# EXPRESSION OF NOROVIRUS P PARTICLE CHIMERIC-HBV ANTIGENS AS INTRANASAL HEPATITIS B PROPHYLAXIS AND THERAPEUTIC VACCINE CANDIDATE

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**Abstract** - P particle chimeric of Norovirus, a 24-mer complex of protruding (P) domain combined with foreign antigen insertion shown succesfull performance in vaccine development. In this study, (P) domain were modified with HBV antigenic determinant (HBcAg and HBsAg) inserted into loop 1 ($I_{293}$-$R_{297}$) and loop 2 ($T_{371}$-$D_{374}$), respectively. Structures of P domain modelled with I-TASSER software indicated that antigens were successfully inserted and exposed on the P domain loop surface. P domain coding sequence was subsequently synthesized and expressed in E.coli. The result showed that P domain was successfully expressed, however the domain expressed mainly in inclusion body, and the soluble form was derived by refolding and characterized by EM. This result revealed that P particle presenting Hepatitis B antigen has the potential as a candidate of intranasal Hepatitis B prophylaxis and therapeutic vaccine.

**Keywords** - Norovirus, P Particle, Purification, Intranasal Vaccine, Hepatitis B

## I. INTRODUCTION

Norovirus is a single-stranded RNA virus which is classified into Caliciviridae family [1]. Its capsid is composed of 2 domains, namely shell domain (S) and protruding domain (P). The in vitro expression of this P domain which is the antigenic structure of Norovirus, using prokaryotic cells can form different complexes that is P dimer, 12-mer small P particle and 24-mer P particle [1]. All of three particles are immunogenic, nevertheless the 24-mer P particle is more excellent in inducing the innate, humoral and cellular immune responses, so this P particle has a great potential as a vaccine candidate against Norovirus [3]. Furthermore, P particle is stable and easily produced in prokaryotic cells.

P particle has a superiority with the surface loop structure that can be inserted with another antigen. Thus, this advantage make P particle act as a dual vaccine, vaccine against norovirus and also against the antigen presented on the loop structures. Each P particle consists of 24 P domain monomer. Every P domain has 3 loops, thus the total number of antigen insertion on the loop could be 24-72 copies. Antigens presented in large numbers may directly improve the antigenicity and immunogenicity [2].
The previous research has been done before mentioned that vaccination through intranasal route using P particle presenting rotavirus antigens and influenza virus epitopes evaluated in animal models in pre-clinical studies induced strong immune responses [2]. P particle also can function as an adjuvant in that delivery system due to its big size [4]. To utilize this potential, P particle can be used in the development of intranasal prophylaxis and therapeutic vaccine for hepatitis B by presenting its antigens on the surface loops of P particle. Despite of the fact that the previous Hepatitis B vaccine has already been able to reduce 90% infection rate in humans after this injected vaccine was introduced to populations, there is still needs for the improvement of the vaccine effectiveness using different approaches to develop safer and more effective hepatitis B vaccines and also to overcome the disadvantages of current vaccines. The prevalence of hepatitis B infections is still high nowadays in developing countries, including Indonesia [7]. Hepatitis B chronical infection causes severe diseases such as liver cirrhosis, hepatocellular carcinoma and other complications [5]. Hepatitis B intranasal vaccine has several advantages comparing to the injected vaccines type, first, it does not need the 3 times injections [6]. Second, previous studies showed that hepatitis B intranasal vaccines in a different platform could strongly induce an effective immune responses 450 times bigger. Third, intranasal vaccines target the mucosal immune system in the NALT tissue (Nasal-associated lymphoid tissue). NALT tissue may induces all the 3 different immune systems, mucosal, humoral and cellular immune responses that cannot be induced through intramuscular injections.

In order to develop intranasal prophylaxis and therapeutic hepatitis B vaccine, in this research, firstly, the in silico design of hepatitis B antigen

insertion into loop structures of P domain was generated. The designed was subsequently synthesized and overexpressed.

