ALP in patients were high (36.31±12.00, 47.92±21.00, and 100.24±45.92) respectively, than controls as shown in table (3). The sensitivity of ALP(83%) and AST(60%) scored highestthan ALT (42%) with significant difference. Based on specificity, ALT(73%) and ALP(56%) scored highest specificity than AST (46%) with significant difference, as shown in table (4). Regarding to liver function test, patients had elevated levels of ALT, AST, and ALP parameters. In Duhok, Saadi *et al*. [1] found increasing ALT levels in chronic hepatitis B were substantially linked with detectable HBsAg, HBeAg, and HBeAb levels. In Najaf, Baqer Almayali and Hussein[19] showed high levels of ALT, AST, and ALP parameters in liver patients than controls, and these results compatible to our results. This means that a higher ALT level indicates a more aggressive response to the replicating HBV and, as a result, more hepatocyte damage[20]. The elevated levels of (ALT, AST, ALP) were most likely to be within the ‘active stage’ of infection, which indicate the presence of intrahepatic necroinflammation and can be linked to liver damage.

**Table 3:** Comparative of liver functions tests between study groups.

| Groups | | N | Mean± SD | P value |
|---|---|---|---|---|
| ALT | Patients | 70 | 36.31± 12.00 | P<0.05* |
| | Controls | 30 | 24.71± 9.73 | |
| AST | Patients | 70 | 47.92± 21.00 | P<0.01* |
| | Controls | 30 | 24.15± 9.61 | |
| ALP | Patients | 70 | 100.24± 45.92 | P<0.001* |
| | Controls | 30 | 61.84± 15.36 | |

*Significant difference between proportions using Pearson Chi-square test at 0.05 level.

**Table4:** Sensitivity and Specificity of liver function parameters.

| Variables | AUC | Std.Error | P value | Sensitivity% | Specificity% |
|---|---|---|---|---|---|
| ALT | 0.339 | 0.043 | P<0.01* | 42 | 73 |
| AST | 0.582 | 0.052 | p>0.05 | 60 | 46 |
| ALP | 0.815 | 0.034 | P<0.001* | 83 | 56 |

* Significant difference between proportions using Pearson Chi-square test at 0.05 level.

The present study revealed there is significant different (p<0.05) between positivity of HBV detected by PCR and study groups(reviewers and controls), where it was found the positivity of HBV of reviewers was high 14(8.20%) than control group 0(0.00%), while 156 (91.80%) were negative, as shown in table (5). Similar results were obtained by Al-Mhanah and Abood [21] found from 80 patients infected only 33 samples (41.25%) were positive for PCR. In Iran from (150) infected patients only 28(18.7%) samples were positive [22] while, in Turkey from (170) infected patients only 108(63.5%) samples were positive [23].

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**Table 5:** Detection of HBV-DNA by conventional PCR.

| Variables | | | Groups | | Total | P value |
|---|---|---|---|---|---|---|
| | | | Reviewers (n=170) | Controls (n=30) | | |
| PCR | Positive | N | 14 | 0 | 14 | P<0.05<br>RR=1.19 (1.01-2.04)<br>OR=5.72 (2.99-8.17)<br>Sn= 8% Sp.=100% |
| | | % | 8.20% | 0.00% | 7.00% | |
| | Negative | N | 156 | 30 | 186 | |
| | | % | 91.80% | 100.00% | 93.00% | |

Abbreviations; relative risk (RR), odd ratio (OR), sensitivity (Sn), specificity (Sp).

The Nested PCR products after amplification using specific primers have been presented to the gel electrophoresis, which showed the bands, as shown in the figures (1) and (2).

