Cloning of open reading frames encoding for wildtype and G145R HBsAg of hepatitis B virus in *Escherichia Coli* Jm109

T. Rostinawati¹, D.S. Retnoningrum, S.A. Lestari²

¹Faculty of Pharmacy, Padjadjaran University, Indonesia
²School of Pharmacy, Institut Teknologi Bandung, Indonesia

Abstract

**PURPOSE:** A point mutation in DNA encoding for “a” determinant of HBsAg resulting in a glycine (G) to arginine (R) substitution at position 145 (arginine-145 variant or G145R mutant) has recently been found in Hepatitis B isolates (HBV) from actively, passively and unimmunized individuals. *In vitro* immunologic studies have shown that this mutant showed significant reduced binding to wildtype HBsAg-induced antibody. This phenomenon is called vaccine escape mutant. The purpose of the research was to construct open reading frames (ORF) encoding wild-type and G145R mutant HBsAg in a cloning vector in *Escherichia coli* JM109 as an early step to obtain recombinant protein to develop a diagnostic kit for G145R HBsAg detection. **METHODS:** ORF encoding for wild-type and G145R HBsAg was each amplified by polymerase chain reaction (PCR) using HBV DNA as templates. PCR product was ligated into pGEM-T and the ligated product was transformed into *E. coli* JM109. The white and ampicillin resistant transformants were screened and characterized by migration, PCR, double restriction analyses and nucleotide sequencing of inserted DNA. **RESULTS:** Result of migration analysis showed that the recombinant pGEM-T migrated slowly than that of non recombinant plasmid. PCR analysis using T7 dan SP primers produced a DNA band of about 500 base pairs (bps) in size, close to its theoretical size (545 bps). Restriction analysis using *BamH*I and *Nde*I gave a DNA insert of smaller than 500 bps in size, close to its theoretical size of 339 bps and a DNA band of liner pGEM-T of 3000 bps. Analysis of nucleotide sequencing of the DNA insert using BLAST program showed that DNA fragment for both ORFs have 99% homology to those corresponding wild type and mutant HBsAg (accession number of AB113220.1 and AB113238.1, respectively). Result of nucleotide sequence alignment showed that there was a proper mutation at the codon 145 i.e. GGA → AGA. **CONCLUSION:** Two recombinant pGEM-Ts each carrying wild-type and G145R mutants of HBsAg gene in *E. coli* JM109 were successfully constructed and confirmed by nucleotide sequencing.

**Keywords:** Hepatitis B virus, G145R HBsAg, vaccine escape mutant

Introduction

Hepatitis B virus (HBV) is an enveloped hepatotropc DNA virus. Acute and chronic HBV infection causes significant liver diseases and it is estimated that more than 300 million individuals word wide are chronically infected with HBV. At area with high prevalence like African, Chinese and South-East Asia (including Indonesia) more than semi population is infection, and more than 8% is chronic carrier the virus. Furthermore, persistent viral infection leads to chronic active hepatitis, liver cirrhosis and the development of hepatocellular carcinoma. Disease prevention of Hepatitis B is way that is very effective and vaccination HBV is mandatory program at many states. WHO has released vaccination protocol of hepatitis B to be applied in the world. Currently HBsAg which is surface protein of HBV has been made commercial recombinant vaccine and as diagnostic kit to infection HBV(1).

The HBsAg amino acid sequence contains a highly conformational, hydrophilic domain from positions 100 to 160 referred to as the ‘a’ determinant. The ‘a’ determinant conformational epitopes are stabilized by backbone of conserved disulfide-bonded cysteine residues. Alteration of residues in the ‘a’ determinant can result in reduced antigenicity and reduced levels of protein expression. Mutation at codon 126,129, 130, 141, and 144 reported has caused change of small antigenicity at protein S. Reported also already happened point mutation at gene encoding ‘a’ determinant HBsAg causing substitution of amino acid glisin (G) by arginin (R) at position 145 (variant arginin-145 or G145R). The mutation generates vaccine escape mutant that is case where individual which have been vaccination HBsAg still suffering infection of hepatitis B. Epitope containing amino acid 145 is has the character of immunodominant and change at this amino acid would will cause increasing epitope antigenity character causing cannot be neutralized by anti-HBsAg which previously has been formed .This mutation causes HBV mutant no longer be
recognized by antibody which has been formed at the time of immunization so that this virus still infection of man can who has been vaccination (2,3,4). At other research is reported also existence of mutation at DNA causing change of amino acid metionin to become valine at codon 133 showing degradation of antibody reactivity induced by recombinant vaccine HBsAg (5).