## II. MATERIALS AND METHOD

### Design of P domain chimeric protein

The loops locations of the Norovirus GII.4/2008 (GenBank No. KC990829)[10] of P domain were determined using the sequences reference from Norovirus GII.4/1997 (VA387) (GenBank No. JQ478407)[11] that has been previously studied [12]. The determination of the loop locations was pursued by comparing the two 3D models of P domain secondary structure prediction from online application I-TASSER. This programme is based on the algorithm principle to obtain accurate structure estimation [13]. The in silico designs of hepatitis B antigen insertion were located on 2 P domain loops, those are loop 1 and loop 2. Hepatitis B antigen sequences were taken from NCBI GenBank (www.ncbi.nlm.nih.gov) with accession number of AB466417[14] and KM526748[15], each of the sequence has a CDS encoding "a" determinant HBsAg (surface antigen) and 18-27 HBcAg epitope (core antigen) of Hepatitis B genotype B sub-genotype B3 which is the predominant genotype distributed widely in Indonesia[14].

### Construct of P domain chimeric protein encoding gene

DNA fragments encoding the designed of NoV chimeric P domain with HBV antigen insertion were synthesized by Gene Script and inserted into pET29b vector. The pET29b-HBcAg-HBsAg plasmid was transformed into E.coli BL21(DE3).

### Expression of P domain chimeric protein

Transformants were subsequently cultured at 37 $^{o}$C with the presence of 80 µg/mL kanamycin. To induce the chimeric protein, Isopropyl-β-D-thiogalactoside (IPTG) was added to the final concentration of 0.2 mM after the $OD_{600}$ reached 0.6-0.8 and cells were continued to be cultured at 25 $^{o}$C for 4 hours. The cells were then harvested by centrifugation at 5000 xg for 10 min. Pellet was subsequently weighed and every 0.1 g wet weight cells were resuspended in 40 µl of loading buffer (buffer SDS page) and boiled for 10 minutes. SDS PAGE was performed with 12 % acrylamide gels in denaturing condition according to Laemmli protocol

### Unfolding and Refolding of chimeric protein

E. coli BL21(DE3) harboring expression plasmid was induced by IPTG according to protocol above. The cell was harvested by centrifugation at 5000 x g, 4°C, 10 minutes. 0.3 gram of cell pellets was given 2.5 ml of lysis buffer (10 mM Phosphate buffer, 10mM EDTA, 10 mM NaCl, 1mM PMSF, pH 8.5) before sonicated for 20 minutes. The precipitate was solubilized by solibilization buffer (50 mM phosphate buffer, 100 mM NaCl, 1 mM β-mercaptoethanol, 8 M urea, pH 8.5) at 4°C overnight. Solubilized protein was centrifuged at 12,000 x g for 30 minutes at room temperature. The supernatant was refolded with refolding buffer (100 mM Phosphate buffer, 0.2 M EDTA, 500 mM NaCl, and 1% urea). Refolding was done by dilution method until solubilized protein was diluted 20 times. Refolded protein was ultracentrifuged at 400 x g for an hour.

## III. RESULT AND DISCUSSION

Loop structures at the distal part of P domain that can be inserted with antigens located at the sequence $I_{293}$-$H_{297}$ (loop 1), $T_{371}$-$D_{374}$ (loop 2) and $D_{391}$-$T_{394}$ (loop 3) [12]. Loops are the strategic place for foreign antigen insertion and presentation. After inserting the antigens, when it is expressed, the antigens would appear on the surface of P particle, so indeed it will directly interact with the molecules from immunity systems. The insertion into loop 1 and loop 2 was well designed as showed in picture 1 and the structure prediction result represented that antigens were exposed on the surface of P domain. This design has been compared with the model designs of P domain inserted with influenza and Rotavirus antigens [2] (picture 2). At glance, all antigens were successfully exposed on the surface of P domain without changing the original structure of P domain. CDS sequence of HBcAg and HBsAg epitope after inserted into loop structures of P domain at DNA level made the P domain sequence formed a new frame with the length of amino acids sequence became 477 aa. The insertion was in frame therefore would not change the translation of the original P domain protein.

