

## Detection of hepatitis-B virus genotype B among patients with HBV-related chronic liver disease by using nested polymerase chain reaction

Anwar Salih Saihood

Microbiology Dept. Medicine College, Al-Qadisiya University

### تحديد النوع الجيني (ب) لفايروس التهاب الكبد الفايروسي نمط (ب) في مصول المصابين باستخدام تقنية السلسلة المتبلورة (PCR)

انوار صالح صيهود

جامعة القادسية, كلية الطب, قسم المايكرو بايلوجي

#### المستخلص

**الهدف:** لتحري وتحديد فيروس التهاب الكبد نوع بي في المرضى الذين ظهروا نتائج مصلية موجبة للمضاد (HbsAg) في مدينة الديوانية, العراق.

**طريقة العمل:** تم اختبار 30 مصل من مرضى مصابون بفيروس التهاب الكبد المتعلق بمرض الكبد المزمن لتحري عن فيروس التهاب الكبد نوع بي باستخدام تفاعل سلسلة البلمرة المتداخل مع نوعيين متخصصين من البادئات للحمض النووي لفيروس التهاب الكبد.

**النتيجة:** تم تحديد فيروس التهاب الكبد نوع بي بنسبة (30:1) (3,3%) من العينات المصلية المفحوصة, حيث كان 281bp ناتج تفاعل سلسلة البلمرة المتداخل يشفر للمنطقة الغير مشفرة المتخصصة في للحمض النووي لفيروس التهاب الكبد نوع بي.

**الاستنتاج:** ان تفاعل سلسلة البلمرة المتداخل هو طريقة بسيطة وسهلة الاستخدام خصوصاً في بلدنا حيث ان جميع التقارير حول التمييز الجيني مازالت لاحد الان غير مدروسة, والدراسة الحالية وجدت بان النوع الجيني العام لفيروس التهاب الكبد هو نوع بي.

#### Abstract

**Aim:** To detect the genotype B of Hepatitis-B virus in patients seropositively for HbsAg in Al-Dewayia city, Iraq.

**Methods:** Sera from 30 patients with HBV-related chronic liver disease were tested for HBV genotype B using Nested Polymerase Chain reaction toward two sets of type specific primers of HBV genome.

**Results:** The genotype B was detected in (6/30) samples at (20%) of tested sera, where the PCR product was 281bp, which encode to untranslated region (UTR) specific region of HBV genome.

**Conclusion:** The Nested Polymerase Chain reaction is a simple method and easy to use, especially in our country where not all of the reported genotypes until now detected, and the present study established that the common genotype of HBV was B.

#### Introduction

Originally, Hepatitis is a systemic viral disease that infected the liver and can be recognize by lysis and necrosis of Hepatitis cells with acute liver inflammation (1).

Hepatitis B virus is considered as one of the most significant global health problems and approximately, two billion people in the world have been infected by hepatitis B virus and is responsible for more than one million deaths annually (2). Hepatitis B virus is a member of the hepadna virus family and transmitted as a blood born pathogen, which contains a small, circular, partially double stranded segment of DNA of approximately 3-2 kb in size (3). Current approaches to genotyping HBV are multiple such as sequencing and phylogenetic analysis (4); differential hybridization (5). Nested and multiplex PCR (6). Thus, the influences of HBV genotypes on clinical outcomes need to be clarified. Recent studies have shown that virological characteristics and clinical manifestations may differ even among HBV isolates of the same genotypes (2). Several studies were reported that some patients with acute liver diseases due to viral agent, despite testing negative for hepatitis B surface antigen (HbsAg) and hepatitis B core antibody immunoglobulin (anti-HbcIgM) had detectable hepatitis B virus (HBV) DNA in the serum of liver using polymerase chain reaction (PCR) assays (7). Efficient extraction of nucleic acids from clinical sample is highly determinant for the reliability and performance of any molecular diagnostic assay (8).

The presence of HBV DNA in serum is a reliable marker of genomic viral replication and infectivity (9) and molecular biology techniques show a powerful tool for few genomic copies and more are widely used for diagnostic and research purposes (10). This study to detect and identify the genotype B virus in patients' serum of seropositively HbsAg by using Nested polymerase chain reaction.

## **Materials and Methods**

### **Specimen collection**

A Thirty blood samples were collected from patients with Hepatitis B virus-related chronic liver disease infection, which previously diagnosed as seropositive for HbsAg who attending the Dewaniya Teaching Hospital from July to October, 2012. Patients have received antiviral treatment, histories like alcohol consumption, diabetes or drug abuse or any positive serology for hepatitis A, C were also excluded. A 5ml of blood samples were collected in sterile test tubes by using sterile syringe, then the tubes centrifuged at 1500 rpm for 15 minutes, 1ml serum placed in sterile eppendorf tube and directly transported by ice bag into molecular biology laboratory in college of medicine to perform the viral DNA extraction.

