

# IDENTIFICATION OF HEPATITIS B VIRUS BY POLYMERASE CHAIN REACTION TECHNIQUE: PERFORMANCE STUDY AND COMPARISON WITH ENZYME LINKED IMMUNOSORBENT ASSAY

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## ABSTRACT

### *Background*

Diagnosis of hepatitis B virus (HBV) infection is routinely based on the serological assay of hepatitis B surface antigen (HBsAg) detection. However, detection of HBV DNA has been documented from HBsAg negative samples. Occult hepatitis B virus infection is generally defined as the detection of HBV DNA in the serum or tissues of subjects who have negative test for HBsAg.

### *Objectives*

The aim of this study was to determine the rate of occult HBV infection among HBsAg negative subjects and the introducing of PCR as a diagnostic tool for HBV.

### *Methods*

Serum samples from thalassemic patients and blood donors, previously tested for HBsAg by ELISA technique, were examined for the presence of HBV DNA by PCR in Kurdistan Technology and Scientific Research Establishment Center. PCR has been used due to its high specificity and sensitivity.

### *Results*

HBV DNA was detected in 11 (100%) thalassemic patients, who had detectable HBsAg while from 29 HBsAg negative blood samples, 7 samples (24.14%) were positive for HBV DNA. A significant difference was observed between PCR and ELISA tests in detecting HBV markers. Statistically, ELISA had showed (61%) sensitivity when compared to PCR technique in detecting PCR positive HBV DNA sera samples. However, it showed (100%) specificity in detecting PCR negative HBV-DNA samples. Furthermore, no significant association was observed according to sex effects on the incidence of HBV infection.

### *Conclusions*

These results indicated that HBV DNA was observed in HBsAg negative patients. In addition, the present study showed that using of PCR in detection of the virus in patient's samples is more sensitive than the ELISA assay.

**Keywords:** *Hepatitis B Virus, PCR, ELISA.*

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## INTRODUCTION

Hepatitis B virus (HBV) is an enveloped, circular, partially double-stranded, DNA virus, of approximately 3200 bp DNA genome. It is a member of the Hepadnaviridae family <sup>(1)</sup>. The virus replicate by reverse transcription process, the HBV DNA template is transcribed by cellular RNA polymerase to pregenomic RNA, which is reversely transcribed to DNA by viral polymerase <sup>(2)</sup>.

The HBV infection is one of the major human health problems worldwide <sup>(3)</sup>, affecting an estimated 2 billion persons. Of those infected with HBV, 400 million remain chronically infected globally. Annually, up to one million of this population dies due to the consequences of this infection such as cirrhosis and hepatocellular carcinoma (HCC) <sup>(4)</sup>. HBV infection is common in patients with hereditary hemolytic anemia, particularly thalassemia, who require frequent blood transfusions <sup>(5)</sup>. Currently, HBsAg screening test is used to reduce transfusion-transmitted HBV <sup>(6)</sup>. However, previous studies have shown that HBV DNA can be detected in the absence of HBsAg, this so-called occult HBV infection <sup>(7, 8)</sup>. So by introducing the PCR technique, the diagnosis of occult infection can be reduced significantly. This is of major importance in the context in screening blood donors. The diagnostic PCR techniques are recognized as methods for identification of a large and still growing number of pathogens. Many clinical and public health diagnostic laboratories have implemented PCR technique due to its sensitivity and accuracy in comparison with traditional methods. PCR may have a significant impact on detection, therapy monitoring and resistance testing of different viruses. Therefore, it is of major importance for the routine diagnostic laboratory to report accurate and reliable results <sup>(9)</sup>.

The aims of this study were identification of HBV in serum of thalassemic patients and blood donors by PCR technique, evaluating the sensitivity and specificity of ELISA test in comparison with PCR, and determining the rate of occult HBV infection among HBsAg negative serum samples.