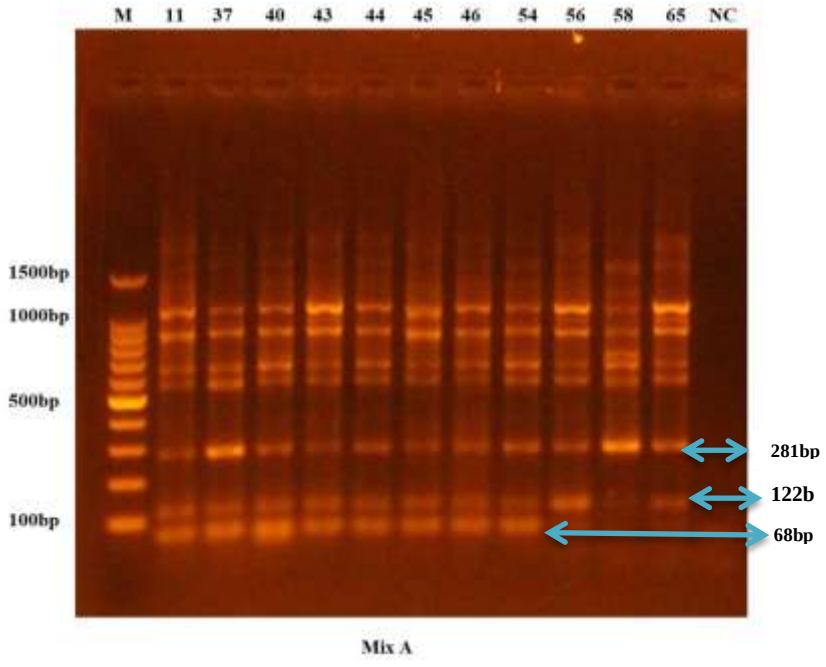


**Figure1:** Results of the amplification of MixA primes for Genotyping Hepatitis B virus (A, B, C) from serum samples were Electrolized on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lane (11-54) Hepatitis B virus Genotyping A at PCR product 68bp, (11-56 and 65) Hepatitis B virus Genotyping C at PCR product 122bp and Lane (11-65) Hepatitis B virus Genotyping B at PCR product 281bp.

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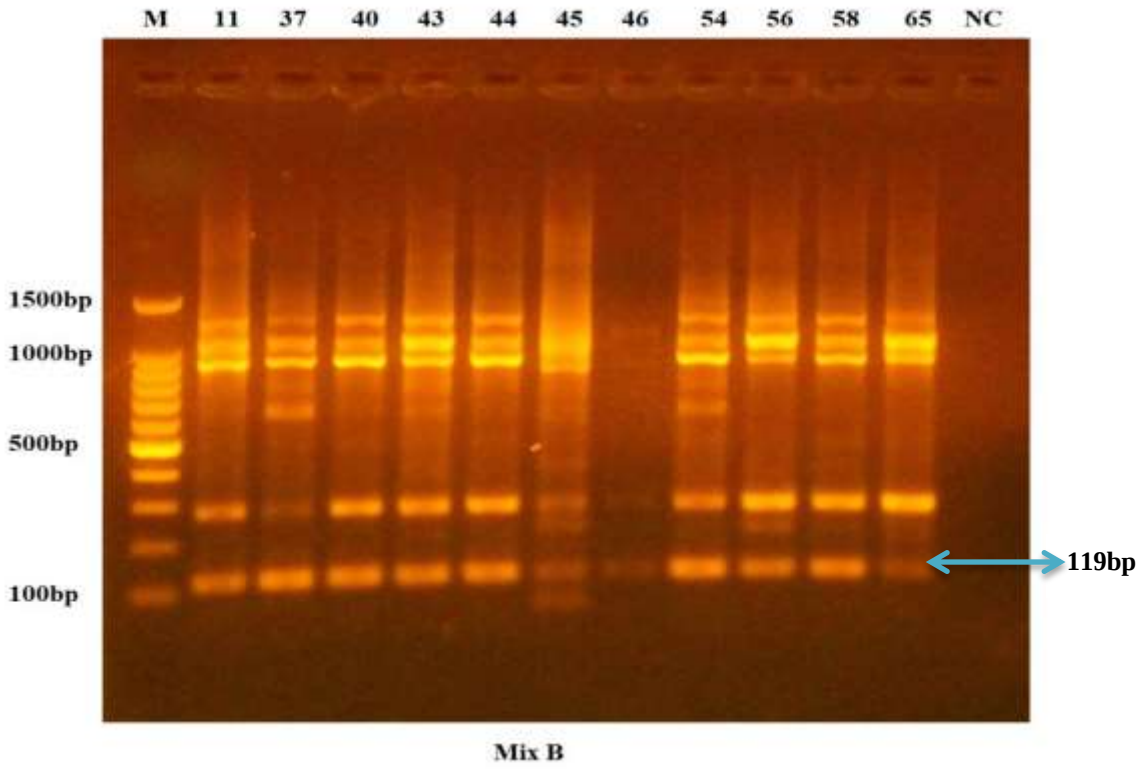


**Figure2:** Results of the amplification of MixB primers for Genotyping Hepatitis B virus (D,E,F) from serum samples were Electrolyzed on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100bp ladder marker. Lane (11-65) Hepatitis B virus Genotyping D at PCR product 119bp.

