

# Determination Genotype D Of Hepatitis B Virus Amongst Patients In Mosul-Iraq

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**Abstract: Background:** Hepatitis means inflammation of liver, caused by several viruses and agents. Hepatitis B virus is one of many types of viruses can infect liver and causes inflammation and in sometimes reaching to cirrhosis and hepatocellular carcinoma. **Objective:** the aim of this study is to detect the genotype of hepatitis B virus from patients in Mosul. **Methods:** We evaluate 25 patients presumably with HBV in acute and chronic cases whom have HBsAg positive. Viral DNA was extracted from patients' serum. The genotypes was detected using Real time PCR. **Results:** The Real time PCR based methodology was standardized illustrated that 22 (88%) of samples perceived genotype D while the other 3(12%) samples conferred negative result for genotypes B, C, or D. **Conclusion:** The standardized Real time PCR assay is a rapid and accurate method for detection and differentiation of HBV genotypes that are more frequent in mediterranean East and Asia. This method can be applied in the clinical practice.

**Key word:** HBsAg, Real time PCR, CHB, ELISA, HBeAg, anti-HBs, anti-HBe.

## Introduction:

Hepatitis B virus is a serious global public health problem. The World Health Organization (WHO) has estimated that over 350 million people worldwide are chronically infected with HBV (2). This infection can be transmitted through sexual intercourse, parenteral contact or vertical transmission (mother-to-child), and blood transfusion. Severely cases of HBV can lead to chronic liver disease, including cirrhosis and hepatocellular carcinoma (3). Acute disease typically occurs in the infected adolescents or adult who have not been vaccinated. Chronic HBV(CHB) infection can be define as the presence of hepatitis B surface antigen (HBsAg) in the serum of an infected individuals for at least six months or as the presence of HBsAg in a patient who is negative for immunoglobulin M antibodies to the hepatitis B core antigen (anti-HBc) (4). HBV is the smallest DNA virus with 3200 base pairs, which contains four overlapping genes encoding the viral envelope (S and pre S), nucleocapsid (Precore and Core), polymerase with reverse transcriptase enzyme and X proteins (6). The clinical relevance of such genotype is yet unclear. However, because the HBV-induced disease is the resultant of virus-host interaction, the disease characteristics may be influenced by the genotypes of the virus. Interaction between hepatocyte genome and HBV genome may also vary according to the prevalent HBV genotype (1). Detection of HBV genotype is very essential to clarify the pathogenesis, rout of infection and virulence of the virus (7). The HBV genotypes are variable that could potentially influence the outcome of chronic HBV and the success of antiviral therapy. Varieties of methods have been used, including whole or partial genome sequencing, genotype-specific PCR amplification, line probe assay, enzyme-linked immunoassay as well as serological methods. Whole-genome sequencing is the "gold standard," and it is particularly accurate for detecting recombinant viruses (11, 10). The aim of this study is to detect the genotype of hepatitis B virus due to that some genotypes like A and D are more virulence with less response of treatment especially in the Middle East countries.

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## Materials and methods:

This study was done among populations who were infected (patients) or under suspicion infected persons with hepatitis B virus in Mosul city and its suburbia. A total of 25 serum samples were collected from patients with hepatitis B virus. These patients were in different cases with acute or chronic hepatitis according to clinical manifestation and serological tests with ELISA and Immunochromatography. The serum was separated and stored in multiple marked clean tubes at (-20 ° C) for both ELISA and for PCR assays.

### Detect HBsAg by ELISA:

Bioelisa is a direct immunoenzymatic method of the sandwich type for anti-HBs antibodies coated to microplate wells using to detect HBsAg of Biokit- Spain commercial kit.

### Confirmation detection of HBsAg:

To confirm detection of HBsAg, we used Multi –HBV Markers Test Device which detects 5 markers of HBV (HBsAg, anti-HBs, HBeAg, anti-HBe, and anti-HBc). This kit consists of 5 chromatographic strips. Each strip detects a certain HBV marker. A 60µl of serum sample was added to each well and simultaneously timing start. The results were recorded after 15 minutes.

