

PCR Based Diagnosis of Hepatitis B Virus

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Abstract.- The detection of viral genome through the use of polymerase chain reaction (PCR) has made possible a new step in viral diagnosis. The main advantages of PCR are its extreme sensitivity and the possibility to develop rapid assays using non radioactive probes. The amplification of hepatitis B virus (HBV) DNA sequences in sera for the diagnosis of HBV is an important application of PCR with regard to HBV. A set of primer B2833S 5' GGGTCACCATATTCTTGG 3' and B170AS 5' GT CCTAGGAATCCTGATG was tested for the amplification of HBV genome from the plasma derived HBV patients and a cloned HBV. Annealing temperature and units of Taq polymerase were optimized. Annealing temperature 55°C was found best for B2833S and B170AS. It was established that one unit of Taq polymerase amplify optimally in a reaction mixture of 25 µl. This procedure will be more reliable evidence of the presence of Hepatitis B virus.

Key words: PCR, HBV, amplification of DNA.

INTRODUCTION

Hepatitis B disease is caused by a virus called hepatitis B virus (HBV). HBV was first discovered by Blumberg *et al.* in 1965. HBV is a small virus about 42 nm in diameter composed of envelope containing hepatitis B surface antigen (HBsAg) and internal nucleocapsid structure (Feitelson, 1992; Sharma *et al.*, 2005). The nucleocapsid consists of the hepatitis B core antigen (HBcAg) and the viral genome. HBV is a DNA virus with 3200 bp partially double stranded circular genome (Blum *et al.*, 1989).

Viral hepatitis, especially caused by infection with hepatitis B virus (HBV), is major public health problem. About 300 million people are yearly infected by hepatitis B virus. In Taiwan, about 20% of the population have hepatitis B surface antigen in the serum (Chen and Sung, 1978). In China about 0.5-1 million new cases appear every year (Tiollais *et al.*, 1985). In Pakistan, hepatitis B disease is highly endemic and the carrier rate in the population is one-tenth (Malik and Akhtar, 1995).

Epidemiological studies have clearly shown the importance of HBV in hepatocellular carcinoma and liver cirrhosis, which are the major causes of mortality (IARC, 1994). HBV is a significant cause of post transfusion hepatitis and a major cause of chronic hepatitis and hepatocellular carcinoma in

South Asia and Japan (Blumberg and London, 1985). In USA there are about one million carriers of HBV, 25% of whom develop chronic active hepatitis and 4000 die due to HBV related cirrhosis annually, while an additional 800 persons die each year due to HBV related hepatocellular carcinoma (Tiollais *et al.*, 1985).

For the detection of HBV various serological markers have been shown to be sensitive and convenient but these are not always good indicators of viral activity (Shih *et al.*, 1990; Qian *et al.*, 2005). The main drawbacks of the traditional methods are the involvement of long time required and their low sensitivity. Viral DNA is the more reliable evidence of the presence of virus. Conventionally HBV DNA is detected by dot or spot hybridization having sensitivity at 0.1 to 1 pg (3×10^4 - 3×10^5) of HBV DNA (Shih *et al.*, 1990) while polymerase chain reaction (PCR) can detect even a single copy of the gene (Kaneko *et al.*, 1989). Since the PCR was developed in 1988 (Saiki *et al.*, 1988), it has found a wide application in molecular biology and is now regarded as irreplaceable tool in many laboratories.

MATERIALS AND METHODS

Samples collection and processing

Blood samples were collected from clinically diagnosed HBsAg positive patients from Services Hospital and Jinah Hospital, Lahore Pakistan. EDTA was added to a final concentration of 7.0 mM and plasma was extracted by centrifugation with in 24 hours and stored at -20°C as 100 µl aliquots.

Extraction of HBV DNA

Viral DNA was extracted from the blood plasma using the method of Persing *et al.*, (1993) with some amendments. To 100 µl of blood plasma in an eppendorf tube 100 mM Tris pH8, 0.75M NaCl, 35mM EDTA, 2.5% SDS, and 0.4µg Proteinase K enzyme were added. Tubes were wrapped in parafilm and incubated at 56°C for 2 hours. An equal volume of phenol:chloroform (1:1) was added and centrifuged at 12000 xg, the aqueous phase was collected and repeated this step twice more. Volume of the aqueous phase was estimated and HBV DNA was precipitated with ethanol in the presence of carrier. Tubes were stored at -20°C overnight. Nucleic acids were recovered by centrifugation at 12000 xg for 30 minutes and resuspended in 15 µl TE (Tris-EDTA) buffer.

PCR amplification

A set of primer (B2833S and B170AS) was designed and checked for PCR amplification.

Sequences of the primers (5'-3')

B2833S (2816-2833 nt)	GGG TCA CCA TAT TCT TGG
B170AS (170-187 nt)	GTC CTA GGA ATC CTG ATG

Times for PCR were:

Step I	94 °C for 1 minute	(Denaturation)
Step II	55 °C for 1 minute	(Annealing)
Step III	72 °C for 2 minutes	(Extension)

Number of cycles 20-25.

Total volume of PCR reaction was 25 µl and consisted of 20 µl of master mix (9 mM Tris-Cl pH8.3, 45mM KCl, 2.25mM MgCl₂, 180mM of each dNTP and 0.9µM of each primer), 3 µl of HBV DNA and 2 µl (1 unit) of Taq DNA polymerase.

