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Developing Strategies for Early Detection of Hepatitis B Infection

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Abstract

Hepatitis B caused by hepatitis B virus (HBV) 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. 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. Polymerase chain reactions (PCR) assays are often employed to detect these viruses in blood. While most of these assays generally rely on detection of HBV Surface Antigen (HBsAg) or hepatitis b core IgM Antibody (anti-HBc IgM), recent developments have made possible the detection of HBV DNA in blood. These assays, however, necessitate the requirement of adequate controls to distinguish true from false negative results. To address this issue, we have developed positive controls for Hepatitis B virus using molecular biology techniques. These controls can be used in PCR assays for detection of HBV by competitive amplification, thereby allowing detection of false negatives. The controls developed in this study were successfully tested with virus-seeded blood sample concentrates.

Keywords: HBV; pBS; Controls

Introduction

Hepatitis B is a devastating infectious disease affecting the liver that is caused by the hepatitis B virus (HBV). It is capable of causing both acute and chronic infections. No symptoms can be detected in many cases during the initial infection. These symptoms spontaneously develop which can be seen as vomiting, yellow fever, abdominal pain and dark urine. Mode of transmission is generally through blood and other body fluids [1]. Contact with infected fluids during childhood or infection during birth is the most common cause that results in chronic hepatitis B. Intravenous drug use and sexual intercourse may also lead to transmission of the virus. About a third of the population is infected by hepatitis B virus at some point in their life [2]. Over 8,00,000 patients die due to hepatitis B each year, majorly due to liver diseases [3]. Therefore, early detection and routine surveillance of virus are the routes to management, control and eradication of this disease. Many molecular-biology techniques are available for detection of virus, PCR being the most widely adopted. However, one problem associated with any molecular detection assay is the potential for false negative results. A false negative result may appear due to many cases such as PCR inhibitors, poor DNA extraction, and target DNA degradation before amplification or even manual errors in setting up a reaction [4-15]. Therefore, the presence of positive control will ensure the efficient run of the PCR as well as help in monitoring the PCR procedure [16,17]. Ultimately the positive control can be monitored by resolving the reaction products on an agarose gel [12]. In this study, we have developed positive controls by amplifying the surface antigen gene present in the HBV genome. The positive control used is a 208 bp long DNA. Compared to nested PCR as the reference standard, the real-time assay has a sensitivity of 99.5%, specificity of 100%, positive predictive value of 100%, and negative predictive value of 99.6% for the detection of viruses. Our assay uses species-specific forward primers in combination with a conserved reverse primer and largely overcomes primer competition for the minor species DNA. With a blind panel of clinical samples, we successfully identified the species in 12/16 mixed infections. This assay was further validated with 97 blood samples and demonstrated a specificity and sensitivity for single infections of 100% compared with nested PCR as the "gold standard.

Materials and Methods

HBV viruses and viral DNA extraction

Blood infused with HBV virus was collected in an EDTA vacutainer. The tube was centrifuged at 25000 rpm to separate RBCs and the separated plasma was transferred in a separate eppendorf and stored at -80°C. Viral DNA was extracted by taking 200 µl of the plasma with the help of Invitrogen's viral DNA kit by Life Technologies using the manufacturer's instructions. The viral DNA was eluted in 40 µl of elution buffer. For long term use, extracted DNA was preserved at -80°C whereas for short term use, it was preserved at -20°C.

Preparation of positive controls of viral DNA

The positive control DNA template was constructed using two oligonucleotide primers which were custom made. Each primer consisted of a positive strand and a complementary negative strand, so that it can be easily annealed in a DNA fragment [5]. The first pair consisted of Forward (+), AAAATTCGCAGTCCCCAAC and the forward (-), TTTTAAGCGCAGGGGTTG. The second pair consisted of Reverse (+) and the reverse (-), with sequences GTCCCGTGCTGGTAGTTGAT and CAGGGCACGACACCATCAACTA respectively (Figure 1). The primers were designed to have binding sites for HBV S-gene forward(S-gene+151 AAAATTCGCAGTCCCCAAC) and the S-gene reverse (S-gene -323 ATCAACTACCAGCACGGGAC) in the S-gene forward (+) and the S-gene reverse (-) respectively. The complementary primers were denatured at 95°C and reannealed at

58°C. The primers were fashioned in such a manner that they could be used in a blunt end ligation using a single restriction site.

tggcc***aaaattcgcagtccccaac***ctccaatcactcaccaacctcttgtcctccaacttg
tcctggctatcgctggatgtgtctgcggcgttttatcatattcctcttcatcctgctgctatgc
ctcatcttcttgttggttcttctggactaccaaggtatgttgcccgtttgtcctctacttccaggaa
c***atcaactaccagcacgggacc***atgcagaacctgcacgattcctgctca

Figure 1: Nucleotide sequence of S-gene. The former boldface represents the forward primer (+) and latter represents the reverse primer (-).