## MATERIALS AND METHODS

### *Samples collection*

Serum samples were collected from 40 subjects; 28 (70%) and 12 (30%) were obtained from thalassemic patients and healthy blood donors, respectively. The samples were collected from the period between June

to August 2008. The serum samples were previously screened for the presence of HBsAg using Sandwich ELISA method at the laboratories of Hiwa Hospital, Sulaimani, Iraqi Kurdistan Region. It should be noted that some of the blood donors were from the family of thalassemic patients.

### *HBV DNA Extraction & PCR amplification:*

DNA was purified from 100 µl serum samples using a commercial DNA purification kit as described by the manufacturers (DNP Kit, CinnaGen, Tehran, Iran). The resulting supernatant which contains the purified DNA was transferred to another tube and stored at -30°C, until amplification of the viral genome.

For amplification of viral DNA, 10 µl of extracted DNA were added to 15.4 µl of the reaction mixture, containing 0.4 µl Taq DNA Polymerase, and 15 µl 1X PCR Mix which contained 10X reaction buffer, MgCl<sub>2</sub>, dNTP, loading buffer, and the specific primers. The primers 5'-TAT GTT TCC CTC CTG CTG CT-3' (forward primer) and 5'-CCC CCA ACT CCC AAT TCT AT-3' (reverse primer), were utilized for PCR. This pair of primers was targeted to amplify 353 bp of the highly conserved region of the S gene (CinnaGen, Tehran, Iran). The thermocycler was programmed as described by the CinnaGen company including one cycle of 93°C for 60 sec, 61°C for 20 sec and 72 °C for 40 sec. Then, followed by 35 cycles of 20 sec at 93 °C, 20 sec at 61°C and 40 sec at 72 °C. HBV genome provided by the same manufacturer was used as positive control.

### *Detection of PCR product*

For analyses of the PCR amplification 10 µl of amplified samples was electrophoresed on a 2% agarose gel made in TBE buffer and visualized by UV illumination after ethidium bromide (10 mg/ml) staining. Positive and negative controls were also treated as samples. The amplified (353 bp) DNA fragment was verified with 100 bp DNA ladder to serve as an indicator for the sizes of the bands.

### *Statistical Analyses*

Chi-square analysis was performed to establish the association between the rate of HBV infection and measured factors. Subsequently, the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of markers was calculated to differentiate between the validity of ELISA and PCR. *P* values less than 0.05 were considered statistically significant.

## RESULTS

### Descriptive Analysis of Patients Data

The results of the PCR technique test showed that only 12 (42.8%) thalassemic patients had HBV DNA in their serum while 6 (50%) of the blood donors had HBV DNA positive results (Table 1). These results indicate a high incidence of viral marker in both thalassemic patients and blood donors. There were no significant differences between the incidence of HBV infection in the thalassemic patients and blood donors

The total group of 40 patients and subjects included 29 males and 11 females. All apparently healthy blood donors examined in this study were males. The results of the PCR assay showed 13 (44.83%) HBV DNA positive and 16 HBV DNA negative in 29 males, but in 11 females, 5 (45.45%) of them had HBsAg positive.

Our investigation clearly indicated that the prevalence of HBV infection was not found to be significantly associated with sex.

### Comparative Evaluation of PCR Detection Results with ELISA Method

The present study indicated that there was significant difference between PCR and ELISA test in detecting HBV markers. Statistically, ELISA showed 61% sensitivity when compared to PCR technique in detecting PCR positive HBV-DNA sera samples. However, it showed 100% specificity in detecting PCR negative HBV-DNA samples (Table 3). The positive predictive value of this technique was found to be 100% whereas its negative predictive value was 76%. Therefore, the agreement rate was 82.5%.