The current study demonstrated that each genotype contributes a certain amount to the genotype mixture where the ratio was as follows: genotype D (93%), B (79%), C (64%), A (57%), E (21%), and F (0%), as shown in figure (3). HBV genotyping method developed based on PCR amplification assay utilizing type specific primers capable of recognizing six primary HBV genotypes (A-F) [10]. The main result of this study was the detection of genotype D in almost all HBV carriers (93%) followed by genotype B that constituted (79%) while F genotype scored least percentage (0%) and genotype F was not found in any case. These results consistent to results Abbood *et al*. [24] that showed genotype D scored highest percentage (94%) than others HBV genotypes. Dawood *et al*. [25] who showed the genotype D scored highest percentage (100%), and these results close to our results.

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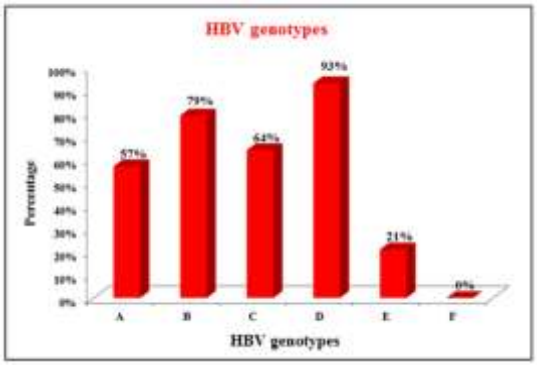


**Figure 3**: Percentage of each Genotype of HBV detected by nested PCR.

Based on HBV genotypes mixture, the present study revealed the mixture genotype A+B+C+D and D+E scored highest percentage (50% and 14%), while F genotypes was lowest percentage (0%), as shown in figure (4). The percent distribution of HBV genotypes among patients was determined to be one single genotype E (7.1%), with the remaining samples showing mixed infection of genotypes as follows: A+B+C+D(50%), A+B+D(7.1%), B+C+D(7.1%), D+E(14.3%), B+D(7.1%), C+D(7.1%), whereas genotype F was not found in any patient (0%). This results of mixed genotype consistent with result of Abbood and Al-Mhanah [25] who found the most patients have mixed genotype of HBV. Also, Al-Suraifi *et al.*[7] discovered that no single genotype was identified, that all of these samples demonstrated mixed genotype infection, and that the HBV genotypes were distributed among patients as follows: A+B+C+D+E (77.77%), A+B+D+E (16.66%), A+B+C (2.77%), A+B+E (1.38%) and A+D+E (1.38%), while genotype F was not detected.

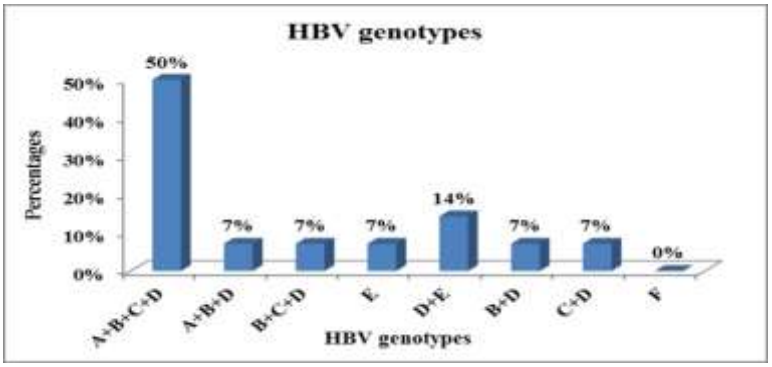


**Figure 4:** Percentage of each HBV genotype mixture.

## Conclusions

The biochemical parameters (ALT, AST, ALP) gave high mean level in HBV patients than controls; ALP and AST scored highest sensitivity than ALT, while based on specificity, ALT and ALP scored highest specificity than AST. The HBV genotype D was the most frequent among the investigated sample of HBV patients from Diyala. These results contradict findings of previous studies in which the D genotype was predominantly observed.

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