academicJournals

Vol. 7(1), pp. 1-7, January, 2015
DOI: 10.5897/JBSA2011.021
Article Number: 398802849921
ISSN 2141-243X

http://www.academicjournals.org/JBSA

Journal of Bioinformatics and Sequence Analysis

Full Length Research Paper

Primer designing for PreS region of hepatitis B virus from the most conserved patches of hepatitis B virus genome

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Receive 21 December, 2011; Accepted 8 May, 2014

The most conserved regions for 15 hepatitis B virus complete genome of different subtypes were aligned using PCGENE software CLUSTAL to design a new pair of primer that can bind to each subtype of hepatitis B virus (HBV), to amplify PreS region of HBV genome. A pair of primer from these conserved patches was selected using software PRIMER and named as Nhepf1 and Nhepr1. Nhepf1, forward primer bound 2362-2385 nucleotides and Nhepr1, reverse primer bound 260-283 nucleotide amplify 1.12 Kb region of HBV genome that contain PreS sequence. The pair of primer was optimized for PCR. Nhepf1 and Nhepr1 annealed well at 50°C to subtype adw2 (American), adr4 (Japanese) and Pakistanian patient derived HBV DNA without any nonspecific bands. The results were found to be highly reproducible with greater accuracy.

Key words: Hepatitis B virus (HBV) genome, HBV conserved region, polymerase chains reaction (PCR) primers, primer designing.

INTRODUCTION

There is prevalence of hepatitis B virus in an estimated 500 million person in the world, the frequent occurrence of this infection in close contacts of carriers, perinatal vertical transmission from mother to infant and high incidence of insidious chronic liver disease or cancer of the liver among the carriers, signify a major public health problem of worldwide concern (Abbas, 2007). Hepatitis B viral genome (3200 bp) has four known genes, S encoding the viral surface protein, C encoding core protein, P DNA polymerase encoding gene and X gene (Lai et al., 1991). Virus envelope proteins designated PreS1, PreS2 and S carries neutralization epitopes and plays an important role in virus/cell interactions (Wong et al., 2004). At least five epitopes have been mapped within PreS region (Kuroki et al., 1990) and it contains two directly or indirectly binding sites to hepatocytes that constitute the first step in the infection (Dash et al., 1992). Antibodies to PreS region are considered to play a major

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role in clearance of HBV. Various naturally occurring mutations in the PreS and S genes have been described, including deletions and point mutations leading to subtypic changes (Okamoto et al., 1987 and Lai et al., 1991).

There are at least four subtypes (adw, adr, ayr and ayw) of HBV that can be recognized by antigenic differences in the antigen (Ono et al., 1983; Abbas et al., 2006; Abbas, 2009). HBsAg has distinct geographical, epidemiological and anthropological settings (Courouce-Pauty et al., 1983; Abbas et al., 2008). The considerable number of HBV isolates with divergent nucleotide sequences and the partially double stranded characteristic of HBV impose the need for extreme care in the choice of primers for both full length and fragment amplification (Zhang et al., 2007).

Subtypes provide useful epidemiological marker of HBV. The mutation rate of HBV is estimated to be 100 times higher than that of other DNA viruses. Since HBV infection persists for many decades in patients, base changes can accumulate over time (Drosten et al., 2000). One in every ten HBV isolates is slightly different at the nucleotide level from other all sequenced isolates, that is, >10% sequence heterogeneity in HBV (Lauder et al., 1993; Zhang et al., 2007). It is hence crucial to study sequence variation of envelope protein of Pakistanian HBV isolates and to plan a noval strategy for designing a new set of primers with the anticipated use for clinical diagnosis of HBV after the selection of the most conserved region of HBV genomic DNA of different subtypes. The aim of the current investigation was to design such a pair of primer which could perfectly bind to all different genotypes and subtypes of HBV, and would be helpful in detection and DNA amplification of HBV isolated from areas (as Pakistan) that have no previous HBV sequence data.

