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Research Article

CLONING OF HBsAg GENE IN REPLICASE BASED VECTOR

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ABSTRACT

The HBsAg gene was cloned in pAlpha vector in right orientation which can be utilized to further work for use in developing DNA vaccine.

Key words: Cloning, HBV, surface antigen, pAlpha vector

INTRODUCTION

The hepatitis B virus (HBV) is cause of liver disease which occurs worldwide. The preventive HBV vaccine has to some extent reduced the incidence of disease. However large number of chronic HBV cases still need of treatment (Chen *et al.,* 2016). The qualitative hepatitis B surface antigen (HBsAg) testing has served as a diagnostic tool for individuals infected with HBV (Liu and Yao, 2015). Neutralizing or diagnostic antibodies against the HBsAg are directed towards its highly conserved major hydrophilic region (Genkay *et al.,* 2017). It chronically infects 250 million people worldwide, resulting in nearly one million deaths annually (Tsai *et al.,* 2018). Interferon alpha (IFN-α) is able to inhibit the replication of this virus, and the sustained and stable expression of IFN-α at appropriate level may be beneficial to HBV clearance. Gene therapy plays a more and more important role in clinical practice (Yu *et al.,* 2013).

HBV itself was among the first viruses to be detected by assay of its DNA genome and IgM antibodies against the HBV core antigen were the first to be selectively detected by the anti-μ capture assay. The cloning and sequencing of the HBV genome in 1978 paved the way to understand the viral life cycle, and allowed development of efficient vaccines and drugs. Today's hepatitis B vaccine was the first vaccine produced by gene technology. Among the problems that still remain today are the inability to achieve a complete cure of chronic HBV infections, the recognition of occult HBV infections, their potential reactivation and the incomplete protection against escape mutants and heterologous HBV genotypes by HBV vaccines (Gerlich, 2013). The

present work was taken to clone the HBsAg gene from an Indian isolate.

MATERIALS AND METHODS

HBsAg gene

It was synthesized by GenScript,USA containing 688 bp initially cloned in pUC57 vector at *EcoR*V site. It was released by digestion with *EcoR*I and *Hind*III, run on 1% agarose gel electrophoresis and the gene insert was cut out. Melted the agarose at 70^0C for 5 min, make up the volume to 400 µl with TE. Remelted at 70^0C, added equal volume of phenol and mixed vigorously for 30 seconds. Kept on ice for 5 min, centrifuged and extracted again the aqueous phase twice with buffered phenol. Added 3 volumes of ethanol to aqueous phase and kept overnight at -20^0C. Warmed and centrifuged for 5 min to pellet DNA. Dissolved the pellet in 50 µl nuclease free water and added 5 µl 3M sodium acetate and 165 µl chilled ethanol. Kept at -20^0 C overnight, pellet and washed the DNA and resuspended in 50 µl nuclease free water. Checked on agarose mini gel to ensure that extraction was successful.

Blunting was done by preparing the reaction mix in 0.5 ml microfuge tube, nuclease free water 2 µl, RE cut DNA 10 µl,10 mM dNTPs mix 5 µl, T4 DNA Polymerase buffer 2 µl, T4 DNA Polymerase (6 U) 1 µl, total 20 µl , mixed by tapping, spin briefly and incubated at 30^0C for 1 h. Then dephos*Sph*orylated and purified by phenol-chloroform-isoamyl alcohol extraction.

pAlpha vctor

It was available at BTS Institute of Biotechnology. *Stu*I was chosen to create blunt end. The 50 µl reaction mixture contained nuclease free water 22 µl, pAlpha vector DNA (100 ng/µl) 20 µl, 10X NE buffer 4(NE Biolab) 5 µl, StuI (NE Biolab, 10,000U/ml) 3 µl in a microfuge tube. It was incubated at 37°C overnight and then the linearised plasmid was checked and quantitated on 1% agarose gel electrophoresis, gel extracted and quantified. The dephos*Sph*orylation was done as per Sambrook and Russel (2001).

Cloning

For blunt end ligation of pAlpha vector and HBsAg gene fragment, a 10 µl reaction mixture was prepared containing DW 1 µl, 10X ligation buffer 1 µl, pAlpha vector (100 ng/µl) 0.5 µl, HBsAg gene (150 ng/µl) 4 µl, 30% PEG-8000 1.5 µl, T4 DNA ligase **(**20U/µl) 2 µl. Mixed, centrifuged and incubated at 37ºC for 30 min. One step competent cell preparation and transformation was done as per Chung *et al*., (1989) to transform *E.coli* DH5 alpha. The selected clones were processed for plasmid DNA isolation by TELT method (He *et al*., 1990). The concentration and purity of plasmid DNA was determined spectrophotometrically by taking absorbance at 260nm and 280nm in Eppendorf Biophotometer D30 using micro cuvette which shows reading of DNA concentration in µg/ ml as well as its purity.

RE analysis

The presence of gene in right orientation in the recombinant plasmid was confirmed by restriction endonuclease analysis. The HBsAg gene sequence (688bp), pAlpha vector sequence (10799bp) and pAlpha-HBsAg recombinant plasmid DNA sequence (11547bp; 10799 + 748bp gene insert removed from pUC57 vector after digestion with *EcoR*I+*Hind*III) were analysed by DNAstar (Mapdraw) software to select restriction enzymes for determining the right gene orientation. The

*Sph*I and *Xba*I restriction enzymes were found useful in determining right orientation of the gene - gene in right orientation will yield two fragments of 11444bp and 103bp on digestion with *Sph*I + *Xba*I; fragment of 1090bp and 103bp will be obtained if the gene is in wrong orientation. The 103 bp fragment will not be visible in gel since it is too small size. The recombinant plasmid was double digested with *Sph*I and *Xba*I to confirm the right orientation of HBsAg gene.