**Table 1. Comparison of PCR Result between Thalassemic Patients and Blood Donors.**

Group	PCR		Total
	Positive PCR	Negative PCR	
Thalassemic Patients	12	16	28
Blood Donors	6	6	12
<b>Total</b>	18	22	40

*P* value= 0.677

0.677 <sup>NS</sup>

**Table 2. Statistical Analysis of the PCR Result according to Gender.**

Gender	PCR		Total
	Positive PCR	Negative PCR	
Male	13	16	29
Female	5	6	11
<b>Total</b>	18	22	40

*P* value= 0.971

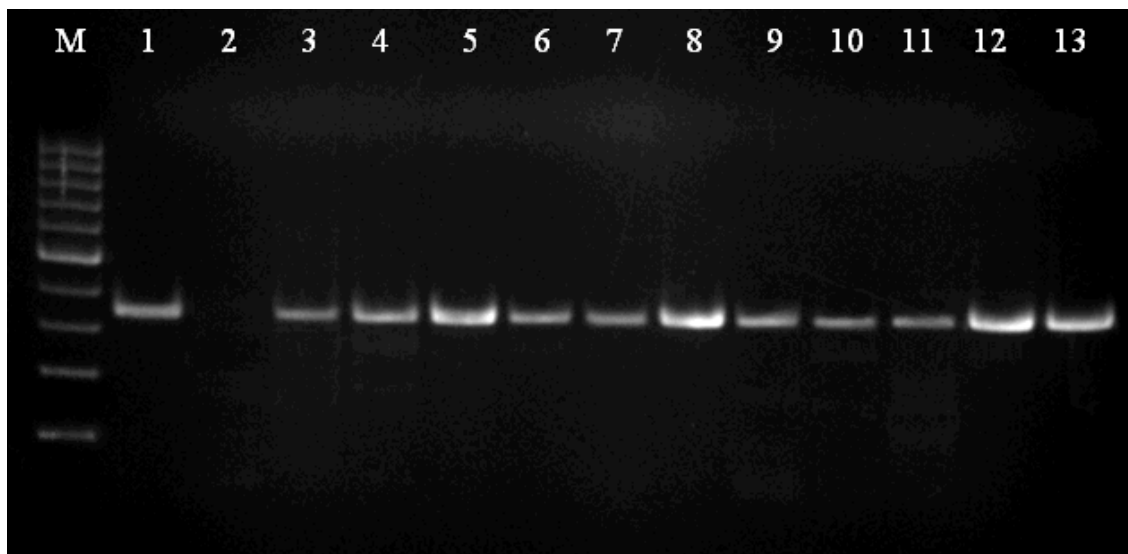
0.971 <sup>NS</sup>

**Table 3. HBV DNA Detection in HBsAg Seropositive and Seronegative Patients.**

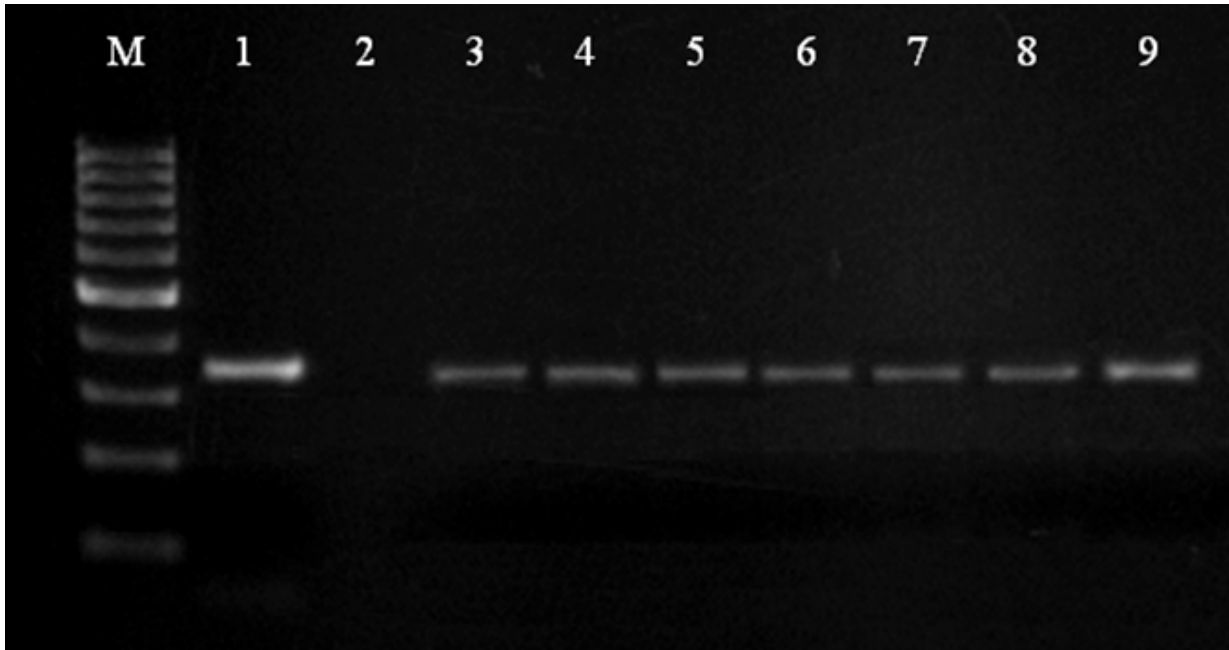
Validity		PCR		Total
		Positive	Negative	
<b>ELISA</b>	Positive	11	0	11
	Negative	7	22	29
<b>Total</b>		18	22	40
<b>Chi value</b>		8.54**		

According to hypothesis proposed by Brechot *et al.* (2001) <sup>(10)</sup>, the serum samples were divided into two specific groups: HBsAg positive and HBsAg negative individuals. Out of the total, 11 HBsAg positive and 29 HBsAg negative sera obtained from thalassemic patients and blood donors were tested. It found that all (100%) of the HBsAg positive sera were positive for

HBV DNA, (figure 1). It was apparent that 7 (24.14%) of the 29 HBsAg negative sera were found to be positive for HBV DNA, (figure 2). For confirmation, the above 7 seronegative samples which were positive for viral DNA were amplified again, and identical results were observed.



**Figure 1. PCR profile of HBsAg positive blood samples. The bands demonstrate presence of HBV infection. Lane M: 100bp DNA ladder, Lane 1: Positive control, Lane 2: Negative control, Lanes 3 to 13 represent amplification results of eleven patients who were positive for HBsAg.