MATERIALS AND METHODS

Primer designing

Eight sequences were collected from GenBank and seven were selected and edited from research papers and fed into computer (PCGENE Release 6.5, 1991 Intelligenetics Inc.). Two sets of subfiles, for each of the fifteen sequences, one having 200 nucleotides stretch from 2300/2307 to 2500/2507 positions and other of 300 nucleotides stretch from 155/157 to 455/457 positions, were created using SEQIN (PCGENE). Fifteen subfiles for each portion were compiled in a library file using FILE (PCGENE). These library files were used in CLUSTAL (PCGENE), to align fifteen patches of HBV genome. The most conserved regions found in this way were subjected to biological software PRIMER version 0.5 (MIT USA, 1991) for primer selection.

DNA preparation

An American clone adw2 gifted by Dr. Aleem Siddique (NIH USA) and a Japanese clone kindly provided by Dr. Okubo (Japan) were prepared as in Sambrook et al. (1989). HBV DNA from sera of HbsAg positive patients were extracted as described in Abbas et al. (2005).

PCR amplification

The cloned DNA as well as patient derived HBV DNA were used for PCR amplification using 1 μM of each Nhepf1 and Nhepr1 with 10 mM Tris-Cl (pH 8.3), 2.5 mM $MgCl_2$, 50 mM KCl, 200 μM each dNTP, 2U *Taq* polymerase per 100 μl reaction (Persing et al., 1993). The mixture was denatured at 95°C for 2 min, annealed at 50°C for 2 min, and elongated at 70°C for 2 min for 35 cycles. PCR product was resolved on 1% agarose gel according to Sambrook et al. (1989).

RESULTS AND DISCUSSION

Fifteen (15) sequences of different genotypes and subtypes were collected and aligned to find conserved regions to design a pair of primer. Eight nucleotide sequences of hepatitis B virus were collected from Genbank with accession numbers were M32138, X75658, X75657, X04615, X75664, X75665, X75663, X75656 and seven selected from research papers (Okamoto et al., 1986; Norder et al., 1994; Tong et al., 1990; Ono et al., 1983). Two regions which were reported to be conserved (Lauder et al., 1993) were selected. The 200 nucleotides conserved patch was from the end of core region overlapping with start of P gene at 2300/2307-2500/2507 position. This patch gave only one region of 37 nucleotides (Figure 1) after alignment of library file through CLUSTAL, which was adopted for forward primer selection.

The 300 nucleotide conserved patch selected for reverse primer was from first hydrophobic region of surface antigen at 155/157-455/457 position. The alignment of library file through CLUSTAL of this patch gave three stretches of 27 nucleotides each (Figure 2). First stretch was chosen for reverse primer selection.

The above two selected regions which were found absolutely conserved and consensus to each other by CLUSTAL were used to select forward and reverse primer respectively by PRIMER selection (Figure 3). After examining 37 forward primers it accepted 9 forward primers, similarly after examining 27 reverse primers, it accepted 10 reverse primers. Finally, one pair of 24-mer each was selected AS given below:

Nhepf1 GTCCCCTAGAAGAAGAACTCCCTC Tm= 62.0°C
Nhepr1 CCCTAGAAAATTGAGAGAAGTCCA Tm= 60.1°C

Forward primer possesses one GC clamp while reverse primer possesses two GC clamps at its 3' end. GC

```
=========================================================================PC/GENE===

*****************************************
* MULTIPLE SEQUENCE ALIGNMENT forward primer. *
*****************************************
Setting of computation parameters
================================
K-tuple value  : 2
Gap penalty    : 5
Window size    : 10
Filtering level: 2.5
Open gap cost  : 10
Unit gap cost  : 10
Transitions are: WEIGHTED twice as likely as tranversions.
Setting of other parameters
==========================
The alignment was done on 15 Nucleic acid sequences.
Character to show that a position in the alignment is perfectly conserved: '*'
Character to show that a position is well conserved: '.'

Alignment
B4          ATGCCCCTATCTTATCAACACTTCCGGAGAATACTGTTGTTAGACGAAG-    49
B8          ATGCCCCTATCTTATCAACACTTCCGGAGAATACTGTTGTTAGACGAAG-    49
B10         ATGCCCCTATCCTATCCACACTTCCGGAAACTACTGTTGTTAGACGACG-    49
B12         ATGCCCCTATCCTATCCACACTTCCGGAAACTACTGTTGTTAGACGACG-    49
B2          ATGCCCCTATCTTATCAACACTTCCGGAAACTACTGTTGTTAGACGACG-    49
B18         ATGCCCCTATCTTATCAACACTTCCGGAAACTACTGTTGTTAGACGACG-    49
B6          ATGCCCCTATCTTATCAACACTTCCGGAAACTACTGTTGTTAGACGAAG-    49
B14         ATGCCCCTATCTTATCAACACTTCCGGAAACTACTGTTGTTAGACGTCG-    49
B24         ATGCCCCTATCTTATCAACACTTCCGGAAACTACTGTTGTTAGACGACG-    49
B26         ATGCCCCTATCTTATCAACACTTCCGGAAACTACTGTTGTTAGACGAAG-    49
B28         ATGCCCCTATCTTATCAACACTTCCGGAAACTACTGTTGTTAGACGACG-    49
B22         ATGCCCCTATCCTATCAACACTTCCGGAGACTACTGTTGTTAGACGACG-    49
B16         ATGCCCCTATCTTATCAACACTTCCGGAGACTACTGTTGTTAGACGACG-    49
B30         ATGCCCCTATCTTATCAACACTTCCGGAGACTACTGTTGTTAGACGACG-    49
B20         ATGCCCCTATCTTATCAACACTTCCGGAAACTACTGTTGTTAGACGACGG    50
            *********** **** ***********.* ***************. *

B4          -----AGGCAGGTCCCCTAGAAGAAGAACTCCCTCGCCTCGCAGACGAAG    94
B8          -----AGGCAGGTCCCCTAGAAGAAGAACTCCCTCGCCTCGCAGACGAAG    94
B10         -----AGGCAGGTCCCCTAGAAGAAGAACTCCCTCGCCTCGCCGACGAAG    94
B12         -----AGGCAGGTCCCCTAGAAGAAGAACTCCCTCGCCTCGCAGACGAAG    94
B2          -----AGGCAGGTCCCCTAGAAGAAGAACTCCCTCGCCTCGCAGACGAAG    94
B18         -----AGGCAGGTCCCCTAGAAGAAGAACTCCCTCGCCTCGCAGACGAAG    94
B6          -----AGGCAGGTCCCCTAGAAGAAGAACTCCCTCGCCTCGCAGACGAAG    94
B14         -----AGGCAGGTCCCCTAGAAGAAGAACTCCCTCGCCTCGCAGACGAAG    94
B24         -----AGGCAGGTCCCCTAGAAGAAGAACTCCCTCGCCTCGCAGACGAAG    94
B26         -----AGGCAGGTCCCCTAGAAGAAGAACTCCCTCGCCTCGCAGACGAAG    94
B28         -----AGGCAGGTCCCCTAGAAGAAGAACTCCCTCGCCTCGCAGACGAAG    94
B22         -----AGGCAGGTCCCCTAGAAGAAGAACTCCCTCGCCTCGCAGACGAAG    94
B16         -----AGGCAGGTCCCCTAGAAGAAGAACTCCCTCGCCTCGCAGACGAAG    94
B30         -----AGGCAGGTCCCCTAGAAGAAGAACTCCCTCGCCTCGCAGACGAAG    94
B20         GACCGAGGCAGGTCCCCTAGAAGAAGAACTCCCTCGCCTCGCAGACGCAG   100
                  ************************************ **** **
```