The 6 µl recombinant plasmid DNA was mixed with 1µl *Sph*I and *Xba*I each, 10X buffer 1 µl and 1 µl nuclease free water. The reaction mixture was vortexed, spun and incubated in a waterbath at 37°C overnight. Two µl 6X loading dye was added, mixed and then electrophoresed on 1% agarose gel using 1X TAE buffer system containing ethidium bromide 0.5µg/ml final concentration at initially 50 V for 5 min and then at 100 V and visualised under UV transilluminator (UVP) and photographed. The released fragments after digestion were compared against 1 kb DNA ladder.

Sequencing of gene

The sequencing of gene insert in pAlpha vector was done using BGH reverse primer to confirm the gene and its orientation**.**

RESULTS AND DISCUSSION

The 688bp HBsAg gene was synthesized and cloned initially in 2710bp pUC57 plasmid by *EcoR*V. It was digested with *EcoR*I and *Hind*III to release the gene insert (Fig. 1). This gene insert, size 748bp because of additional RE sites bases, was cloned by blunt end ligation at StuI site of pAlpha vector. Ten transformed colonies were processed which yielded plasmid DNA. These DNA were digested with *Sph*I and *Xba*I to select the clone with right gene orientation (Fig. 2 and 3). It was sequenced to further confirm that the gene was in right orientation.

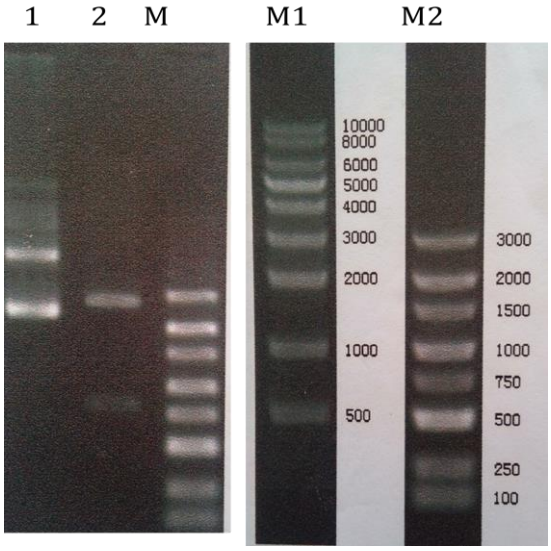

Figure 1. pUC57-HBsAg plasmid DNA Lane M,M1: KB DNA ladder ,M2: DL3000 ladder; 1: pUC57-HBsAg plasmid DNA unut; 2: pUC57-HBsAg plasmid DNA digested with *EcoR*I and *Hind*III.

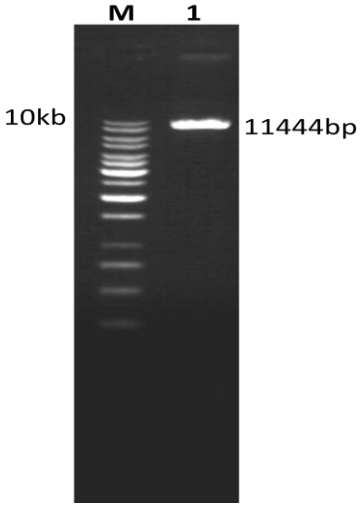

Figure 2. pAlpha-HBsAg
Lane M: DNA MW ladder;
1: rDNA cut with *Sph*I+*Xba*I yielding 11444bp fragment suggesting right orientation of the gene.

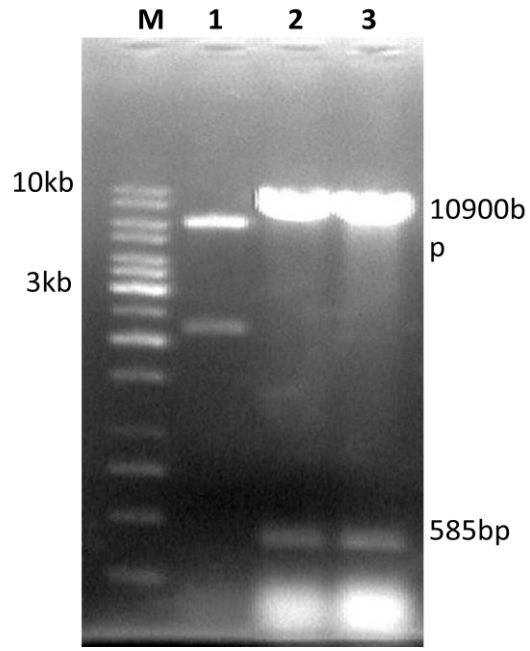

Fig.3. pAlpha-HBsAg.
Lane M: DNA MW ladder; 1: Uncut rDNA; 2,3: rDNA digested with *Sph*I+ *Xba*I yielding 1090bp and 585bp fragments suggesting wrong orientation of the gene.

A pcDNA-HBs-IL-18 recombinant plasmid was constructed which contained HBV surface antigen linked to murine interleukin-18, i.e.. Its immunogebnicity was studied in mice and found to be immunogenic. An eukaryotic expression plasmid pcDNA3.1-S containing HBsAg gene was constructed and the accuracy of the construct was confirmed by restriction enzyme digestion and DNA sequencing (Liang *et al.*, 2014). It is evident that our work is in agreement with the work of earlier workers and the recombinant plasmid can be used for further immunogenicity trials.

CONCLUSION

The HBsAg gene from an Indian isolate was cloned in a replicase based Alpha vector which could be further use in immunogenic trials.

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