

Molecular Diagnosis in Differentiating Active and Inactive Forms of Hepatitis B Virus Carriers

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Abstract

Background & objectives: Introducing a nucleic acid test program is aimed to diagnose and reduces the risk of viral infection or transmission. DNA assay for HBV can detect infection in the windows period, chronic occult infection and can discriminate between active and inactive HBV infection. This cross-sectional study designed to diagnose, analyze HBV infection and to differentiate active from inactive infection based on viral DNA detection. **Methods:** Blood samples were collected from 256 patients previously diagnosed on the clinical ground as hepatitis B seropositive in Erbil Central Lab. The viral nucleic acid quantitative assessment was done for the collected samples using RT-PCR. Q-square was performed for statistical analysis. **Results:** Out of 256 collected blood samples 93 (36.3%) showed HBV-DNA positive titers above 50 IU/ml. Among positive subjects, 67 (72.04%) was categorized as inactive carriers (< 2000-20.000 IU/ml HBV-DNA titers). **Conclusions:** The data produced from this study confirmed the importance of the RT-PCR technique in sensitivity and reliability as a superior diagnostics tool specifically in differentiating active from inactive HBV carriers.

1- Introduction

Hepatitis B virus (HBV) is a worldwide risk health problem, about two billion people have HBV serologic markers [1]. On the clinical ground, more than 360 million people are chronically infected [2]. In many countries, molecular techniques have been introduced as routine screening methods [3]. Screening for HBV via DNA amplification is efficient in minimizing HBV transmission via blood and blood products [4]. Periodic monitoring of blood HBV-DNA is of paramount importance in the management of chronic hepatitis B infection [5]. Molecular techniques can be used for differentiation between active and inactive HBV infection. Active HBV carriers with high viral load is a remarkable risk factor for both incidence and mortality of HCC [6],[7], whereas inactive HBV carrier denotes the low rate of HBV reactivation, HCC, progressions to cirrhosis and good prognosis [8]. The viral genome is a 3.2 kb circular partially duplex DNA molecule with the circularity conserved by 5' ends. The genome is made only from condensed coding regions with four open reading frames (ORFs) [9]. For quantification of HBV DNA laboratory measures how many units of the viral DNA are found in a ml of blood (international units per milliliter or IU/ml). The viral DNA levels range from thousands up to millions, referred as a high rate of HBV replication. However, levels less than 2,000 IU/ml referred to as an "inactive" infection or undetectable [10],[11]. During HBV infection, markers can be detected in sera samples using immunoassays. For example, patient sera that contain anti-HBs indicates immunity from past infections or vaccinations, and a patient with anti-HBe antibodies in their sera is considered as a spontaneous resolution of infection or therapy-induced improvement [12 and 13]. One of the serological methods using for diagnosis of HBV is enzyme immune assays (EIAs). ELISA test is a qualitative serological test for the diagnosis of HBsAg human serum.

However, DNA amplification using RT-PCR is potentially a more sensitive assay [14]. RT-PCR for example used as a molecular technique for detection and quantification of the viral genome, which can also discriminate between active and inactive infection [15]. The aim of this study was to discriminate between active and inactive HBV infections based on DNA titers.

2- Materials and Methods

For this cross-sectional study, blood samples were collected from 256 patients suspected on the clinical pictures as viral hepatitis, referred to Erbil Central Laboratory, from June to September 2017 for HBV DNA screening. Some patients were referred because of a history of Jaundice confirmed serologically using HBs Ag testing. Other patients were referred as follow up for assessment of viral load and treatment response. Both sexes were included (154 males and 102 females); with age range between 20-60 years. This project was approved at the first site by the Scientific and Research Ethics Committee at the College of Health Sciences, Hawler Medical University/ Iraq.