P particle acts as an adjuvant to modulate the immune responses against hepatitis B. Antigens presented with P particle will interact with the NALT system as a part of mucosal immunity systems. The mucosal surface contains a lot of lymphoid elements, including B cells, T lymphocytes, plasma cells and several cellular elements that are sensitive to antigens [27]. The main effector cells on mucosal surface are CD4+ and CD8+ T lymphocytes alongside protective antibodies produced by activated B cells. T lymphocytes are estimated to be as many as 80% of the entire mucosal lymphoid cells population [26]. Antigens delivery system through intranasal route make it to have a unique interactions with dendritic cells in tonsils organized in a mucosal surface with density to be 500 cells/mm$^2$ [5]. In addition, beside the advantage of intranasal vaccine to reduce the patients discomfort due to injection, this intranasal hepatitis B theoretically could become a preventive vaccine as the protective secretory IgA is also induced on all mucosal immune systems, that will protect human from hepatitis B infection when it is transmitted via

sexual activity, as well as the therapeutic vaccine to eliminate virus-infected cells after mucosal T lymphocytes are activated so it will trigger cellular immune responses at a high level. Mucosal immune responses induction for T lymphocytes could be expected to overcome the down regulation of systemic immune systems because of chronical hepatitis B infection [5] [28].

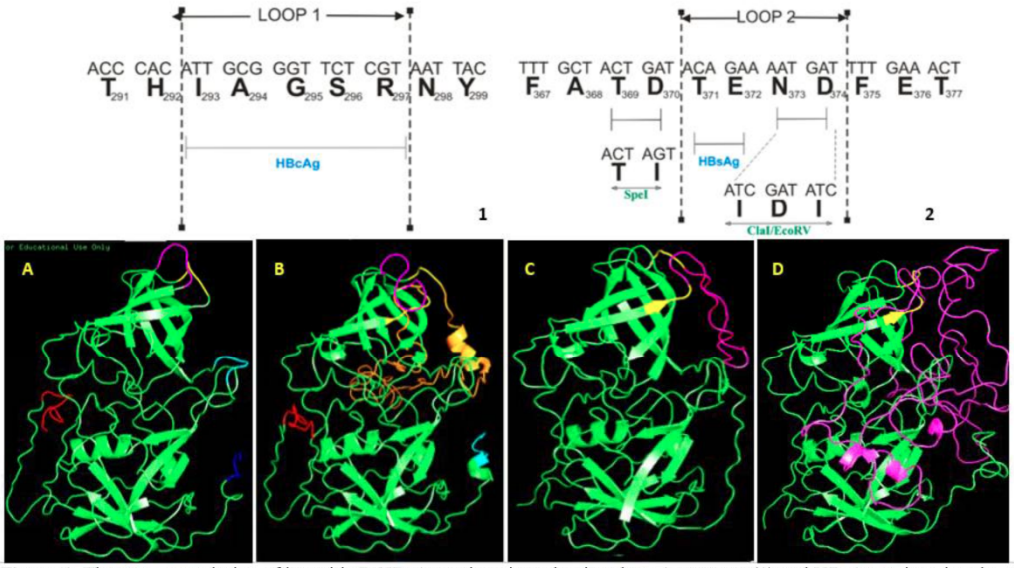



**Figure 1**. The sequences design of hepatitis B HBcAg antigen insertion into loop 1 structure (1) and HBsAg antigen into loop 2 (2) as well the modelling results of protein secondary structure (by I-TASSER) for P domain protein (A) along with its loops locations (loop 1 in yellow, loop 2 in pink, loop 3 in light blue) and His-tag location (in red) and Cysteine-tag (CNGRC) in dark blue. B is the model of P domain structure with HBcAg antigen insertion (in pink) and HBsAg antigen insertion (in orange). C and D are the model of P domain structures inserted with influenza virus antigen and Rotavirus VP8 antigen respectively (in pink).

