COMPARISON STUDY OF TWO PCR PROTOCOL (DIRECT AMPLIFICATION AND NESTED) FOR QUALITATIVE MEASUREMENT OF HEPATITIS B VIRAL DNA

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ABSTRACT

This study investigated thirty patients with chronic hepatitis B who were admitted to Hepatology and Gastroenterology Teaching Hospital in Baghdad, aged from 20-65 years during the period from beginning of February 2008 to the end of February 2009. The patients were suffering from different clinical symptoms with previous risk factors for transmission of HBV infection, also blood samples were collected from thirty healthy HBs Ag carrier was discovered accidentally through attending blood bank for donation of blood, aged from 18-52 years and served as a control group. A cross sectional study was conducted in the period between beginning of February 2008 to the end of February 2009. The establishment of the molecular techniques for identified HBV DNA in chronic patients and carrier group gives an obvious image especially when uses the Nested PCR. The current results confirm that a nested PCR protocol is more sensitive method to detect HBV DNA than PCR direct protocol. The positively of HBV DNA in carrier group and chronic group reached to 46.7% and 30.0%, respectively by employing direct protocol. Whereas, HBV DNA is 100% in both groups by Nested protocol with significant difference (p=0.001).

Key words: Hepatitis, HBV DNA, healthy HBV carriers.
دراسة مقارنة لتقنية تفاعل البلمرة المتسلسل (المباشر والمترددة) المستخدمة في تشخيص نوعي دنا فييروس إلتهاب الكبد البائي

أمينة نعمة النثويني 1 علي رجب عمر 2 محمد عبد الدايم صالح 3

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الخلاصة

شملت الدراسة 30 مريضاً مصاباً بإلتهاب الكبد الفييروسي المزمن نوع (ب) و30 فرداً من الأصحاء الحاملين للمستضد السطحي لفييروس إلتهاب الكبد نمط (ب) من المراجعين للمستشفى التعليمي لأمراض الجهاز الهضمي والكبد في بغداد ومصرف الدم المركزي في بغداد لمدة من الأول من سبتمبر 2008 وليالي نهاية سبتمبر 2009. تراوحت أعمار المرضى المصابين بإلتهاب الكبد الفييروسي المزمن نوع (ب) بين 20-65 سنة، وتكارا أعمار الأصحاء الحاملين للمستضد السطحي لفييروس إلتهاب الكبد نمط (ب) بين 18-52 سنة. وكان المرضى المزمنين يعانون من أعراض مختلفة مع وجود عوامل خطرية سابقة لحدوث الإصابة. أكثت النتائج الحالية بأن تقنية تفاعل البلمرة المتسلسل نوع Nested أكثر هماية من تقنية تفاعل البلمرة المباشر في تشخيص دنا الفييروس، إذ كانت نسبة الحمض النووي الريبوزي المنقوص الأوكسجين 100% لدى المرضى المزمنين و thảليهم الأصحاء باستخدام تقنية تفاعل البلمرة المتسلسل نوع Nested ونسبة 0% لدى المرضى المزمنين والأصحاء الحاملين على التوالي باستخدام Nested P=0.001.
INTRODUCTION

The polymerase chain reaction (PCR) is an in vitro technique which allows the amplification of a specific deoxyribonucleic acid (DNA) region(1,2,3). Moreover, the diagnostic technique is not influenced by the antiviral therapy administered to the patient, so it is possible to detect the viral DNA also during the first days of treatment with a specific antiviral therapy (4,5,6).