### Determination of genotypes:

The QIAamp DNA extraction mini kit (QIAGEN GmbH, Germany) was used for DNA extraction from serum samples according to the manufacturer's instructions. The extracted DNA was used for amplification of HBV DNA by Real time PCR. This kit is used for detection of HBV genotypes B, C & D in serum or plasma (Vacunek, Spain, HD-0006-03). The real time PCR was applied twice. For the first time, the master mix prepared for (5) samples was (182µl) (180µl of reaction mix and 2µl for enzyme mix). The second real time PCR prepared for (20) samples was (728µl) (720µl of reaction mix and 8µl of enzyme mix). All the master mixes prepared in the (1.5ml) eppendroff tube using micropipette. The tubes were applied in the (Applied Biosystem device, USA) after preparing the system which performed the following protocol in the instrument according to the manufacturer's instruction: 37°C for 2min --1cycle. 94°C for 2min --1cycle. 94°C for 10sec, 62°C for 40sec. Fluorescence measured at 62°C 40 cycles. Selection of fluorescence channels-FAM HBV genotype.

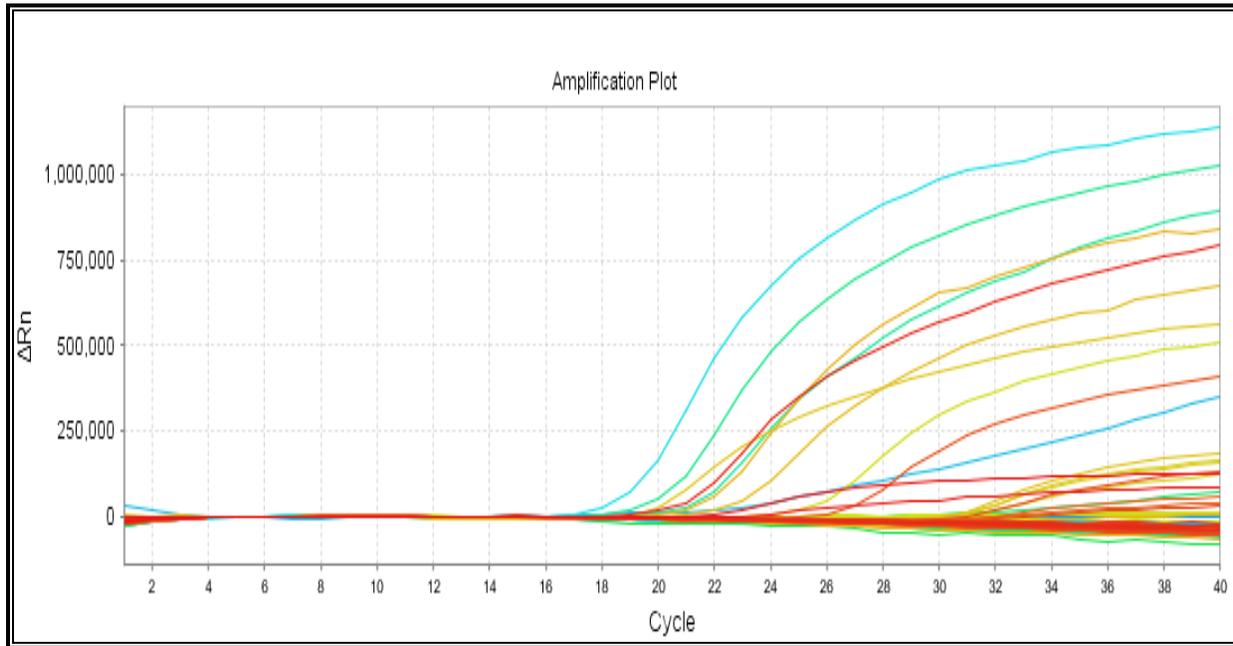
**Data Analysis and Interpretation:**

Below is the analysis of data according to the manufacturer's instruction: Ct Value Negative or Positive 1- UNDET Negative"— 2- ≤38 Positive "+" 3- 38~40 Re-test. If it is still 38~40, then Negative"—

