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Cloning and Nucleotide Sequence analysis of Pre-S1 Surface Antigen Region of Hepatitis B Virus

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Abstract.- Nucleotide sequence of the Pre-S1 region of the surface antigen of hepatitis B virus (HBV) from local population has been determined. Viral DNA extracted from blood plasma of two blood samples NA007 and NA013 from Services Hospital, Lahore was amplified as 1.1 Kb and 0.6 Kb fragments using two different sets of primers (NHBF1 and NHBR1; B170AS and B2833S). The amplified fragment was subcloned in pGEM-T vector. Pre-S1 region of the two samples was sequenced by non radioactive dideoxy chain termination method. Pre-S1 region nucleotide sequence of both samples were found homologous with the Pre-S1 region of reported subtype "adr4". Sequence Alignments showed that NA007 and NA013 were 96% to 100% identical with various adr subtypes (genotype C) and had 9.8% to 23.4% nucleotide variations with other subtypes/genotypes.

Key words: Hepatitis B virus, PreS surface antigen, PCR amplification.

INTRODUCTION

Hepatitis B virus is the smallest known DNA virus that can infect man. It is serious health problem with more than 500 million carriers. It is the major cause of liver cancer and 62% of all reported cancers are caused by HBV (Wai and Fontana, 2004; Raffa *et al.,* 2007). IARC (1994) reported South Asia as the area of intermediate endemicity (2-7%), while reports from Pakistan shows 10-30% endemicity (Malik and Akhtar, 1995; Malik *et al.,* 1995). Hepatitis B virus has a complex genomic organization. It has four overlapping reading frames S, C, P and X, which encode seven polypeptides or viral proteins. Three envelope proteins *viz.,* major (HBsAg), middle (PreS2 + HBsAg) and large proteins (PreS1+PreS2+HBsAg) are found in the serum and can induce protective antibodies (Neurath *et al.,* 1989; Nunez *et al.,* 2001; Madalinski *et al.,* 2004; Abbas *et al.,* 2007). Pre-S region of surface gene is highly immunogenic (Milich *et al.,* 1985; Madalinski *et al.,* 2004) and is most hypervariable part of the HBV genome. Immunogenic response of Pre-S region is able to protect from HBV infection (Millich, 1989; Kazaks *et al.,* 2004). The Pre-S region has five known epitopes (Kuroki *et al.,* 1990; Mimms *et al.,* 1990) and two hepatocellular binding sites (Pontisso *et al*., 1989). Brind *et al.* (1997) have also provided *in vivo* evidence for reinfection of the Liver by HBV particles lacking PreS envelope protein expression.

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Based on sequence heterogeneity, hepatitis virus has eight genotypes A-H that are geographically distributed (Okamoto *et al.,* 1988; Norder *et al.,* 1993; Stuyver *et al.,* 2000; Arauz-Ring *et al.,* 2002; Ong *et al.,* 2005). Significant heterogeneity (greater than 10%; reviewed in IARC monograph, 1994) in the envelope proteins of HBV, reducing the efficacy of passive immunity reagents and also allows carriers of Asian HBV isolates to go undetected with current serological detection kits because most of these kits are developed with European and American HBV isolates. This heterogeneity also reduces the efficacy of vaccines (developed with non Asian HBV isolates) for Asian population. So to find out the variation among the Pre-S1 surface antigen region the Pre-S1 region of Pakistanian HBV isolates was sequenced and compared with other reported sequences.

MATERIALS AND METHODS

Patient history

Two clinically diagnosed HBsAg positive patients blood samples were collected from Services Hospital, Lahore. Patient designated NA007 was female (15Y) while NA013 (55Y) was male.

According to the medical record of the hospital NA007, admitted for endocartitis, belonged to Lahore while NA013 with severe condition of blood vomiting was from Faisalabad.

The HBV DNA was amplified by PCR using external set of primers. PCR amplified product was run on 0.8% agarose gel and 1.1 kb band was eluted by Wizard PCR purification system or Sigma's Nucleiclean Kit. The gel purified DNA was cloned in pGEM-T vector, specially designed for the cloning of PCR product because this vector has T overhangs while *Taq* polymerase adds A overhangs at 3' end of PCR product. The plasmid DNA containing 1.1 kb insert was isolated by minipreps and confirmed by restriction digestion and PCR amplification.

Sampling and extraction of HBV DNA

Blood samples were collected from clinically diagnosed HBV positive patients from Services Hospital, Lahore, Pakistan. EDTA was added to a final concentration of 7.0 mM and plasma was extracted by centrifugation. Viral DNA was extracted from the blood plasma using the method of Persing *et al.* (1993). 10 µl of extracted DNA was used for PCR.