Five ml of venous blood were collected using EDTA tubes and centrifuged at 1000rpm for 5 minutes and patients' plasma was separated. Viral HBV DNA was extracted from a 200 μ L aliquot of serum using a Qiagen mini blood kit (Qiagen, Hilden, Germany) according to manufacturer instructions. Viral loads of HBV DNA were estimated by RT-PCR (HBV RG PCR Artus Germany). For RT-PCR reaction a 50 μ L reaction mixture was used, which contains: 30 μ L of Master-mix (Buffer, dNTP, primer, probe, and enzymes) and HBV RG inhibition control mixed with 20 μ L of DNA template. HBV-Taq I forward primer: CAA CCT CCA ATC ACT CAC CAAC and HBV- Taq 2 reverse primer: ATA TGA TAA AAC GCC GCA GAC AC were used [16]. RT-PCR in which Rotorgene 3000 was then used to detect HBV-DNA according to the manufactures instructions. The RT-PCR cycling parameters consisted of denaturation at 95°C for 15 seconds, 55°C for 30 seconds and 72°C for 15 seconds. The results were considered positive if a signal was detected in cycling A FAM, whereas no signal detection indicated as negative results. The quantitation of HBV DNA was performed for all blood samples and the results were considered positive and significant if viral load was more than 1×10^5 viral copies/ ml. Titers less than 50IU were considered as negative for HBV DNA. The screened results were categorized according to positive HBV DNA titers into inactive, gray zone and active carriers.

3- Statistical Analysis

For statistical analysis, SPSS 23.0 was used. HBV titers expressed as means \pm SE. Comparisons between active and inactive carriers T-tests were depended. Comparisons between multiple groups were performed by ANOVA. P values \leq 0.05 was considered as statistically significant.

4- Results

The results of the present study delineated the frequency of HBV-DNA among screened patients. Accordingly, HBV-DNA was positive in 36% of the tested patients; the frequency of HBV-DNA positive results constituted 42% and 27% for males and females respectively (Table 1). The distribution of HBV-DNA titers revealed the highest positively rate at titer 101-500IU/ ml and > 20.000 IU/ml (20% and 23% respectively) (Table 2). On the other hand, Table (3) showing the distribution of means of HBV-DNA titers, and statistically the differences were highly significant ($p \leq 0.001$). The discrimination of active and inactive HBV infection among HBV-DNA blood samples was based on three HBV-DNA titers (8). Thus, inactive carriers (≥ 50 -2000 IU/ml); Gray zone carriers (2001-20.000 IU/ml) and active carriers (> 20.000 IU/ml) were categorized among the total HBV-DNA positive blood screened (Table 4). Thus, the frequency of HBV infection as inactive, gray zone (as extended inactive) to active group revealed 46%, 21%, and 30% respectively. Table 5 delineated highly significant differences between the mean titers of HBV-DNA inactive and active carrier ($p \leq 0.05$).

Table 1: Frequencies of HBV DNA positivity according to the gender.

Gender	Positive PCR	Negative PCR	Total	P Value
Male	65 (42.20%)	89 (57.79%)	154	≤ 0.001
Female	28 (27.45%)	74 (72.54%)	102	
Total	93 (36.3232%)	163 (63.67%)	256	

Table 2: Percentages of HBV DNA according to titer range among patients

HBV DNA PCR titers IU/ml						
< 50 IU	50-100	101-500	501-1000	1001-2000	2001-20.000	> 20.000
21	4	23	9	9	21	27
(18.42%)	(3.50%)	(20.17%)	(7.89%)	(7.89%)	(18.42%)	(23.68%)

Table 3: Mean HBV DNA titers among positive patients screened.

PCR titers IU/ml						pValue
≥ 50-100 n=4	101-500 n=23	501-1000 n=9	1001-2000 n=9	2001-20.000 n=21	> 20.000 n=27	
71.25 ± 5.66	284.60 ± 21.38	755.0 ± 39.95	1425.3 ± 70.95	3767.14 ± 405.32	120936819± 36526296.47	0.001

Table 4: Percentage of active and inactive HBV infection among patients according to titers.

Inactive carriers	Gray zone carriers	Active carriers
≥ 50-2000 IU/ ml	2001-20.000 IU/ ml	> 20.000-990885600 IU/ ml
45 (48.38 %)	21 (22.58%)	27 (29.03%)

Table 5: Mean HBV DNA titers in active and inactive patients.

PCR HBV DNA titers		PValue
Inactive carriers n=66	Active carriers n=27	
1599.53 ± 225.70	120936819.9± 36526296.47	≤ 0.01

