

# PCR Amplification of Hepatitis B Virus in the Limiting Dilution Format

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**Abstract.-** Hepatitis B virus has eight genotypes based on sequence heterogeneity and at least four HBV genotypes are present in Pakistan. If infected patient has mixed infection of different HBV genotypes, PCR amplification will result the product that may be hybrid of different HBV DNA molecules. Sequence analysis of PCR product amplified from such samples is often unreliable due to ambiguities in sequencing gel/electropherogram. In order to establish HBV DNA amplification from a single HBV molecule in a sample using limiting dilution format, two blood samples (NA004 and NA012) were collected from Lahore hospitals. Viral DNA was extracted from blood plasma and a regions of 1.1 Kb and 0.6 Kb were amplified by using two different sets of primers (NHBF1 & NHBR1 and B170AS & B2833S). Two limiting DNA dilutions, 1:256 and 1:4096 were found effective to get PCR product from a single HBV DNA molecule.

**Key words:** PCR amplification, HBV genotype, mixed infection, quasispecies, sequence heterogeneity.

## INTRODUCTION

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

Viral hepatitis, especially caused by infection with HBV is a major public health problem. About 300 million people are yearly infected by HBV. 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; Wai and Fontana, 2004;

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Marcellin *et al.*, 2005). HBV is a significant cause of post transfusion hepatitis and a major cause of chronic hepatitis and hepatoma 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).

There are at least thirteen subtypes, eight genotypes and more than 121 strains exist as quasispecies so far (Okamoto *et al.*, 1988; Arauz *et al.*, 2002; Norder *et al.*, 1993; Stuyver *et al.*, 2000; Olinger *et al.*, 2006 Tadokoro, *et al.*, 2006) that are geographically distributed (Table I). According to genotype HBV virulence and pathogenicity differ in each location (Lok *et al.*, 2000; Kidd-Ljunggren *et al.*, 2002; Hayashi *et al.*, 2007). Genotyping helps to clarify the routes of infection with and virulence of the virus. Especially, examination of sequence diversity among different isolates of the virus is important since variants may differ in their patterns of serological reactivity, replication of the virus, activity of the liver disease, prognosis and response to treatment (Gao *et al.*, 2007). Patient infected with genotype C have a more aggressive phenotype than those with genotype B (Orito *et al.*, 2001; Kao *et al.*,

2000ab). However, isolates of HBV within the same genotype can cause different clinical manifestations *e.g.* between subgroups Ba (asian) and Bj (Japanese) (Sugauchi *et al.*, 2003).

**Table I.- Geographic distribution of HBV genotypes**

Genotypes	subtypes	Geographic Distribution
A	<i>Adw, adw2, ayw1</i>	US, North Europe, Sub-Saharan Africa
B	<i>Adw2, ayw1</i>	China, Indonesia, Vietnam, Japan
C	<i>Adr, ayr, adr4, adrq</i>	China, Korea, Japan, Veitnam, South Asia
D	<i>awy, ayw2, ayw3, ayw4</i>	Mediterranean, Middle East, South Asia
E	<i>Ayw4</i>	W Sub-Saharan Africa, south to Angola
F	<i>adw4, adw4q</i>	Polynesia, New World - Brazil, C. & S. America
G	<i>adw4, adw2</i>	New World - Brazil, N. & S. America, France
H	<i>adw4</i>	Central America

Now a days only PCR is the quick, effective and most reliable tool for viral diagnosis and recovery of DNA for cloning, sequencing or site specific mutagenesis etc. For isolating single viral sequence to enable the analysis of heterologous samples Brown and Simmonds, in 1995 proposed a method of limiting DNA dilution for HIV positive blood samples. During this study the assay was tried for PCR amplification of hepatitis B virus from the blood samples of the chronic HBV patients who had the probability of bearing quasispecies.

## MATERIALS AND METHODS

### *Samples collection and extraction of HBV DNA*

Blood samples were collected from clinically diagnosed HBV 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 at 5000x g for two minutes in a microfuge. Aliquots of 100 µl plasma were made and stored at -20°C. All the steps were carried out in Type IIB biosafety hood.

Viral DNA was extracted from the blood plasma using the method of Persing *et al.* (1993). To

100 µl of blood plasma in an eppendorf tube, 120 µl of 5x Proteinase K digestion buffer (100 mM Tris pH8, 0.75M NaCl, 35mM EDTA, 2.5% SDS) plus 20 µl Proteinase K enzyme (20 µg/ml) plus 360µl deionized (alpha Q millipore) autoclaved water. Tubes were wrapped in parafilm and incubated at 56°C for 2 hours in a rotating incubator. An equal volume of phenol:chloroform (1:1) was added, mixed vigorously and centrifuged at 12000 xg, the aqueous phase was collected and the extraction repeated with phenol:chloroform twice. Volume of the aqueous phase was estimated and 1/10th volume of 0.1mg/ml glycogen was added. NaCl concentration was increased to 0.3 M and 2.5 volume of chilled ethanol was added. Tubes were stored at -20°C over night or at -70°C for 30 minutes and centrifuged at 12000x g for 30 min. Supernatant was aspirated and the pellet was air dried after washing twice with 70% ethanol. The pelleted DNA was resuspended in 15 µl TE.