PCR amplification

Two sets of primers were used. The first pair of primers NHBF1 (GTC CCC TAG AAG AAG AAC TCC CTC) and NHBR1 (CCC TAG AAA ATT GAG AGA AGT CCA) 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 external primer (Abbas, 2001). Second set of primers B2833S (GGG TCA CCA TAT TCT TGG) and B170AS (GTC CTA GGA ATC CTG ATG) was selected from a published source (Thiers *et al.*, 1988) and used for sequencing of Pre S region of HBV by dideoxy chain termination method (Sanger *et al.*, 1977).

The denaturation was done at 94°C for 1 minute, annealing was done at 55 or 50°C for 1 minute, and extension at 72°C for 2 minutes for 20-25 cycles.

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 $MgCl_2$, 180mM of each dNTP and 0.9µM of each primer), 3 µl of HBV DNA and 2 µl (1 unit) of Taq DNA polymerase.

Sub cloning

The PCR product of external primers was run on 0.8% low melting agarose gel and the bands of interest was purified from the gel slice through Wizard DNA purification system (Promega/ Madison, WI, USA) or Sigma's Nucleiclean Kit. This gel purified DNA was cloned in pGEM-T vector, especially designed for cloning PCR products. The following ligation reaction was set up: Ligase 10X buffer, 1 µl; PGEM-T vector, 1 µl (50 ng); PCR product, 3 µl (30 ng of 1.1 kb), and T4 DNA ligase, 1 µl (3 units). Later water was added to make the volume 10 ml. The contents were mixed by finger flicking, centrifuged briefly and then incubated at 4°C overnight. 2 µl of ligation reaction was added to an eppendorf tube containing 50 µl of competent cells, incubated on ice for 20 minutes and then heat shocked at 42°C for 2 minutes. Later 1 ml of LB medium was added and incubated at 37°C for 1 hour in incubator shaker. Later serial dilutions were prepared and plated onto LB-Amp./IPTG/X-Gal plates.

DNA sequencing

Non-radioactive dideoxy sequencing was carried out according to the manufacturer's instructions (Silver sequence kit; Promega/Madison, WI, USA). The sequencing was performed to characterize the pre-S1 region of two Pakistanian HBV isolates. The sequencing reactions were resolved on a 0.2 mm thin 6% polyacrylamide gel (PAGE) (Sambrook *et al.*, 1989). After resolving the gel was silver stained according to the manufacturer's instructions (Silver sequence kit; Promega/Madison, WI, USA). The silver stained image of the gel was stored as computer file by a video imaging system and the DNA sequence was read manually and stored as a computer file.

RESULTS

Figure 1 shows PCR product of ten clones

from HBV isolates # NA007 and NA013 each, having 1.1Kb inserts.