**



**Figure 2. Gel electrophoresis of PCR product obtained from HBsAg negative blood samples. Lane M: 100bp DNA ladder, Lane 1: Positive control, Lane 2: Negative control, Lanes 3 to 9 represent amplification results of seven patients who were negative for HBsAg.**

## DISCUSSION

The present study indicates that the increased risk of HBV may be related to an increased exposure to risk factors such as frequent blood transfusions to the thalassemic patients, and the high prevalence of HBV in blood donors may be due to familial contact between the thalassemic. It should be noted that the present study included limited number of the samples. So, the high rates of HBV infection in the risk groups and other factors may be related to this problem.

According to gender, the result of this study is in agreement with that of Kurien *et al.* (2005) <sup>(11)</sup>, who found that the prevalence of HBV infection is not associated with gender. In contrast, Ahmad *et al.* (2006) <sup>(12)</sup> reported that the frequency of HBV infection was slightly higher in males than in females.

It has been reported that PCR is an accurate, sensitive and highly specific method for detection of pathogenic microorganisms. Thus, it is now accepted as the gold standard for detecting nucleic acids from a number of organisms and it has become an essential tool in the

research laboratory <sup>(13,14)</sup>. These hepatitis B serologic tests are needed as a back up to DNA-based methods

like PCR as gold standard for evaluating their sensitivity and specificity <sup>(15)</sup>. Comparison of the sensitivity and

specificity between PCR and ELISA for detecting HBV-DNA and HBsAg were carried out by many researchers. Therefore, in this study the comparison of ELISA test sensitivity and specificity with PCR methods were carried out.