### *PCR amplification*

Two sets of primers were used for PCR. The first pair of primers (NHBF1 and NHBR1) was selected from the conserved region of HBV genome near S gene. A region of about 1.12 kb from nucleotide No. 2362 to 283 was amplified by using this set of primer (Abbas, 2001). This pair was termed as external primers. Second set of primers (B2833S and B170AS) was selected from a published source (Thiers *et al.*, 1988).

### *Preparation of limiting dilutions*

The HBV DNA of two Pakistanian isolates was amplified by using single DNA molecule as template for PCR. Limiting dilution of the HBV DNA was carried out according to the scheme outlined by Brown and Simmonds (1995). This limiting dilution scheme uses a statistical method to predict that when the HBV DNA dilution employed contains only a single HBV DNA molecule then only 1 out of 5 PCR reactions will turn out to be positive. To the HBV DNA isolated from 100 µl of patient plasma, an equal amount of deionized water was added to prepare 1:2 dilution. Fifteen µl of this was dispensed into 5 PCR tubes each having three µl of this 1:2 diluted DNA while to the remaining

DNA solution (1:2 dilution) an equal volume (15  $\mu$ l) of deionized water was added. So the resultant dilution was 1:4. It was dispensed in the same way for another round of PCR and to the remaining DNA 15  $\mu$ l of water was added to generate a 1:8 dilution. In this way dilutions were prepared upto 1:4096 dilution and 5 PCR amplifications were carried out from each dilution. Positive and negative controls were set for each dilution to avoid any false negative or false positive results.

#### Sequences of the primers (5'-3')

NHBF1 (2362nt- 2382nt)	GTC CCC TAG AAG AAG AAC TCC CTC
NHBR1 (260nt-283nt)	CCC TAG AAA ATT GAG AGA AGT CCA
B2833S (2814nt- 2831nt)	GGG TCA CCA TAT TCT TGG
B170AS (168nt-185nt)	GTC CTA GGA ATC CTG ATG

Times for PCR were:

Step I	94°C for 1 minute	(Denaturation)
Step II	55 or 50°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  $\mu$ l and consisted of 20  $\mu$ l of master mix (9 mM Tris-Cl pH8.3, 45mM KCl, 2.25mM MgCl<sub>2</sub>, 180mM of each dNTP and 0.9 $\mu$ M of each primer), 3  $\mu$ l of HBV DNA and 2  $\mu$ l (1 unit) of Taq DNA polymerase.

## RESULTS AND DISCUSSION

#### Patient history

Blood samples were collected from clinically diagnosed HBsAg positive patients from Lahore Services and Jinnah hospitals. Patient designated NA012 was female of 22 years while NA004 was male of 50 years. According to the medical record of the hospital both NA012 and NA004 were chronic patients and suffering from liver cirrhosis.

#### PCR amplification in limiting dilution format

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. Annealing temperature for the external set of primers was 50°C (Abbas, 2001).

Limiting dilutions were performed with an external set of primers (NHepF1 and NHepR1) amplifying a region of about 1.1 Kb. Purpose of these dilutions was to get DNA amplification from a single molecule of HBV DNA. The PCR was performed for a set of five reactions, because it is reported (Brown and Simmonds, 1995) that when one out of five PCR products is positive, the probability that it may be amplified from a single DNA molecule is 90%. During these limiting dilutions one sample of HBV DNA (NA004) gave one positive result out of five PCR amplifications at 1:256 dilution (Fig 1). Sample designated as NA012 gave 2/5 positive PCR product at 1:4096 dilution.

1:256 dilution

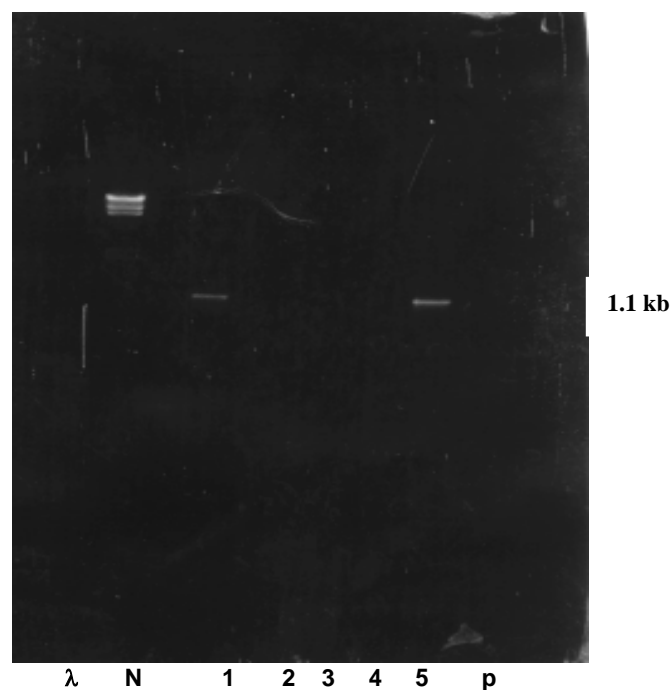


Fig 1. PCR amplification with limiting dilutions of hepatitis B virus DNA.  $\lambda$ , Lambda Hind III marker; N, negative control; P, positive

