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Development of Positive Control for Hepatitis B Virus

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ABSTRACT

More than 2 billion people are believed to be infected with Hepatitis B virus at some point in their life and thus Hepatitis B is regarded as the most common form of chronic hepatitis worldwide. Around 15-40% of people infected with HBV develop HBV-related complications and approximately 25% die as a result of these complications. Major complications include cirrhosis and hepatic carcinoma. It is estimated that approximately 65 million people developing such complications will die of liver diseases. It is therefore essential to develop rapid screening methods for detection of this virus in early stages. Since HBV is infectious and spreads through blood, semen and other body fluids, it will be easy to detect the presence of this virus in these body fluids. This detection of virus can be confirmed if we have a standard for comparing it with, referred to as positive control. Thus, by using PCR techniques we isolated HBV from blood samples and developed positive control for the same through simple molecular biology techniques.

INTRODUCTION

Various blood tests are available to detect HBV from blood which all uses the Hepatitis B blood panel. This panel generally consists of three tests - Test for Hepatitis B surface antigen HBsAg, test for Hepatitis B surface antibody HBsAb or anti-HBs and test for Hepatitis B core antibody HBcAb or anti-HBc. Of these three tests, the test for surface antigen directly detects the virus, further the surface antigens can be found in the blood within 6-16 weeks of infection whereas the antibodies require 60-150 days to develop, and thus is one of the earliest serological markers of HBV detection[1].

Another major advantage of targeting the surface antigen is that the gene coding for it, the S-gene, is quite conserved in all hepatitis B genotypes[2]. Due to the presence of three in-frame start (ATG) codons, the long open reading frame S-gene may be divided into three sections: pre-S1, pre-S2 and S. In this work, we targeted the S-gene by first identifying the conserved region in S-gene which is retained in all the hepatitis B genotypes using the NCBI Blast tool. After identifying this region, suitable primers were designed according to criteria's mentioned by Integrated DNA Technologies Primer Quest Tool. Once the primers were obtained, molecular cloning was carried out to develop positive controls.

MATERIALS AND METHODS

Invitrogen's viral RNA/DNA kit by Life Technologies, Qiagen's PCR Purification kit, Qiagen's Miniprep kit for plasmid isolation, Qiagen's Gel extraction kit, Ligase buffer, Taq polymerase, Taq buffer, T4 ligase, PEG, Luria Agar, Luria Broth, X-gal, Ampicillin, IPTG (iso-propyl thiogalactoside), Thermofisher's 100bp and 1 kb DNA ladder.

Isolation of Viral DNA from blood

10 ml of blood was collected from a Hepatitis B confirmed patient in an EDTA blood vacutainer. It was allowed to stand for few hours so as to allow its contents to settle and then centrifuged at the speed of 2000 rpm to obtain plasma. 200ul of this plasma was then used to obtain the viral DNA using the Invitrogen's viral DNA kit. The DNA obtained was then quantified using UV spectrophotometer.

PCR amplification and purification

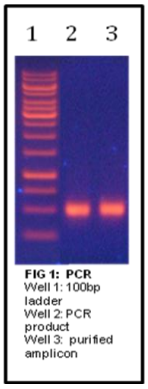

FIG 1: PCR
Well 1: 100bp ladder
Well 2: PCR product
Well 3: purified amplicon

Polymerase Chain Reaction was set up using the isolated DNA. Gene-specific primers targeting the specific region on the S-gene were used for amplification. Other requirements of PCR such as Taq buffer, $MgCl_2$, dNTPs, Taq Polymerase were also provided. Temperatures suitable for denaturation, annealing and renaturation were calculated using the Tm of primers and a PCR of 35 cycles was set up. The amplified product of the gene specific primers was checked for the size of 207bp on 2% agarose gel (with Ethidium bromide) using 100bp ladder. This product was then purified using PCR purification kit so as to remove impurities (Fig 1).

Restriction Digestion of Vector

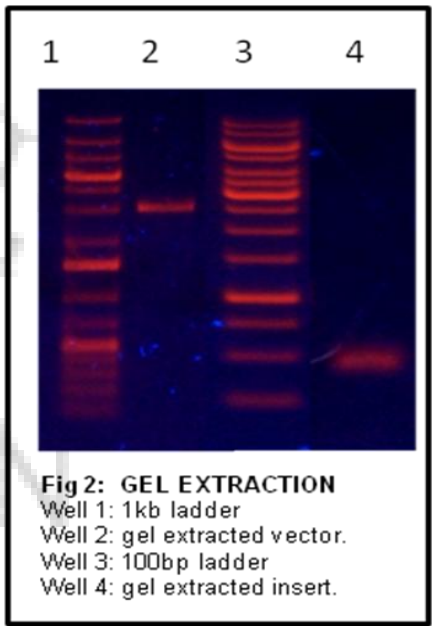

Fig 2: GEL EXTRACTION
Well 1: 1kb ladder
Well 2: gel extracted vector.
Well 3: 100bp ladder
Well 4: gel extracted insert.

The vector used for cloning was pBluescript KS II +. This phagemid is readily available and has certain advantages in cloning: pBluescript, like pUC vectors, have a multiple cloning sites inserted in the Lac Z' gene, thus offering blue-white screening as an advantage for the selection of positive clones. pBluescript also has an origin of replication of the single stranded phage f1, which is related to the M13 phage vectors. This translates into that a cell harboring a recombinant phagemid if infected by f1 helper phage that supplies the single stranded phage DNA replication components; it will produce and package single stranded phagemid DNA. Further, this phagemid is flanked by two different RNA polymerase promoters, a T3 promoter at one end side and a T7 promoter at the other end. This is key because it enables one to isolate the double-stranded phagemid DNA and transcribe it *in vitro* with either of these two phage polymerases to produce pure RNA transcripts to coincide with either of the two different strands [3].