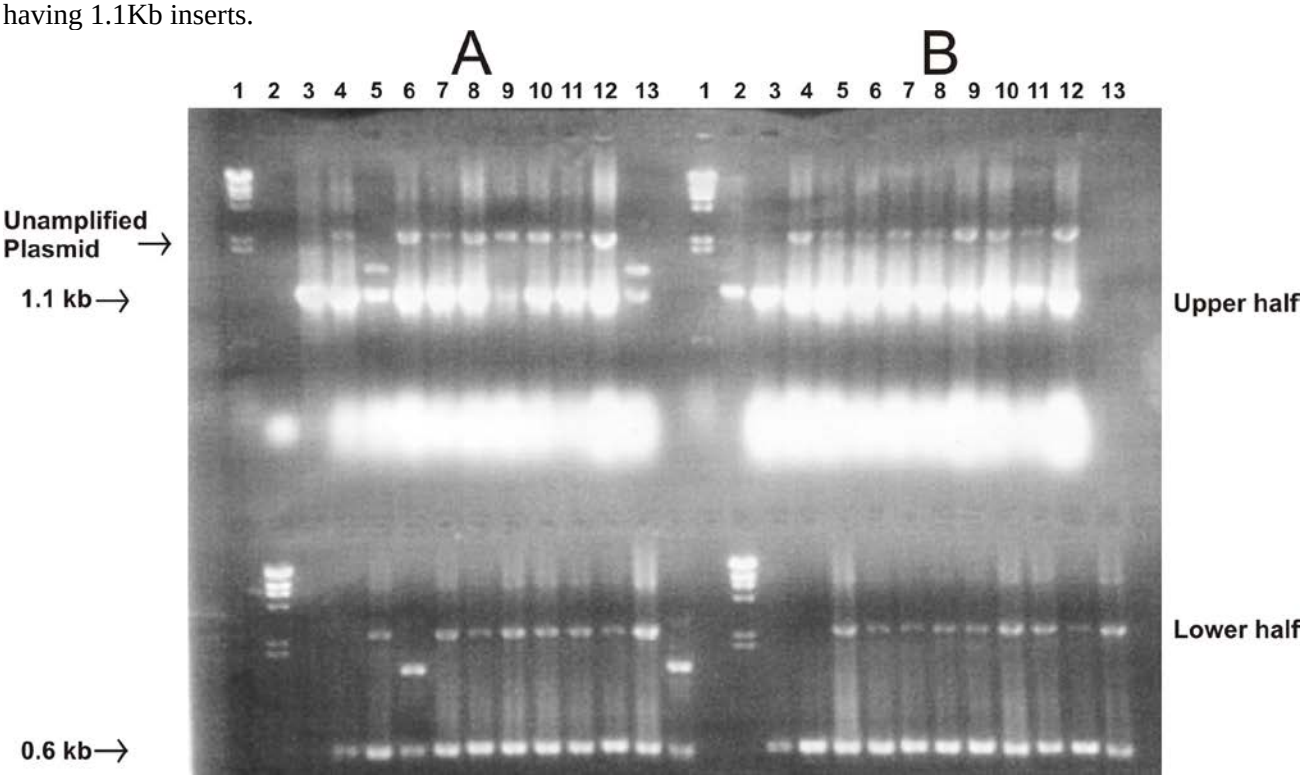

Fig. 1. PCR confirmation of NA007 (Panel A) and NA013 clones (Panel B) having 1.1Kb (upper half) and 0.6 kb inserts (lower half), using specific primers in each case.

The agarose gel in panel A and B consists of two halves. The upper half of the gel shows PCR products of NHBF1 & NHBR1 primers. Lane 1 shows the λ HindIII size marker, lane2 is the negative PCR control, lane3 has the positive PCR control and lanes 4 to 13 contain PCR products of ten clones derived from Pakistanian HBV isolate # NA007. The panel B has λ HindIII size marker in lane 1 and HBV positive PCR control in lane 2. Lanes 3 to 13 contain PCR products of ten clones derived from Pakistanian HBV isolate # NA013. The lower half of the gel is loaded with PCR products using nested primers B170AS and B2833S. Negative PCR control next to the λ HindIII size marker is clear and all ten clones from Pakistanian HBV isolates # NA007 and NA013 show a 0.6 Kb DNA fragment which confirms the identity of the cloned HBV DNA.

Amplified product containing PreS region was sequenced in both sense and antisense directions using one of the internal set of primer (B170AS and B2833S). Nucleotide sequence (357bp) of the PreS1 region of NA007 and NA013 was read manually from the silver sequencing gel (sequence gels not shown). HBV PreS1 sequence was fed into PC/GENE program to get its amino acid sequence (Fig. 2).

Sequence variation analysis

PreS1 sequence from samples NA007, and NA013 were found similar to adr4 after homology/ variation analysis through PCGENE. Sequence alignment through the NCBI Blast search facility revealed that Pakistanian HBV PreS1 sequence showed 96.6% with ayr, 90.2% with adw2, 86.8Adw, 82.4% with ayw, 79.4% with ayw4. Both of these sequences varies with other subtypes follows the order adw4 > ayw4 > ayw > adw > adw2 (Table I). The variations due to vaccine were excluded in these cases, as the patients were not vaccinated. There may be other reasons such as

persistent of HBV infection in chronic state. These mutations may be correlated to cirrhotic state, as these are common in two patients with liver cirrhosis.

Fig. 2. DNA and amino acid sequences of PreS1 region of NA007 and NA013.

Table I.- Comparison of sequence with other subtypes/ genotypes

Subtypes (geno)	References	Homology	Variation
ADR4(C)	Fujiyama *et al.* (1983)	100%	0.0%
AYR(C)	Okamoto *et al.* (1986)	96.6%	3.4%
ADRQ(C)	Norder *et al.* (1994)	95.5%	4.5%
Adw2(A)	Valenzuela *et al.* (1979)	90.2%	9.8%
Adw(B)	Okamoto *et al.*(1988)	86.8%	13.2%
AYW(D)	Tong *et al.* (1990)	82.4%	17.6%
AYW(D)	Lai *et al.* (1991)	82.7%	17.3%
AYW4(E)	Norder *et al.* (1994)	79.4%	20.6%
ADW4(F)	Norder *et al.*(1994)	76.5%	23.5%

Interest in the PreS1 region was due to its hypervariability, having point mutations (Lauder *et al.*, 1993). Amino acid sequence encoded by the PreS1 region is found highly immunogenic (Okamoto *et al.*, 1985). Of the total mutations occurring in the HBV genome, 50% occurs in the hypervariable regions, including PreS1. It is reported that some of the mutations affect the clinical course of HBV infection (Tong *et al.*, 2005).

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