HBcAg and HBsAg are hepatitis B major structural immunogenic antigens as a target for activation of immune systems. "a" determinant HBsAg is a component of S protein used as antigens in vaccine that has a function to induce antibody production for neutralizing the viruses[29]. HBcAg can become a preventive or therapeutic vaccine candidate[28]. 18-27 HBcAg epitope (FLPSDFFPSI) is a peptide that clinically has been tested as an immunodominant lymphocyte T cells which can be used without the addition of adjuvant. This epitope is recognized by the lymphocyte T cells when being presented on MHC class I molecules [25]. MHC class I molecules are responsible for antigen presentation to CD8+ T cells to induce the activation of naïve T cells so it can produce effector cells such as cytotoxic T cells in cellular immune responses. CD8+ T cells specific for hepatitis B plays an important role for virus elimination [25]. The unbalance number between Lymphocyte T cells and hepatitis B virus is a scenario that is common in patients with the chronic hepatitis B infection [23]. Therapeutic vaccine using HBcAg epitopes can induce immune response to produce more powerful cytotoxic T cells in order to be able to stop the virus replication and eliminate the virus from the patients [24][25].

Intranasal hepatitis B vaccine that uses the combination of HBcAg and HBsAg in the previous clinical phase 1 study in different platform[23], showed that the vaccine is safe, can tolerate allergic reactions and highly immunogenic in inducing local and systemic immune systems[5]. Anti-HBs and anti-HBc appeared with high titter on the subject. Therefore, it would be expected in the further research to do the construction of antigen insertion into P domain loops, also the protein expression, purification and pre-clinical studies to prove the hypothesis that the hepatitis B antigens presented on the surface loop of P particle when administrated via intranasal route can induce the protective immune responses.

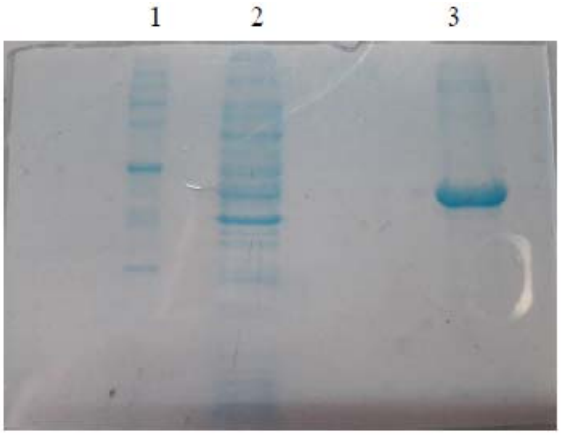



**Figure 2. Solubilization of P domain inserted with HBV antigens. Lane 1: Mw Marker, Lane2: supernatant after sonication, Lane 3: soluble P domain-inserted with HBV antigens after unfolding**

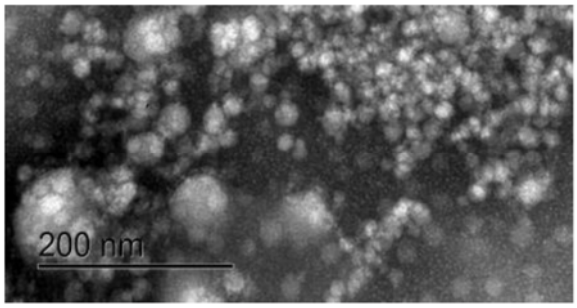



**Figure 3. Transmission Electron Microscopy of refolding P particle NoV GII.4-chimeric HBV**

The designed P domain chimeric HBV antigens coding sequence was subsequently synthesized, ligated into pET24a and cloned into E.coli BL21(DE3). With codon optimization, P domain chimeric HBV antigens has been successfully expressed. Since most protein was expressed into inclusion bodies, protein was subjected to be unfolded and refolded again. Figure 2 shown that P domain chimeric HBV antigens has been solubilised after unfolding (lane 3). Refolding experiment is being pursued, and some of soluble protein was characterized by electron microscopy

## IV. CONCLUSION

P domain with HBV antigenic determinant (HBcAg and HBsAg) inserted into loop 1 ($I_{293}$-$R_{297}$) and loop 2 ($T_{371}$-$D_{374}$), respectively, and most protein was expressed into inclusion bodies, subjected to be unfolded and refolded again and some of soluble protein was characterized by electron microscopypurified and characterized. Our resutl indicates that the P particle presenting hepatitis B antigens has the potential as a candidate of intranasal hepatitis B vaccine.

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