**Results:**

A total 25 of serum patients were used in this study obtained positive results for HBsAg using Bioelisa kit. For confirming

detection of HBsAg, only 22(88%) of serum patients that were used in ELISA assay got positive results, while these cases got different results for other HBV markers. Real time PCR results for all 25 samples as shown below with PCR curve reaction table for cut-off value: 22 (88%) of serum samples obtained the genotype D. 3 (12%) obtained negative result for any genotypes B, C, or D. When repeated tests, only 3 patients got negative result for ELISA and PCR test.



**Figure (1):** Result plots of Real time PCR: Cyan line: Genotype D, Lime green line: Genotype C, Red line: Genotype B, yellow line: control genotype D, Fuchsia line: control genotype C, Yellow line: control genotype B.

**Table (1):** Cut-off value for samples, positive and negative controls: CI+B= control + genotype B, CI+ C= control + genotype C, CI+D= control + genotype D, CL- = negative control, RM B = reaction mix genotype B, RM C = reaction mix genotype C, RM D = reaction mix genotype D, S = sample, Undet. = undeterminant.

No.	Sample name	Target name	Ct mean	No.	Sample name	Target name	Ct mean	No.	Sample name	Target name	Ct mean	No.	Sample name	Target name	Ct mean
1	CI + C	RM C	21.735	21	CI + B	RM B	21.078	41	S 14	RM B	Undet.	61	S 14	RM D	19.382
2	CI + D	RM D	21.575	22	CL -	RM D	Undet.	42	S 15	RM B	Undet.	62	S 15	RM D	37.497
3	CL -	RM B	Undet.	23	S 1	RM B	Undet.	43	S 16	RM B	Undet.	63	S 16	RM D	19.431
4	S 1	RM C	35.898	24	S 2	RM B	Undet.	44	S 17	RM B	Undet.	64	S 17	RM D	30.206
5	S 2	RM C	31.373	25	S 3	RM B	Undet.	45	S 18	RM B	Undet.	65	S 18	RM D	18.966
6	S 3	RM C	22.235	26	S 4	RM B	Undet.	46	S 19	RM B	Undet.	66	S 19	RM D	30.503
7	S 4	RM C	29.77	27	S 5	RM B	38.628	47	S 20	RM B	Undet.	67	S 20	RM D	30.491
8	S 5	RM C	Undet.	28	CI - 2	RM D	Undet.	48	S 21	RM B	Undet.	68	S 21	RM D	30.996
9	S 1	RM D	38.893	29	S 2	RM D	30.018	49	S 22	RM B	Undet.	69	S 22	RM D	32.517
10	S 3	RM D	20.753	30	S 4	RM D	28.287	50	S 23	RM B	Undet.	70	S 23	RM D	23.353
11	S 5	RM D	Undet.	31	CI + B	RM B	22.725	51	S 24	RM B	Undet.	71	S 24	RM D	32.672
12	CI - D	RM D	22.4	32	CI - C	RM C	19.901	52	S 6	RM C	Undet.	72	S 7	RM C	Undet.
13	S 6	RM B	Undet.	33	S 6	RM D	13.842	53	S 8	RM C	Undet.	73	S 9	RM C	31.433
14	S 7	RM B	Undet.	34	S 7	RM D	Undet.	54	S 10	RM C	Undet.	74	S 11	RM C	Undet.
15	S 8	RM B	Undet.	35	S 8	RM D	33.482	55	S 12	RM C	Undet.	75	S 13	RM C	18.576
16	S 9	RM B	Undet.	36	S 9	RM D	31.154	56	S 14	RM C	Undet.	76	S 15	RM C	20.354
17	S 10	RM B	Undet.	37	S 10	RM D	25.566	57	S 16	RM C	Undet.	77	S 17	RM C	Undet.
18	S 11	RM B	Undet.	38	S 11	RM D	7.707	58	S 18	RM C	Undet.	78	S 19	RM C	Undet.
19	S 12	RM B	Undet.	39	S 12	RM D	32.072	59	S 20	RM C	Undet.	79	S 21	RM C	Undet.
20	S 13	RM B	Undet.	40	S 13	RM D	31.978	60	S 22	RM C	20.145	80	S 23	RM C	Undet.