### **Nested polymerase chain reaction**

Nested-PCR assay was performed to detect and determine the HBV genotype B by using two primers set for HBV genome fragment which specific for HBV genotype B include, outer sense primer (5'-TCACCATATTCTTGGAACAAGA-3'), outer antisense primer (5'-CGAACCACTGAACAAATGGC-3'), and the inner sense primer (5'-GGCTCCAGTTCAGGAACAGT-3'), inner antisense primer (5'-CAGGTTGGTGGTACTGGAGA-3'). The assay was carried out according to the method described by Tsai *et al.*, 2009 (11) as follows:

### **Viral DNA extraction**

Genomic DNA of HBV was extracted by using (viral nucleic acid extraction kit, supplied by Geneaid company and done according to manufacturer's instructions as follow:

1) - Sample Preparation; the virus concentrated by adding 150 µl of PP Buffer to 1 ml of serum or plasma, and mixed well. Then let stand at room temperature for 30 minutes, after that centrifuge at 15.000 xg for 15 minutes. The supernatant removed and the viral bullets saved to lysis step.

2)- Lysis step; A 100 µl of LS Buffer mixed with 1 µl of IC (short ds DNA, E3/µl) and vortex. Then 100 µl of the mixture added into the viral bullets, then vortex and incubated at room temperature for 5 minutes.

3)- Nucleic Acid Binding step; A 234 µl of absolute ethanol were added to the mixture from step 2 and mix by shaking 10 sec. Then, the mixture transferred to the VB column and centrifuge at 15.000 xg for 30 seconds. After that, 2 ml collection tube with flow-through discarded.

4- Washing step; 200µl wash buffer were added to VB column and centrifuge at 15.000 xg for 30 seconds.

5- A 50µl release buffer (preheated at 65°C) was added to the center of column matrix to release the viral DNA. Then the tubes let stand at 65°C for 3 minutes and centrifuged at 15.000 xg for 1 minute to elute the purified viral DNA.

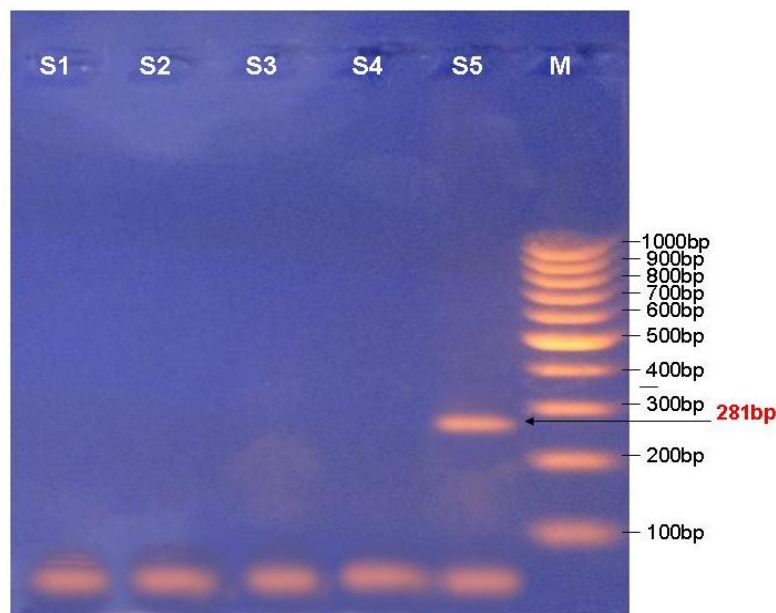
### **Nested PCR master mix preparation and amplification procedure**

PCR reaction mix was prepared by using (AcuuPower PCR PreMix kit Bioneer. Korea), and just added 5ul viral DNA template, 1.5ul outer sense primer, 1.5ul outer antisense primer, and completed to 20ul by PCR water. First amplification stage was carried out in PCR Thermocycler (TECHNE, USA) as follows initial Denaturation 95°C for 5min 35cycles, denaturation at 95°C for 1 minute, annealing at 52°C for 30sec, and extension at 72°C for 1 minute with final extension at 72°C for 10minutes.

After the first amplification, 1ul of PCR product was re-amplified for another 35 cycles by using 100ng of each inner primers and same previous master mix and PCR thermocycler condition. The final PCR products were electrophoresed on a 1.5% agarose gel, stained with Ethidium bromide, and evaluated under UV. light source, where 281bp PCR product detects indicates the HBV genotype B.

### **Results and Discussion**

This study showed that patients with HBV infections -related chronic liver disease attributed predominantly to viral genotype B that constituted (20%) of the total infections. These results, shown by Nested PCR based genotyping, in 281bp product size as HBV genotype B (Fig. 1).



**Figure (1):** Agrose gel electrophoresis of HBV genotype B using Nested PCR with type specific primers, which showed Lane (M) DNA marker 100-1000bp), Lane (S5) positive for HBV in 281bp Nested PCR product, and lane (S4, S3, S2, and S1) negative for HBV genotype.