Preperation of S-gene amplicon

An amplicon of HBV S-gene with a size of 208 bp was generated from the viral DNA using the above primers by setting up a RT-PCR with the help of Qiagen's one step PCR kit. The Qiagen's one step PCR kit was used with a 30 µl reaction mixture along with 1.2 µl of enzyme mixture supplied by the kit (along with hot start Pfu. Pol), Pfu buffer, 10 pmol of each (S-gene forward and S-gene Reverse) primers (0.5 µl), 400 Um (each) of deoxynucleoside triphosphate and 5 mM $MgCl_2$. The thermo cycling conditions for PCR included heat start activation of Pfu Polymerase at 94°C for 5 mins followed by 40 cycles of PCR amplification with each cycle consisting of 30 s of denaturation at 94°C, 30 s of annealing at 60°C, 45 cycles of elongation at 72°C and one final cycle of elongation at 72°C for 10 mins [6]. The PCR was carried out using GeneAmp PCR system 2700 from applied bio-systems. The probes used for monitoring the RT-PCR were Vic and Fam. The amplicon was loaded in a 0.75% agarose gel and checked for its purity and size (208 bp) along with a 100 bp thermo fisher DNA ladder. The gel was cut at the appropriate band size and the amplicon was gel eluted in 35 µl of TE buffer provided in the Qiagen's gel extraction kit and was stored at -20°C for short term use and -80°C for long term use.

Manipulation of viral DNA and vector DNA

Restriction digestion of the vector DNA and agarose gel electrophoresis of DNA were carried out using standard protocols. The vector used was pBlueScript (pBS) KS II+ and the restriction site targeted in the Multiple cloning site of the pBS vector was Eco RV (GAT^ATC). The enzyme was found to cut the site in 15 mins and the reaction was set up accordingly [7,8]. The ligation and cloning of DNA was carried out using T4 DNA ligase from Promega (Madison, WI) according to manufacturer's instructions. Being a blunt end ligation, higher quantities of ligase and insert (amplicon) were added to the reaction mixture, along with Polyethylene glycol (PEG) so as to aid the blunt end ligation. Ligation was set up at lower temperatures than usual (22°C) so as to avoid heat inactivation of PEG [9].

Transformation and screening for positive colonies

The ligation mixture was added to 100 µl of competent Top 10 *E. coli* cells and transformation was carried out using standard protocols. 10 µl of transformation was then loaded on luria agar plate containing 1mg/ml of Ampicillin, 100 mM/ml of Isopropyl beta-D-thiogalactopyranoside (IPTG) and 40 µg/ml of X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside). The plates were incubated overnight at 37°C and based on blue-white screening principle [10] (Figure 2), white colonies were picked and screened for presence of HBV amplicon by setting up a screening PCR. The positive clones obtained were reconfirmed using two pairs of vector specific primers, which were so designed that they amplify the entire cloned region of S-gene of HBV along with some portion of the pBS vector. The first pair consisted of T17 forward (+), TAATACGACTCACTATAGGG and T17 forward (-) ATTATGCTGAGTGTGATATCCC. The second pair consisted of M13 reverse (+), GGAAACAGCTATGACCATGA and M13 reverse (-), CCTTTGTCGATACTGGTACT. The primers were designed to have binding sites for sequence TAATACGACTCACTATAGGG and reverse sequence TCATGGTCATAGCTGTTTCC in the T17 forward (+) and M13 reverse (-) primers, respectively, described above. The amplicon obtained using these primers in a wild type pBS vecor was 201 bp, whereas in a recombinant pBS vector containing the cloned HBV region, the amplicon generated using these primers was found to be 409 bp long (Figure 3). This indicated the successful insertion of portion of S-gene of HBV in the pBS vector.