Detection of amplified sequences

After the amplification, 15 µl aliquot of the reaction mixture was subjected to gel electrophoresis (Southern, 1979) on 1.5 % agarose in Tris-borate-EDTA buffer. Ethidium bromide was added to a final concentration of 0.5 µg/ml to the gel mixture or the gel was stained with ethidium bromide after electrophoresis. The gel was then visualized and DNA bands detected on UV transilluminator and photographed.

Quality controls

Because of the very high sensitivity of PCR, great care was taken to prevent any possible contamination which can give rise to false positives. Beside, both negative control and positive control were used to make the assay more reliable.

Positive control	45 ng of cloned HBV DNA was used as a template.
Negative control	3 µl of distilled water (without any template).

RESULTS

Patient history

Blood samples were collected from fifty clinically diagnosed HBsAg positive patients from Lahore hospitals. Age of the patients was 20-40 years. According to the medical record of the hospital, thirty were in chronic condition, while twenty were in acute phase of the disease.

PCR amplification

The success of PCR depends upon a number of factors like template DNA, primers, dNTPs and magnesium ion concentration in the reaction mixture as well as choice of the polymerase enzyme and primer's annealing temperature. Annealing temperature was optimized for the internal set (B2833S and B170AS) by setting PCR at various annealing temperatures. Annealing temperature 55°C was found best for this pair of primers. Units of Taq polymerase (prepared by CEMB enzyme facility) were also optimized and it was found that viral DNA amplifies optimally with one unit of the enzyme.

The primers amplify a region of 0.5 kilo base (kb) from nucleotide No. 2833 to nucleotide No. 170 (Fig. 1). PCR optimization is necessary step to get the clear PCR band. For this, different concentrations of DNA, magnesium chloride, primers; Units of Taq polymerase and annealing temperatures were checked. One unit of Taq polymerase was chosen best; as we increase unit of enzymes it also increases background smear. Annealing temperature 55°C was found best, at higher temperatures PCR product from patient derived HBV DNA gradually vanished (Figs. 1, 2). After optimization of the PCR conditions,

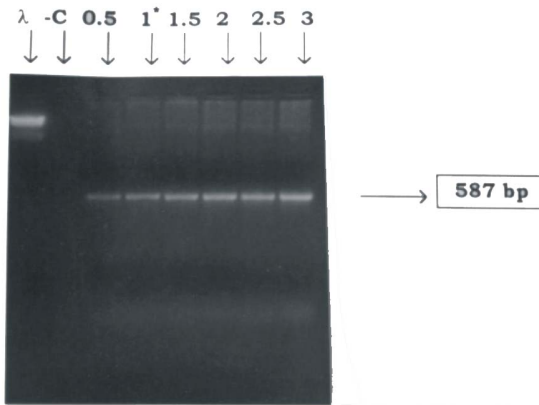


Fig. 1. PCR test for various Taq polymerase Units. λ, Marker; C, negative control; 0.5-3, 0.5-3units of Taq polymerase.

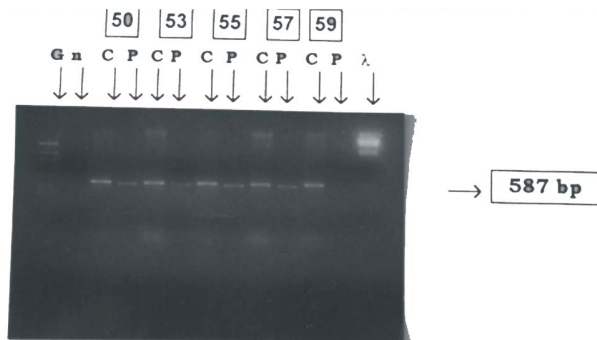


Fig. 2. PCR using different annealing temperatures 50-59°C. λ, Marker; C, Control HBV DNA; P, HBV DNA from patient; n, negative control; G, marker.



Fig. 3. Hepatitis B virus PCR products derived from cloned and patient derived templates. Lanes 1&7, λ marker; Lane 2, positive control; Lanes 3-6 PCR from patients.

amplification with this set of primers gave a right size band of 0.5 kb on agarose gel in two of four patients tested, two were negative for HBV DNA (Fig. 3). With each reaction a positive and negative controls were planned to exclude the risk of false positives or false negatives (Figs. 1, 2). The positive control contained a known quantity (45 ng in 25 µl of the reaction mixture) of HBV DNA cloned. In negative control all of the ingredients were present except HBV DNA but water was added to make the volume 25 µl.

After amplification the PCR product of both the controls and plasma derived HBV DNA was loaded on the same gel and visualized on UV transilluminator. Appearance/presence of band in the negative control represent false positive due to contamination because no HBV DNA was present as a template. On the other hand, no amplification in the positive control represents false negatives due to absence of one or more of the ingredients.

DISCUSSION

The PCR technique provided a novel rapid and sensitive method for detecting HBV DNA (Brechot, 1993). The competitive PCR test described here provides a sensitive method for the detection of HBV particles from serum samples. One of the major drawback of PCR is its over sensitivity. This over sensitivity results in false positives (Norder *et al.*, 1990). The problem of false positives can be eliminated by choosing appropriate controls and care during handling (Brechot, 1993). In this study Japanese clone was used as a positive control and reaction mixture containing no DNA was used as a negative control. False negatives may also occur due to some inhibitors or with primers which are not designed for that subtype or designed for that subtype but due to mutations in the primer flanking region (Jalava *et al.*, 1993), mutations occur even with in the same subtype of HBV isolated from a single donor (Ono *et al.*, 1983).

A few copies of HBV DNA can be detected by PCR alone, provided that the virus DNA purification procedure can be simplified and the cost becomes lower, the PCR may become an indispensable tool for screening of HBV DNA in

patients with various liver diseases.

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