The distribution of HBV genotype B in liver disease which also explained by Zekri *et al* 2007(12). Who indicate that among the 22 patients who had an acute form of liver disease, genotype B showed predominance over the other genotypes with the following values respectively: 2 cases (9%) A, 10 (45.5%) genotype B, 3 (13.6%) genotype C and 3 (13.6%) genotype D. The distribution of HBV genotype B in acute forms of liver disease was higher than that seen in chronic forms of the disease suggesting an association of genotype B with more severe acute forms of liver disease. A similar result was observed in the study of Imamura *et al.* (13) who showed that genotype B was more prevalent in patients with FH and AH. They attributed this result to the possibility that Genotype B virus may have the motifs that strongly bind to HLA class I molecules, thereby resulting in activation of a stronger immune response and a more liver damage.

The genotyping of HBV is important to clarify the route and pathogenesis of the virus. In particular, the examination of sequence diversity among different isolates of the virus is important, because variants may differ in their patterns of serologic reactivity, pathogenicity, virulence, and response to therapy (14). Although there are several methods for HBV genotyping (e.g., RFLP, multiplex PCR, and DNA sequencing) (6), the nested PCR method described in this study provides a rapid and sensitive way for distinguishing HBV genotypes (21). This method may be suitable for large-scale clinical and epidemiologic studies, especially in Iraq and it is the first study of genotyping of hepatitis B patients with very good results.

## References

- 1- **Sherlock, S., and J. Dooley. (1997).** Diseases of the liver and biliary system, 10th ed. Blackwell Science, Oxford, England.
- 2- **Schaefer S. (2005).**Hepatitis B virus: significance of genotypes. *J Viral Hepat* ; 12: 111–124.
- 3- **Bowden, S. (1997).** Hepatitis B: treatment for the 21st century. *Antivir. Chem. Chemother.* 8:77–82.
- 4- **Ohba K, Mizokami M, Ohno T, Suzuki K, Orito E, Lau JY, et al.(1995).** Relationships between serotypes and genotypes of hepatitis B virus: genetic classification of HBV by use of surface genes. *Virus Res.* 39: 25 – 34.
- 6- **Kirschberg O, Schulttler C, Repp R, Schaefer AF. (2004).** A multiplex-PCR to identify hepatitis B virus-genotype AF. *J Clin Virol.* 29: 39 – 43.
- 7- **Suzuki C, Yamashita S, Korenaga M, Uchida K, Tanigawa K, Kimura T, Kayano K, et al.(1998).** Detection of hepatitis B virus DNA in liver by polymerase chain reaction for the diagnosis of fulminant hepatitis B. *Hepatol Res.* 12(1):23-30.
- 8- **Kramvis A. , S. Bukofzer, and M.C. Kew.(1996).** “Comparison of hepatitis B virus DNA extraction from serum by the QIAamp Blood Kit, GenReleaser, and the phenol-chloroform method”, *Clinical Microbiology*, 34: pp. 2731-2733.
- 9- **Gobbers E., T. A. M. Oosterlaken, M. J. A. W. M. VAN Bussel, R. Melsert, A. C. M. Kroes, and E. C. J. Claas. (2001).** “Efficient Extraction of Virus DNA by NucliSens Extractor Allows Sensitive Detection of Hepatitis B Virus by PCR”, *Journal of Clinical Microbiology.*, 39 pp. 4339–4343.
- 10- **Bergallo M., C. Costa, G. Gribaudo, S. Tarallo, S. Baro, A.N. Ponzi, and R. Cavallo. (2006).** “Evaluation of six methods for extraction and purification of viral DNA from urine and serum samples”, *New Microbiologica.*, 29: pp.111-119.
- 11- **Tsai C. Y, Lin C. L, Lin S. C, and Liou S. w. (2009).** Detection of Hepatitis B Virus in the Aqueous Humor of a Hepatitis B Virus Carrier. *Ophthalmologica.*223:93–95  
hepatitis C virus infection. *Lab Invest.* 2000; 80: 851 – 856.
- 12- **Zekri, A. N., Hafez, M. M., , Mohamed, N.I., Hassan, K.Z, El-Sayed, M.H., Khaled, M.M, and Mansour, K.(2007).** Hepatitis B virus (HBV) genotypes in Egyptian pediatric cancer patients with acute and chronic active HBV infection. *BioMed Central.* 4:3-7.
- 13- **Imamura T, Yokosuka O, Kurihara T, Kanda T, Fukai K, Imazeki F, Saisho H. (2003).** Distribution of hepatitis B viral genotypes and mutations in the core promoter and precore regions in acute forms of liver disease in patients from Chiba, Japan. *Gut.* 52:1630-1637.
- 14- **Okamoto H, Tsuda F, Sakugawa H, Sastrosowignjo RI, Imai M, Miyakawa Y, Mayumi M. (1988).** Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. *J Gen Virol .* 69:2575-2583.

15- Soguero C, Campo E, Ribalta T, Sanchez-Tapias JM, Siaz JC, Bruguera M. (2000). Assessment of genotype and molecular evolution of hepatitis C virus in formalin-fixed paraffin-embedded liver tissue from patients with chronic hepatitis C virus infection. *Lab Invest.* 80: 851 – 856.