Figure 1. Multiple sequence alignment for forward primer.

```
*********************************************
* MULTIPLE SEQUENCE ALIGNMENT FOR REVERSE PRIMER. *
*********************************************
Setting of computation parameters
================================
K-tuple value  : 2
Gap penalty    : 5
Window size    : 10
Filtering level: 2.5
Open gap cost  : 10
Unit gap cost  : 10
Transitions are: WEIGHTED twice as likely as tranversions.
Setting of other parameters
===========================
The alignment was done on 15 Nucleic acid sequences.
Character to show that a position in the alignment is perfectly conserved: '*'
Character to show that a position is well conserved: '.'
Alignment

B1          GGGGTTTTTCTTGTTGACAAGAATCCTCACAATACCACAGAGTCTAGACT   100
B17         GGGGTTTTTCTTGTTGACAAGAATCCTCACAATACCACAGAGTCTAGACT   100
B5          GGGGTTTTTCTTGTTGACAAAAATCCTCACAATACCACAGAGTCTAGACT   100
B13         GGGGTTTTTCTTGTTGACAAGAATCCTCACAATACCACAGAGTCTAGACT   100
B3          GGGGTTTTTCTTGTTGACAAAAATCCTCACAATACCGCAGAGTCTAGACT   100
B7          GGGGTTTTTCTTGTTGACAAAAATCCTCACAATACCGCAGAGTCTAGACT   100
B15         GGGGTTTTTCTTGTTGACAAAAATCCTCACAATACCGAAGAGTCTAGACT   100
B29         GGGGTTTTTCTTGTTGACAAAAATCCTCACAATACCGAAGAGTCTAGACT   100
B21         GGGGTTTTTCTTGTTGACAAGAATCCTCACAATACCGCAGAGTCTAGACT   100
B19         GGGGTTTTTCTTGTTGACAAGAATCCTCACAATACCGCAGAGTCTAGACT   100
B9          GGTGTGTTTCTTGTTGACAAAAATCCTCACAATACCACAGAGTCTAGACT   100
B11         GGTGTGTTTCTTGTTGACAAAAATCCTCACAATACCACAGAGTCTAGACT   100
B23         GGGGTTTTTCTTGTTGACAAAAATCCTCACAATACCACAGAGTCTAGACT   100
B25         GGGGTTTTTCTTGTTGACAAAAATCCTCACAATACCACAGAGTCTAGACT   100
B27         GGGGTTTTTCTCGTTGACAAAAATCCTCACAATACCTCTGAGTCTAGACT   100
            **.**.***** ********.***************. .***********

B1          CGTGGTGGACTTCTCTCAATTTTCTAGGGGGAGCACCCACGTGTCCTGGC   150
B17         CGTGGTGGACTTCTCTCAATTTTCTAGGGGGAGCACCCACGTGTCCTGGC   150
B5          CGTGGTGGACTTCTCTCAATTTTCTAGGGGGAGCACCCGTGTGTCCTGGC   150
B13         CGGGGTGGACTTCTCTCAATTTTCTAGGGGAAGCACCAAGGTGTCCTGGC   150
B3          CGTGGTGGACTTCTCTCAATTTTCTAGGGGGAGCTCCCGTGTGTCTTGGC   150
B7          CGTGGTGGACTTCTCTCAATTTTCTAGGGGGAGCTCCCGTGTGTCTTGGC   150
B15         CGTGGTGGACTTCTCTCAATTTTCTAGGGGGAACCACCGTGTGTCTTGGC   150
B29         CGTGGTGGACTTCTCTCAATTTTCTAGGGGGAACCACCGTGTGTCTTGGC   150
B21         CGTGGTGGACTTCTCTCAATTTTCTAGGGGGAACTACCGTGTGTCTTGGC   150
B19         CGTGGTGGACTTCTCTCAATTTTCTAGGGGGATCACCCGTGTGTCTTGGC   150
B9          CGTGGTGGACTTCTCTCAATTTTCTAGGGGGACTACCCAGGTGTCCTGGC   150
B11         CGTGGTGGACTTCTCTCAATTTTCTAGGGGGACTACCCGGGTGTCCTGGC   150
B23         CGTGGTGGACTTCTCTCAATTTTCTAGGGGGAACACCCGTGTGTCTTGGC   150
B25         CGTGGTGGACTTCTCTCAATTTTCTAGGGGGAACACCCGTGTGTCTTGGC   150
B27         CGTGGTGGACTTCTCTCAATTTTCTAGGGGAAACACCCGTGTGTCTTGGC   150
            **.****************************.*    * . ***** ****
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Figure 2. Multiple sequence alignment for reverse primer.