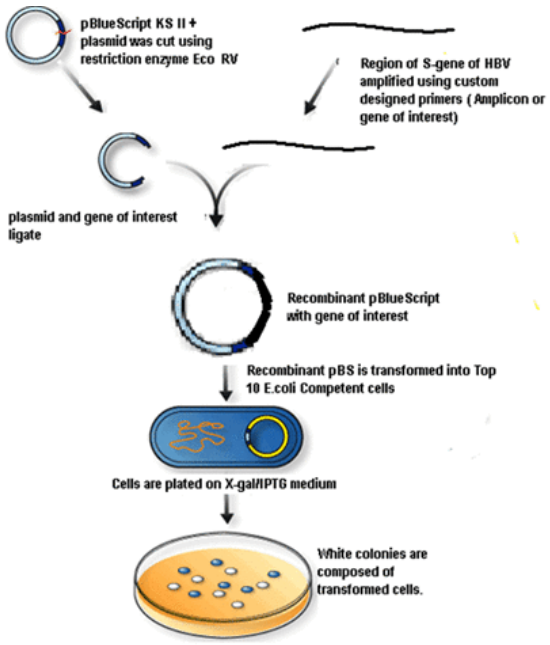

Figure 2: Cloning of Portions of S-gene into pBlueScript KS II +vector and transformation into top 10 *E. coli* competent cells.

Thus, the PCR product(amplicon) obtained after amplification by the custom designed primers was cloned in the multiple cloning site (MCS) of pBlueScript (pBS) KS II+vector using Eco RV restriction site to generate the recombinant plasmid containing the S-gene of HBV. The nucleotide sequence derived from the cloned template in pBS matched to the sequences of the oligonucleotide primers used to

construct the positive control template and was found to be 208 bp long, as expected. The purity of the template DNA was tested on agarose gel electrophoresis. The purified DNA template was then stored in RNAse free Tris-EDTA buffer (pH 8.0) at -80°C. The template DNA used was serially diluted and used in the assay so as to determine the Minimum detection level (MDL).

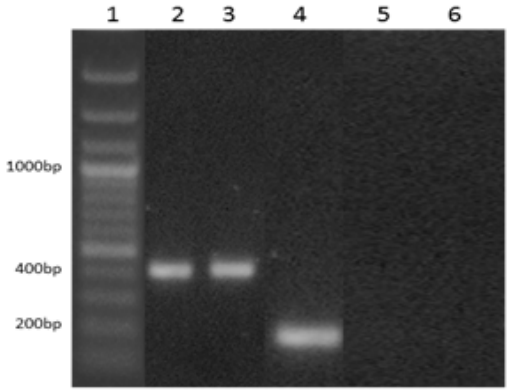

Figure 3: Screening PCR using white colonies and vector specific primers (M13 rev. and t17 fwd.). Lane 1: 100 bp ladder; Lane 2, 3: Recombinant pBS; Lane 4: Wild type pBS; Lane 5: Negative control; Lane 6: Distilled water.

Results

The construction of positive control for Hepatitis B virus was described in materials and methods. The MDL of the positive control was determined to be 0.12 pg for RT-PCR. The stability of the controls was tested under changes in storage conditions. The positive controls were stable for at least six months when stored in Tris-EDTA buffer at -80°C. In the same period, the control was slightly destabilized when stored at -20°C and slightly more destabilized when stored at 4°C-10°C.

Inhibition studies: One of the major problems associated with PCR diagnosis is the presence of PCR inhibitors [6]. The presence of PCR inhibitors was evaluated in blood, serum and plasma. The results showed no inhibition of amplification of viral DNA extract.

Discussion

An extremely important step towards the management control and eradication of Hepatitis B virus in course of Hepatitis B disease is its early detection. PCR is widely used molecular assay for detection of the infection at an early stage [11]. Although PCR is sensitive and specific for is target, there are still concerns with false negative results. In this research, we therefore developed positive controls which can help identify and detect false negative result and thus increase the specificity and sensitivity of the PCR assays [13]. A major advantage associated with the use of these positive controls is that they detect the HBV DNA. The HBV DNA is a reliable marker for detection of hepatitis B Virus [14] which can help in (1) distinguishing active infection from inactive (2) help in monitoring the patient's response to anti-HBV therapy (3) Further, HBV DNA can be detected in blood almost 21 days prior to the appearance of HBsAg in blood, thus facilitating early detection [15]. Thus, developing positive controls is a very economical and time saving method for easy detection of hepatitis B virus.

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