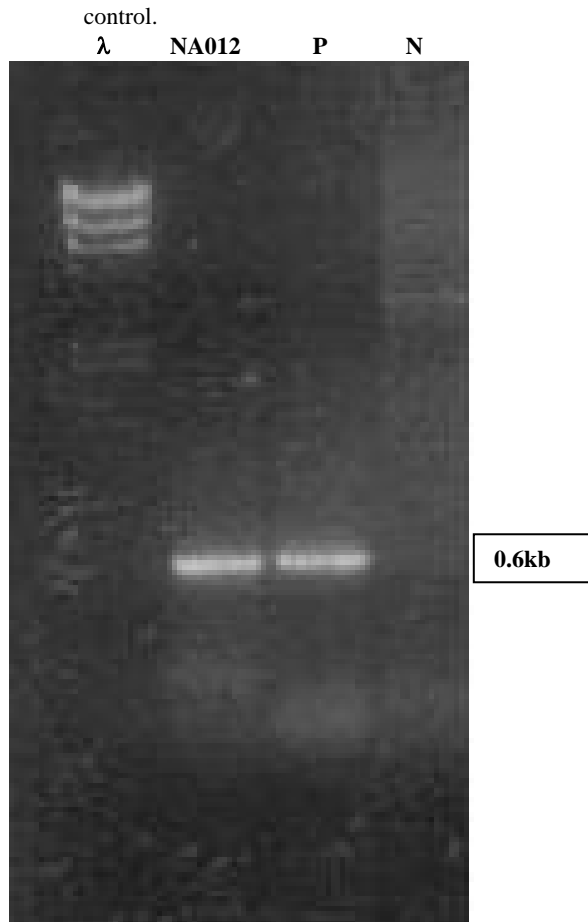


Fig. 2. PCR amplification with primers B170AS & B2833S.  $\lambda$ , Lambda Hind III marker; N, negative control; p, positive control.

Purpose of the PCR amplification in the limiting dilution format was to get homogenous product amplified from a single HBV molecule. Heterogeneity in the HBV genome exists in the same subtype even prepared from a single donor (Ono *et al.*, 1983). Most significant problem with amplification of heterogeneous viral sequences by PCR is artefactual recombination. As reported by Brown and Simmonds (1995) artefactual recombination occurs during the latter cycles of the PCR, where there is insufficient DNA polymerase to complete the synthesis of all primed DNA strands in the allotted time. Partially extended DNA detaches from its template during the following denaturation step, then on cooling anneals to any available complementary strand; synthesis of the partial

sequence is then completed by copying the unwanted sequences. Meyerhans *et al.* (1990) have stated that one out of five sequences are artificial recombinants if a template of about 300 bp is subjected to 25 rounds of PCR amplification. The frequency of recombination increases with the increasing template fragment size. In case of fragment size over 1500 bp, almost all the sequences at the end of the PCR will be hybrid copies of the two, three or more of the original sequences. According to recent review by Brown and Simmonds (1995) when the HBV DNA dilution employed contains only a single HBV DNA molecule then only 1 out of 5 PCR reactions will turn out to be positive. Whereas at a PCR positive frequency of 0.4 (2/5 positive) 77% of PCR product must have come from a single DNA molecule.

For sample NA004, one out of five positive PCR products was selected, obtained at 1:256 dilution. According to the scheme it (1/5 +ve) has a probability of 99% that it has been amplified from a single HBV DNA molecule. In case of sample NA012, after obtaining two out of five positive reactions at 1:4096 dilution, was further diluted to 1:8192. But at this dilution no positive reaction was observed. So two out of five was selected, having 77% probability that it is amplified from a single HBV DNA molecule. DNA from these selected dilutions was reamplified by PCR using the second set of primers (Fig. 2) and used for sequencing and cloning (data not shown).

On the basis of sequence heterogeneity hepatitis virus has eight genotypes A-H and significant heterogeneity has been observed in the sequence of HBV even within the same subtype (Mimms *et al.*, 1990). The assay was already established for HIV, this could be equally effective for HCV or any other virus that showed significant heterogeneity. But a considerable care should be taken while preparing limiting DNA dilutions and PCR amplifications. PCR from a single DNA molecule is very necessary when we need information about all infecting particles for drug prescription or for some sensitive manipulations such as sequencing.

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