5- Discussion

The detection of HBV infection is vital for diagnosis, follow up the study and for controlling spread in community with the limited spectrum of disease range for acute to chronic, often progress to liver cirrhosis and HCC depends on the interplay between viral and host factors [17-19]. Two phases characterize the acute exposure to HBV namely immune-tolerant and immune reactive phase [8]. But some patients remain in an active carrier with serum level of HBV-DNA < 2000 IU/ml; while others progress to HBe antigen-negative chronic hepatitis B [20]; as HBe regarded as markers for HBV replication and increased risk of patient infectivity. The present study revealed HBV DNA positivity in 36.32% patients (previously diagnosed serologically) diagnosed on clinical pictures as HBV infection (Table 1). These results were disagreed with others who reported 60.5%, 71.4% and 82.2% as positive for HBV infection [21-23]. Of these patients, 66 (70.97%) were categorized as inactive carriers. While the remaining were active carriers (29.03%), with DNA viral titers >20,000 IU/ml (Table 5). The impact of genders revealed 42% males and 27% females HBV DNA positivity rate (P value ≤ 0.01) (Table 1), this was disagree with what reported by [23] and agree with [21]. Screening for HBV viral DNA amplification is efficient in minimizing HBV transmission via blood and blood products besides as a diagnostic tool [4]. Four phases characterize chronic hepatitis B (CHB) infection namely immune tolerant, immune clearance, immune control and immune escape [19]. These four phases in CHB infection neither take place sequentially [24] nor occur in all infected people. Of all 4 phases, only patients that require antiviral and immune modulators therapy those in either the immune clearance or immune escape phase [19]. Accordingly, the

selective quantitation using (RT-PCR assay is critical for assessment of serum HBV DNA levels after antiviral therapy; for HBV diagnosis; discrimination between active and inactive HBV infection and for the diagnosis of acute HBV infections [19 and 25]. This holds true for the patients in the present study because some were clinically suspected as viral hepatitis, and others were follow up that needs to discriminate of active from inactive and as treatment response. Especially the HBV DNA levels can shift from low or undetectable to >2,000,000 IU/mL [26] and some inactive carriers could occasionally have HBV DNA levels between 2000-20,000 IU/ml. Thus, single HBV DNA level between 2,000-20,000 IU/ml categorized to be a "gray area" which can refer to both active CHB and inactive carriers [26]. In the present study, 21.64% patients were in the gray zone carriers (Table 4). Accordingly, clinicians should be aware of the importance and significance of serial HBV DNA measurements and lifelong follow-up to confirm maintenance of inactive carrier state [8]. A newly diagnostic criterion has been revised for inactive carrier based on detection of lower ALT upper limits of normal levels of 30 IU/ ml for men and 19 IU/ml for women [26]. But, still, the EASL acknowledges that inactive carriers might exist with DNA levels between 2,000-20,000 IU/ ml [20].

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الخلاصة

الهدف: استخدام برنامج اختبار الحامض النووي لغرض تشخيص العدوى الفيروسية وتقليل انتقالها. ان فحص الحامض النووي الريبوزي منقوص الاوكسجين لفايروس التهاب الكبد نمط B يمكن استخدامه في تشخيص العدوى من خلال فترة النافذة والمزمنة الغامض ايضا، اضافة الى ذلك استخدام هذه الطريقة تودئ الى تشخيص النوع الفعال وغير الفعال للفايروس. اجريت هذه الدراسة المقطعية لتشخيص وتحليل عدوى فايروس التهاب الكبد نمط B والتميز بين الشكل فعال وغير فعال اعتمادا على الفحص النووي المكتشفة.

الطرائق: تم جمع 256 عينة الدم من اشخاص تعرضوا سابقا للاصابة بفايروس التهاب الكبد نمط B وارجعوا مختبر الصحة العامة المركزي في اربيل. تم استخدام طريقة تقنية عكس رد الفعل الناسخ البلمرة المتسلسل RT-PCR لغرض التشخيص الكمي للحامض النووي الريبوزي منقوص الاوكسجين. حلت النتائج الدراسية الحالية اخصائيا باستخدام اختبار Q-square.

النتائج: اظهرت النتائج ان 93 (36.3%) عينة دم من مجموع 256 عينة تحمل قياس تأثير اعلى من 50 وحدة دولية لكل مل من ضمن الاشخاص الحاملين للفايروس. 67 شخص صنفوا على انهم حامل لفايروس اقل 2000-20.000 وحدة دولية/مل.

الاستنتاجات: اظهرت الدراسة الحالية اهمية استخدام تقنية عكس رد الفعل الناسخ البلمرة المتسلسل RT-PCR في حساسيتها وقوتها كاداة معتمدة للتشخيص وخاصة التميز بين الحالة الفعالة وغير الفعالة لحاملي فايروس نمط B.

الكلمات الدالة: التهاب الكبد، الكمية، HBV-DNA، النشط HBV، HBV غير نشط.