This phagemid vector was cut using restriction enzyme Eco RV. A control restriction digestion was performed prior to this and it was found that Eco RV cuts pBluescript in 15 minutes. Once restriction digested, vector pBluescript and purified amplicon of insert were loaded on a 2%

agarose gel (with ethidium bromide) and it was allowed to run at 75 mV for approximately an hour. Once the gel was 3/4th run, the cut vector was checked for a size of 2961bp using a 1kb ladder, whereas the insert was checked for a size of 201bp. After checking for the appropriate size, that portion of gel was cut and the vector and insert were extracted using the Qiagen's Gel extraction kit (Fig 2). A ligation reaction was then set up accordingly.

Ligation and transformation

Since cloning was performed using blunt end ligation, PEG was a pre-requisite, since the addition of polyethylene glycol (PEG) to ligation reactions can promote ligation of blunt-ended fragments by "macromolecular crowding". Further, the amount of ligase and insert was also increased to aid in blunt end ligation. Ligation was allowed to proceed for 1 hour at 22^{o} Celsius. Once the ligation reaction was complete, the ligation mixture was transformed into top 10 strains of *E.coli*. 10ul of this transformation mixture was then plated on X-gal containing agar plates and incubated at 37^{0}C overnight. These plates were prepared using 400ml of Luria Agar so as to contain a final concentration of 40ug/ml of X-gal, 100 ug/ml of Ampicillin and 0.1mM of IPTG. The plated plates were then checked for transformants next day using the blue-white screening principle (Fig 3). White colonies which indicated that the gene must have been inserted in the phagemid were picked up and screened for confirmation.

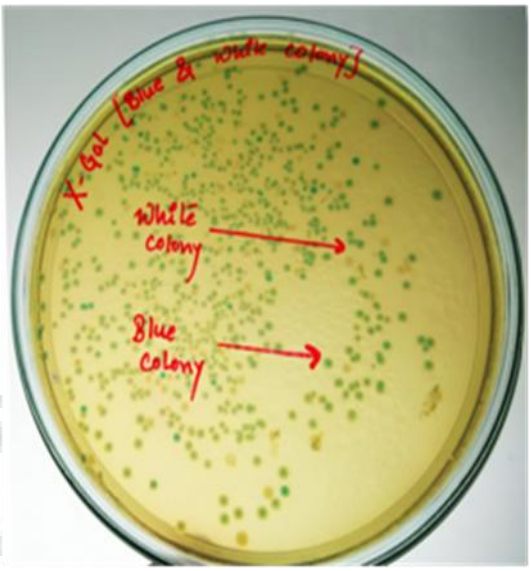

Fig 3: blue-white screening

Screening of white colonies by vector and gene-specific primers

24 white colonies were screened by setting up a PCR using vector specific primers first. The primers chosen to amplify 201bp in wild-type pBluescript, whereas a region of 408bp (201 +207) in transformed pBluescript. This was checked using 100bp DNA ladder.

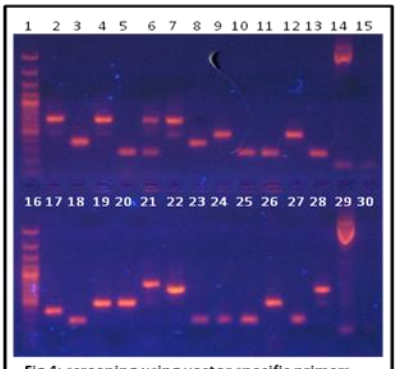

Fig 4: screening using vector specific primers
Well 1,16: 100bp ladder
Well 14,29: negative control
Well 15,30: distilled water
Well 2-13; 17-28: clone 1-12; clone 13-24

The positive clones (clones 2,7,13 as seen in Fig. 4) obtained using vector specific primers (those showing a size of 408bp) were confirmed using gene-specific primers, wherein they amplified a region of 207bp (Fig 5). These clones were confirmed positive and they were picked and grown overnight on a shaker at a speed of 225rpm in 10ml LB media containing 10ul ampicillin. Plasmid isolation was carried out the following day using Qiagen's miniprep kit and the DNA thus obtained was the positive control for HBV.

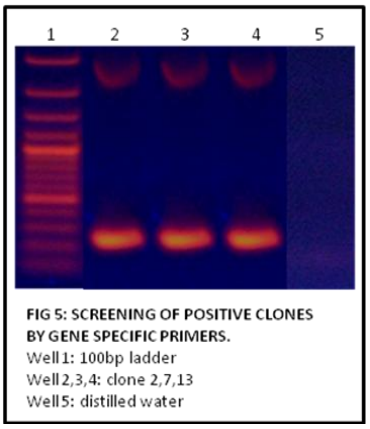

FIG 5: SCREENING OF POSITIVE CLONES BY GENE SPECIFIC PRIMERS.
Well 1: 100bp ladder
Well 2,3,4: clone 2,7,13
Well 5: distilled water

RESULTS AND DISCUSSION

Thus, positive controls targeting a small region of 207bp on the S-gene of Hepatitis B virus were successfully prepared by cloning into pBluescript KS II + using blunt end ligation. This method offers a simple method for development of positive controls which can be done in a week's span. Such positive controls help us to easily confirm patients having a Hepatitis B infection. This method thus, is less time consuming, very economical and requires no special scientific expertise. Furthermore, such positive controls can be prepared for various other viruses and can be used for their easy detection.

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