```
PRIMER PREFERENCES FILE
************************************************************
Analyzing sequence 1 (seq1)
'*' = target, 'X' = restriction site, '?' = N, number = high repeat
homology
              +10         +20         +30         +40
    AGGCAGGTCC CCTAGAAGAA GAACTCCCTC GCCTCGCATG CAGTGGAACT
1   ---------- ---------- ---------- -------*** **********
    TCCGTCCAGG GGATCTTCTT CTTGAGGGAG CGGAGCGTAC GTCACCTTGA
              +10         +20         +30         +40
    CCACAACCTT CCACCAAACT CTGCAAGATC CCAGAGTGAG AGGCCTGTAT
51  ********** ********** ********** ********** **********
    GGTGTTGGAA GGTGGTTTGA GACGTTCTAG GGTCTCACTC TCCGGACATA
              +10         +20         +30         +40
    CTCCCTGCTG GTGGCTCCAG TTCAGGAACA GTAAACCCTG TTCCGACTAC
101 ********** ********** ********** ********** **********
    GAGGGACGAC CACCGAGGTC AAGTCCTTGT CATTTGGGAC AAGGCTGATG
              +10         +20         +30         +40
    TGTCTCTCCC ATATCGTCAA TCTTCTCGAG GATTGGGGAC CCTGCGCTGA
151 ********** ********** ********** ********** **********
    ACAGAGAGGG TATAGCAGTT AGAAGAGCTC CTAACCCCTG GGACGCGACT
              +10         +20         +30         +40
    ACGGTGGACT TCTCTCAATT TTCTAGGGG
201 **-------- ---------- ---------
    TGCCACCTGA AGAGAGTTAA AAGATCCCC
Acceptable regions for a primer:
base 1 to 37
base 203 to 229

Examining 37 forward primers...9 forward primers accepted.
Examining 27 reverse primers...10 reverse primers accepted.
Testing pairs...