## Discussion

Detection of HBV genotype is very significant to clarify the pathogenesis, rout of infection and virulence of the virus (9). For example, Chan et al. indicated that genotype C was associated with more severe liver fibrosis than genotype B probably because of delayed HBeAg seroconversion and prolonged active disease (7, 12). Also, the studying of HBV genotype is more appropriate for investigation of geographic distribution and epidemiologic connections (8). Our study revealed the genotype D of hepatitis B virus for the first time in Mosul city due to several resistance cases for treatment. In this study. We have evaluated 25 patients with acute and chronic HBV. For the first testing assay, all 25 serum samples had positive result for HBsAg using Bioelisa kit. Confirming test using rapid Immuno-chromatography assay for detecting all HBV markers (HBsAg, anti-HBs, HBeAg, anti-HBe and anti-HBc). This type of assay is a unique and recently used in medical labs. This kit is depending on reactions between antigen and antibody, two bands forming for positive result while one band forming for negative result for (HBsAg, anti-HBs, and HBeAg) test while one band forming is positive result and two bands forming are negative results for (anti-HBe and anti-HBc) respectively. Recently, a 22(88%) of serum samples got positive result for HBsAg while 3(12%) got negative result although, all samples got positive result in ELISA. These differences between two results refer that the 3 negative sample shaps related to reduce numbers of HBsAg in the serum. A 22(88%) of total samples got genotype D by demonstrating the cut-off value from threshold of the plots. Other study was done to demonstrate genotype using Line prob assay (INNO- Lip A HBV Genotyping assay). This study found that constituted 100% of the total infections among acute and chronic hepatitis is genotype D in Mosul city (9). Figure (1) shows the amplification plot for samples with positive and negative controls. Different colors of plot lines explained the master mix for each types of genotypes B, C, and D as well as the positive controls of three genotypes and negative control (figure 1). Table 1 shows the Ct value for all samples and controls. We have given examples to describe cycle threshold (Ct) value such in Ct value for reaction mix D of sample (3), here the Ct value is (20.753) which means beginning of exponential phase due to this phase is the optimal point for analyzing data, and according to data analysis of kit, the Ct is under 38 so that reaction mix for sample 3 is positive. Conversely, the plot of reaction mix B for sample (7) which the line remained under cycle threshold and there is no change in amplification line that means lack of DNA amplified, therefore, the Ct value undeterminant by the system so it is a negative result. If we focus on sample (5) which the Ct value for reaction mix B is (38.63), we couldn't evaluate this result as genotype B because the Ct is over than (38) (table 1). The negative results were retested and got the same results for genotyping and also for ELISA because the patients were in recovery. Using genotyping kit till now is vacant in development country like Iraq due to cost effectiveness and less expertise technicians.

## Conclusion:

The main genotype of hepatitis B virus in Mosul and its suburbia is D. Although, identification genotype is vital for clarifying pathogenesis, routs of virulence and epidemiological study. Genotype determination is vital to be applied in clinical practice. The standardized Real time PCR assay is a rapid

and accurate method for detection and differentiation of HBV genotypes that are more frequent in mediterranean East and Asia, for HBV genotypes B, C and D.

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