In case prevalence Asia of vaccine escape mutant is assumed height, remembers spreading of HBV in this region height. In Indonesia, has been found some mutants HBV that is mutant G145R, M133T and insertion of one nucleotide G, at some metropolises in Java. Individual experiencing vaccine escape mutant to experience chronic hepatitis and liver carcinoma even can continue to death.

The purpose of the research was to construct open reading frames (ORF) encoding wildtype and G145R mutant HBsAg in a cloning vector in Escherichia coli as an early step to obtain recombinant protein to develop a diagnostic kit for G145R HBsAg detection. This recombinant protein will be comprehensive detection system to wild type HBsAg which can prevent the happening of giving failure of immunoglobulin hepatitis B (HBIG) at newborn baby from mother which are positive HBsAg and immunoprophylaxis at patient doing transplantation of liver.

**Materials and Methods**

**Strain and reagents**

*E. coli* JM 109. The following reagents were used: PCR kit, pGEM-T cloning vector kit, GFX kit, IPTG, X-Gal, ampicillin, Qiaprep kit, primers FB and RBB (Proligo), primers T7 and Sp6 (Proligo), *BamH*I, *Nde*I, TAE and EtBr.

**Amplification of DNA Encoding Wildtype and G145R Mutant S80-180**

For amplification of the DNA encoding wildtype and G145R mutant S80-180, PCR was performed using S80180fb 5'-CATATgCTgCggCgTTTTATCATC-3' (primer forward) and S80180rbb 5'-gagATCTTAAgCcCGCCGGgACCCAC-3'(primer reverse). The mixture contained 10 μL DNA template, 2 μL primers, 5 μL MgCl₂ 25mM, 1 μL dNTP 20 mM, 0.25 μL *Taq polymerase* 5 U/μL in total volume of 50 μL. The amplification conditions included an initial denaturation for 5 min at 94˚ C for 35 cycles of amplification with denaturation at 94˚ C for 2 min, annealing at 47˚ C for 2 min, extension at 72˚ C for 2 min and follow by final extension at 72˚ C for 10 min. The PCR products were run in 1 % b/v agarose gel in a tank with TAE liquid of electrophoresis.

**Characterization of recombinants pGEM-T**

Recombinants pGEM-T were purified with Qiapreps kit from Qiagen. The purified plasmid products were characterized by using migration analysis (plasmids were run in 1% b/v agarose gel in a tank with TAE liquid of electrophoresis), PCR analysis (using primers T7 and Sp6), double restriction analysis (using *BamH*I and *Nde*I) and nucleotide sequencing analysis of inserted DNA (using DNA sequencer).

**Results and discussion**

DNA encoding wildtype and mutant G145R S80-180 were successfully amplified using primers forward FB and reverse RBB by PCR method. This thing was shown with existence of a DNA band between 214 and 396 base pairs (bps) (theoretical measure result of PCR 345 bps). Clone pGEM-T recombinant had been obtained from selection of white and blue transformant. Recombinants plasmid were isolated and characterized to ascertain existence of an inserted DNA into pGEM-T. Result of migration analysis showed that the recombinant pGEM-T slowly migrated than that of non recombinant so recombinant plasmid contained an inserted DNA.
Figure 1 Result of DNA encoding wildtype and G145R mutant S80-180 PCR using primers FB and RBB DNA encode 1. wildtype S80-180 2. DNA encode G145R S80-180 3. template of HBV 2320 4. pUC/HinfI

Figure 2 Migration analysis of recombinant plasmid and non recombinant plasmid 1. wildtype (S80-180)-(pGEM-T) 2. G145R(S80-180)-(pGEM-T) 3. pGEM-T

Result of characterization with PCR applied primers T7 (primer annealing at promoter T7 pGEM-T with nucleotide sequence 5'-TAATACGACTCACTATAGGG-3') and Sp6 (primer annealing at promoter Sp6 pGEM-T with nucleotide sequence 5'-ATTTAGGTGACACTATAGAAT-3') showed existence of an inserted DNA around 500 bps that is close to its calculation size (545 bps). This thing means existence of an inserted DNA in pGEM-T because gene amplification using those primers was an inserted DNA (345) bps that is added by some nucleotides at pGEM-T limited by primers T7 and Sp6 approximately 200 bps.