HBV PCR can be used for the identification of low level HBV viremia in HBsAg positive subjects with active liver disease without HBV-DNA positivity in standard hybridization technique. It is also useful for the appraisal of HBV infection in liver transplantation, evaluation of antiviral treatment and for the diagnosis of HBsAg in acute or chronic liver disease (7,8,9). Conventional hybridization procedures have now been used widely for the detection of HBV-DNA in serum, tissues, and mononuclear blood cells(10,11). HBV-DNA is the most direct and sensitive test for viral multiplication and semi quantitative tests have been developed, based on liquid hybridization (12). There is a need for more sensitive tests to identify the HBV-DNA sequences, based on the following: (a) hepatitis B surface antigen (HBsAg) positive blood donors or mothers who have been shown to transmit HBV infection despite being positive for antibodies to hepatitis B e antigen (anti-HBe) and HBV-DNA negative in serum(13). (b) some patients with HBsAg positive/anti-HBe positive chronic active hepatitis having active liver disease despite serum HBV-DNA negativity and absence of hepatitis delta virus or hepatitis C virus (HCV) coinfections, autoimmune liver disease, or other causes of liver disease; (c) it is important to have a precise follow up of patients under antiviral treatment, to evaluate partial or complete responses and the subsequent risk of reactivation; (d) PCR is necessary for subsequent sequencing of the amplified products(2). (e) HBsAg negative patients with acute and chronic liver diseases have been shown to contain HBV-DNA sequences in the blood, liver, or mononuclear cells. HBV-DNA has also been shown in blood donors with no HBV serological marker(14). Several studies have highlighted potential use of PCR for HBV-DNA detection (12,13,14). This study aimed to employing two PCR protocol (direct amplification PCR and Nested PCR) to identify the HBV DNA, and comparing them.

MATERIALS AND METHODS

Subject groups include the following:

Chronic hepatitis B patients groups

A total of 30 patient with CHB, who were admitted to Hepatology and Gastroenterology Teaching Hospital in Baghdad, aged from 20-65 years. The patients were suffering from different clinical symptoms with previous risk factors for transmission of HBV infection.

Control groups

A total of 30 healthy HBs Ag carrier was discovered accidentally through attending blood bank for donation of blood, aged from 18-52 years.

Detection of HBV DNA by PCR techniques

AB analitica kit for detection of HBV DNA based on the amplification of the core region was used in the process of amplification.
Three nucleic acid segments are involved in the reaction: double stranded DNA template to be amplified (target DNA) and two single-stranded oligonucleotides" primers "that are designed in order to anneal specifically to the template DNA. The DNA polymerase begins the synthesis process at the region marked by the primers and synthesizes new double stranded DNA molecules, identical to the original double stranded target DNA region, by facilitating the binding and joining of the complementary nucleotides that are free in solution (dNTPs). After several cycles, one can get million of DNA molecules which correspond to the target sequence (14,15).

DNA extraction
The detailed procedure was carried out as has been suggested in the leaflet supplied with the test kit (AB analitica kit, Italy)

PCR direct amplification (1st amplification )
Add to each premixed test tube (colourless tubes):
AB SuperTaq 0.2 μl and extracted DNA 10 μl.

It is important to include in each experiment a negative control to monitor the contamination (add distilled water to the mix instead of extracted DNA) and 10 μl of the positive control included in the kit. Shortly centrifuge and put the test tubes in the thermocycler.

Agarose gel electrophoresis (visualization of the amplification products)
Prepare a 3% agarose gel, (weighting 1.5g of Agarose and adding it to 50ml of 1x TAE). Then mix directly on a parafilm layer
2 μl 6x blue
10 μl amplified product or DNA (MW Marker)

The mixture was loaded in the gel wells and starts the electrophoresis run (100 v. for 45 min.). Then read results on an UV transilluminator. The size of amplified products was determined by comparing with the reference DNA Molecular size Marker included in the kit.

Nested amplification (2nd amplification)
Add to each premixed test tube (green tubes): AB SuperTaq 0.2 μl and Product of direct amplification 1 μl.

Centrifuge shortly and put the HBV test tubes in the thermocycler
The size of amplified products was determined by comparing with the reference DNA Molecular size Marker included in the kit.