Product size range 201-226...
forward primer    7->30  : GTCCCCTAGAAGAAGAACTCCCTC       Tm = 62.0
reverse primer  228->205 : CCCTAGAAAATTGAGAGAAGTCCA       Tm = 60.1
PCR product length:  222, GC = 54%
```

Figure 3. Primer software results.

contents of primers were 54%. Both primers have five self-complementary bases (2.83%), which were not consecutive to each other but occur at irregular intervals. Tm were 62 and 60.8°C, respectively. The annealing temperature of the primers is 10-15° below the Tm of the primers (Desselberger, 1995). Therefore, PCR was tried using newly selected primers at different annealing temperatures ranging from 60-48°C. The optimum temperature selected was 50°C, which was then used for routine PCR. DNA concentrations for cloned adw2 and adr4 were tried from 10 - 500 ng, 50 ng was found best. $MgCl_2$ concentration found best for these primers was 1.5 mM of tested ranges 1-4 mM. Primer (NhepF1 and NhepR1) amplified a fragment of 1.1 Kb of PreS region both in pSHBV and patient derived HBV DNA (Figure 4). HBV clone pSHBV gave 3.7 Kb as the orientation of the HBV insert, vector was amplified along with insert lane 2 and 4 (Figure 4).

Hepatitis B virus is the smallest known DNA virus that cause infection in man. The genome is partially double

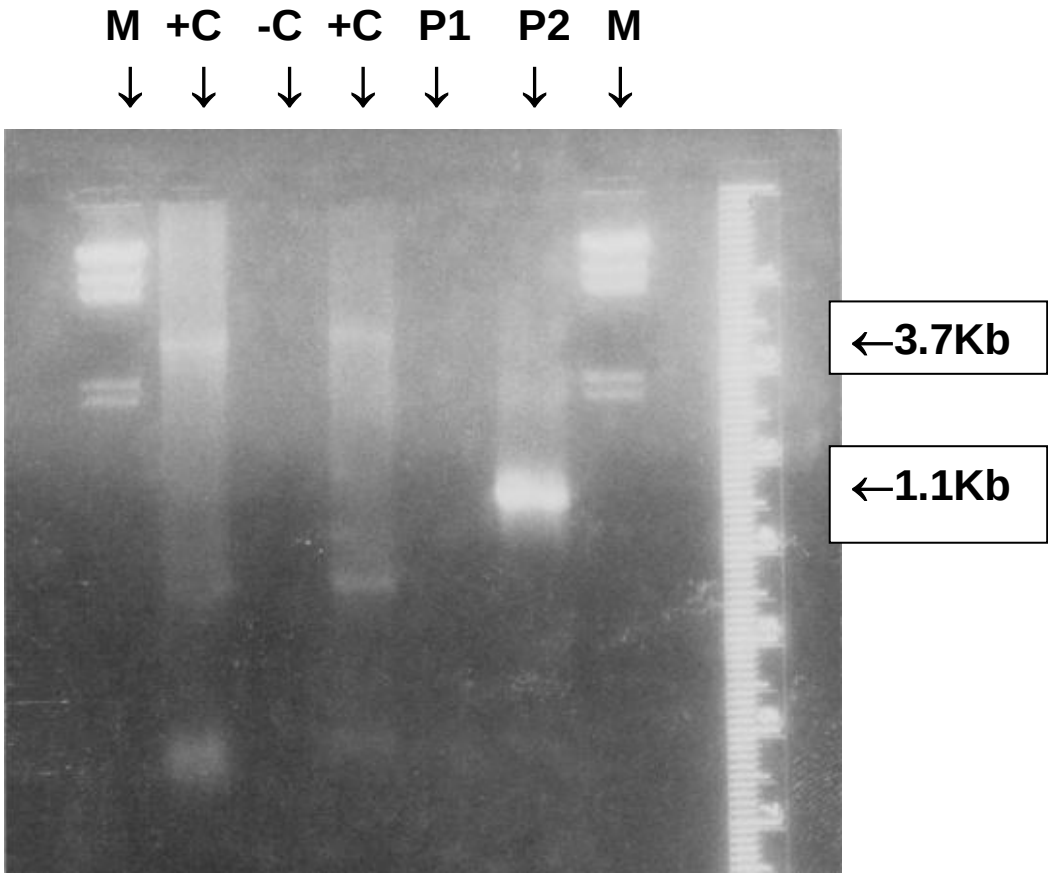

Figure 4. Hepatitis B virus PCR products derived from cloned and patient derived templates using NHepF1 and NhepR1. Lane 1 and 7 (M): λ HindIII marker. Lane 2 and 4 (+C): pSHBV as positive control, Lane 3 (-C): negative control, Lane 5 (P1): patient NA016 PCR, Lane 6 (P2): patient NA012 PCR band.

stranded circular, 3.2 Kb DNA molecule, 0.78 μM in length which corresponds to a molecular weight around 1.6 x 10^6. HBV genome contains an *Eco*RI site which lies 9 bp downstream from 5'end of PreS2 region. A new set of well conserved primers of 24-mer each were selected from consensus regions (the region encoding hydrophobic region of HBV surface gene and the start of the P gene which overlaps the end of gene C).

It is also imperative that the primers should be greater than 18 or 20 mer, in the range of 20-25 mer, so that they may provide better background. The primers should have at least two to three GC clamps at their 3'end to have a strong grip during the positioning and walking of Taq DNA polymerase to extend the primer for polymerization. In the ideal condition, the F and R primers should not have any intra or/and inter complementary bases, but if there is, they should not be greater than 2.5%. The complementary bases should not be consecutive to each other but present at irregular intervals, because all the complementary bases at one site are consecutive to each other, more likely have a chance to come close together quickly and complement each other and may have strong grip as compared to the self-complementation at distance intervals.

We look forward with the hope that this pair can be used for routine clinical diagnosis of hepatitis B virus infection in the diversified geographical world of highly variable strains of HBV. This may prove as the reliable primers for obtaining accurate and confident results.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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