Restriction analysis using BamHI and NdeI produced an inserted DNA with lower than 500 bps in size (theoretical size of 339 bps) and a DNA band of linear pGEM-T of 3000 bps having the same theoretical size.

Analysis to nucleotide sequences of an inserted DNA using BLAST program showed that DNA fragment for gene encoding wild-type and G145R mutant S80-180 have 99%

Figure 3 Result of PCR recombinant plasmid using primers T7 and Sp6 1. 100 bp ladder 2. wildtype (S80-180)-(pGEM-T) 3. G145R(S80-180)-(pGEM-T) 4. G145R(S80-180)-(pGEM-T)

homology with those of gen S (accession number of AB113220.1 and AB113238.1 in GenBank). Result of nucleotide sequence alignment showed that there was a right mutation at the codon 145 i.e. GGA → AGA there were different nucleotides at codon 117, codon 161 and codon 184 on a recombinant pGEM-T wildtype S80-180. At codon 117 happened silent mutation which do not change the amino acid of protein because the mutated codon still codes the same amino acid i.e. serin. At codon 161, the codon of recombinant plasmid codes fenilalanin while the codon of S gene nucleotide sequence codes tyrosin. Both amino acids are included in one groups of aromatic amino acid that having different polar character, fenilalanin has the character of non polar while tirosin is having the character of polar. That difference doesn't cause change of surface probability based on the result of prediction of epitope (visible inexistence of change of graph meaning at amino acid 85 proteins S80-180 or amino acid 117 proteins S HBsAg). At codon 184, the codon of recombinant plasmid codes alanine whereas the codon of S gene nucleotide sequence codes valin. Both amino acids also are included one groups of aliphatic amino acid non polar. Based on the analysis can be assumed that both proteins don't have significant difference conformation.
Result of determination of nucleotide sequence a recombinant pGEM-T G145R S80-180 showed homology 99% with nucleotide sequence HBV access code AB1132201. There were different of nucleotides base sequence at codon 104, codon 145 and codon 184. At codon 104 happened silent mutation which do not change the amino acid of protein because the mutated codon still codes the same amino acid i.e. leucin. At codon 145, the replacement of a single base nucleotide with another nucleotide i.e. glisin→arginin. Arginin is basic amino acid whereas glisin is having the character of nonpolar. Result of epitope prediction of a recombinant pGEM-T G145R showed that an epitope S80-180 mutant G145R was different from an epitope S80-180 wildtype at amino acid 69 of protein S80-180 caused by point mutation glisin becomes arginin.

The G→A 145 substitution alters the projecting loop (aa 139-147) of the ‘a’ determinant such that neutralizing antibody induced by vaccination no longer recognizes the mutated epitope, hence the term vaccine escape mutant. At codon 184, codon of a recombinant pGEM-T G145R codes alanin whereas codon of S gene nucleotide sequence codes valin. Both amino acids are included into one groups i.e. aliphatic amino acid nonpolar.
Hepatitis B virus S gene for surface antigen, partial cds, isolate: Patient

Figure 6  Result of BLAST program analysis of gene encoding G145R S80-180. Query = nucleotide sequence of gene encoding wildtype S80-180. Sbjct = nucleotide sequence of HBV S protein (access code AB113238.1)

Figure 7  Prediction of linear epitope of wildtype and G145R S80-180. 1. G145R (S80-180)-(pGEM-T). 2. wildtype (S80-180)-(pGEM-T)

Conclusions

Result of analyses of recombinants pGEM-T covering migration analysis, PCR analysis, restriction analysis, and determination of nucleotide sequence showed existence of inserts DNA that encode wildtype and G145R mutant S80-180 in pGEM-T cloning vector. Based on the result of analyses this research successfully constructed two clones of recombinant pGEM-T carrying wildtype and G145R mutant of S gene in E. coli JM 109 that had been confirmed by nucleotide sequencing.

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References