RESULTS AND DISCUSSION
The PCR is used for detecting HBV DNA in liver and serum. The PCR has permitted the distinguishment of extremely small quantities of nucleic acid for HBV in serum of patient. It is also useful for the appraisal of HBV infection in liver transplantation, assessment of antiviral treatment and for the identification of HBsAg in acute or chronic liver disease, so the detection of hepatitis B virus DNA is a reliable evidence of the presence of viral agent and its replication (16,17).

The establishment of the molecular techniques for identified HBV DNA in chronic patients and carrier group gives away an obvious image especially when uses the Nested PCR. The current results confirm that a nested PCR protocol is more sensitive method to detect HBV DNA than PCR direct protocol. The positively of HBV DNA in carrier group and chronic group reached 46.7% and 30.0%, respectively by employing direct
protocol whereas, HBV DNA is 100% in both groups by using Nested protocol (Table 1 and Figs. 1, 2).

Table (1): The difference between chronic group and carrier group according to different PCR protocols.

<table>
<thead>
<tr>
<th>Nucleic Acid Test</th>
<th>Cases</th>
<th>Healthy carrier HBV</th>
<th>Chronic HBV</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR direct amplification</td>
<td>Positive Count</td>
<td>14</td>
<td>9</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>% within cases</td>
<td>46.7%</td>
<td>30.0%</td>
<td>38.3%</td>
</tr>
<tr>
<td></td>
<td>Negative Count</td>
<td>16</td>
<td>21</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>% within cases</td>
<td>53.3%</td>
<td>70.0%</td>
<td>61.7%</td>
</tr>
<tr>
<td>Total</td>
<td>Count</td>
<td>30</td>
<td>30</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>% within cases</td>
<td>100.0%</td>
<td>100.0%</td>
<td>100.0%</td>
</tr>
<tr>
<td>Nested PCR of HBV</td>
<td>Positive Count</td>
<td>16</td>
<td>21</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>% within cases</td>
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<td></td>
<td>% within cases</td>
<td>100.0%</td>
<td>100.0%</td>
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</tr>
</tbody>
</table>

Fig.(1): Detection of HBV DNA on 3% agarose gel electrophoresis (direct amplification) for 45 min at 100 volt. (1) DNA marker. (2) HBV positive control. (3) Negative control. (4&5) HBV positive sample (270 bp band).

Fig.(2): Detection of HBV DNA on 3% agarose gel electrophoresis (nested amplification) for 45 min at 100 volt. (1) DNA marker. (2) HBV positive control. (3) Negative control of the direct amplification. (4) Negative control of nested amplification. (5&6) HBV positive sample (258 bp band).
Studies by some researchers showed that HBV DNA among healthy HBV carriers and CHB patients were detected in 44.0% and 37.0%, respectively of the samples using a direct amplification of HBV DNA with detection limit of 8.600 copies/ml(17). Beside that, Pawlotsky(18) had detected HBV DNA by employ commercially available PCR assay with percentage of positivity was 56.0% among CHB patients and 48.0% among carrier HBV group with limit of HBV detection of 6.340 copies/ml. 

On the contrary, Barlet et al.(19) reported that the HBV DNA was detected in 94% of CHB samples and 86% of carrier HBV samples by nested protocol with low level of HBV DNA (560 copies/mL). Zalewska et al.(20) found the positivity of HBV DNA in chronic patients and a symptomatic HBV carrier by using nested polymerase protocol was 89% and 92%, respectively with low detection limit of HBV DNA was noticed (400 copies/ml). Other investigator showed that a non significant difference was noticed between a chronic patients and carrier subject in regard to nested PCR protocol (21).

In the present study, the comparison of the two molecular methods of HBV DNA identification revealed that the second molecular PCR was able to detect very low amount of HBV DNA in serum of patients, therefore this method may be regarded as the most efficient technique.

As a final point, the current results confirmed the importance of this molecular test which leads to increase the chance of diagnosis and decrease the progress of the disease to end-stage of liver disease and hepatocellular carcinoma, therefore the high sensitivity and specificity of PCR permits virus detection soon after infection and even before the onset of disease, as well as early detection may give physicians a significant lead in treatment.

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