Moreover, the results of the present study is supported by several previous studies; Scully *et al.* (1994) <sup>(16)</sup> demonstrated the prevalence HBV DNA in HBsAg negative from 7 of 36 or (19.5%) of those with undefined chronic liver disease, whereas 18.4% of the HBsAg negative samples showed the positivity for HBV DNA by Torres-Baranda *et al.* (2006) <sup>(1)</sup>. Also, the prevalence of occult HBV infection was studied by Shetty *et al.* (2007) <sup>(17)</sup> and was 28% among of HBsAg negative samples. Moreover, in the study of Jafarzadeh *et al.* (2008) <sup>(18)</sup> HBV-DNA was detected in (28.57%) of HBsAg negative samples.

A number of explanations for the persistence of HBV DNA in HBsAg negative samples have been proposed, including the presence of immune complexes in which HBsAg may be hidden <sup>(19)</sup> or may be due to chronic HBV infection, particularly in those with HCV-related chronic hepatitis <sup>(20)</sup>.

Reesink *et al.* (2008) <sup>(21)</sup> illustrated that occult HBV infection may represent as; (i) acute infection in the

window period, (ii) HBV tail end of chronic HBV infection, (iii) persistence of replication at low level after recovery in the presence of anti-HBs, or (iv) occurrence of an escape mutant in the S gene in vaccinated or unvaccinated individuals. Moreover, it has been suggested recently that HIV infection may be a risk factor for occult hepatitis B<sup>(19,22)</sup>.

One of the most important causes of occult HBV infection is the HCV related chronic hepatitis. According to some studies, the reason for disappearance of HBsAg in subjects co-infected with HCV is that the HCV core protein can inhibit HBV<sup>(8)</sup>. HBV viral level in serum is usually less than (104 copies/ml) in patients with HCV infection, which is significantly lower than in those with HBsAg positive<sup>(23)</sup>. Thus, we need a sensitive PCR technique for detection of this form of infection.

Another cause for the occult HBV infection is the mutation in the HBV genome<sup>(9)</sup>. These variants do not express the same antigen coded by HBV and also differ in the serological pattern. However, they show sequence homology at the DNA level<sup>(24)</sup>. In recent years, mutant HBsAg have caused great academic interest, and many analyses and researches have been made for the emergence of HBV mutants with mutations in the “a” determinant of HBV S gene can alter expression of HBsAg and result in immune escape<sup>(2)</sup>. The mutation in the “a” determinant of HBV S gene occur as a drug resistance<sup>(25)</sup> or during the course of chronic hepatitis particularly in patients with chronic hepatitis who develop end stage liver disease (ESLD) and HCC, although the clinical relevance of this association is unclear<sup>(2)</sup>. According to these research results, it is very important to further investigate mutant distributions and clarify mutations in HBV S gene which could cause the changes of antigenicity and immunogenicity of HBsAg. These mutations in the coding region of HBsAg could not be detected in routine serologic assays such as ELISA<sup>(24,26)</sup>.

Furthermore, during the seroconversion phase of acute hepatitis B infection HBsAg marker cannot be detected in the blood. This phase called the “the window period”, which represents a carrier state of the disease. The long seroconversion window period remains a problem and is responsible for most cases of HBV infection in the blood transfusion<sup>(27)</sup>.

In conclusion, current serological tests for HBV screening are insufficient to safeguard the blood supply and that the marginal yield of HBsAg screening test

does not justify its implementation in routine screening of blood donors. Sensitivity and specificity of PCR is an optimal, they represent a first safeguard, particularly for the detection of occult hepatitis B, in order to have a reliable diagnosis, prevent HBV dissemination.

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