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SARS-CoV-2 multi-epitope subunit vaccine proof-of-concept derived from the in-silico study with protein expression in Escherichia coli BL21

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A B S T R A C T

The protein subunit vaccine is the most considerably developed SARS-CoV-2 vaccine, according to the WHO vaccine tracker in 2023. The acceleration of vaccine development in 2 years of eradicating the COVID-19 pandemic is attainable due to the role of bioinformatics. This paper aims to evaluate strategies for developing multi-epitope SARS-CoV-2 recombinant vaccines in silico with high protein expression in *Escherichia coli* vector plasmid. The study was conducted by analysing SARS-CoV-2 epitopes using immunoinformatic tools provided by IEDB, codon optimisation, rare codon analysis, plasmid design, and ribosomal binding site (RBS) analysis were analysed using RNA structure 6.4, gene cloning by *E. coli* DH5α and protein expression by *E. coli* BL21. Each epitope peptide candidate was linked to a flexible linker sequence (GGGGS). Gel Analyzer 19.1 was utilised to determine the protein band of SDS-PAGE. The immunoinformatic study obtained a multi-epitope of the recombinant SARS-COV-2 vaccine with 7 epitopes for HLA-I allele candidates and 4 for HLA-II. It is demonstrated that the candidate vaccine protein was successfully cloned in *E. coli* DH5α and expressed in *E. coli* BL21. The result of this study will benefit the development of a SARS-CoV-2 protein recombinant vaccine that is safe, affordable, and efficient to induce T-cell response.

Keywords: SARS-CoV-2; protein expression; codon optimisation; ribosomal binding site.

Introduction

Since March 2023, there have been 199 COVID-19 vaccine candidates in the pre-clinical stage and 183 in

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clinical development, of which 59 are protein subunit vaccines.[1] Subunit vaccines, which contain only the purified, recombinant, or fragments of an antigen from a pathogen without any pathogenicity, are noticed as the safest type of vaccine.[2] This is an affordable and practical approach to preventing health disorders because it has fewer side effects than live-attenuated vaccines. [3] Most immunisations focus on the immune response by introducing the SARS-CoV-2 spike protein to induce an immune response by neutralising antibodies. [4]

The emergence of various variants of SARS-CoV-2 raises the question of whether it is sufficient to rely solely on neutralising antibodies as a vaccination goal. [5] This question highlighted the importance of introducing CD4 and CD8 T cells in the immune response after COVID-19 vaccination. [5] Despite these reasons, proteins or peptides are generally poor inducers of CD8 + T cell responses, which is the common reason why the subunit vaccine does not elicit a robust immunogenic response like an inactivated viral vaccine to avoid severe adverse effects.[6] For this reason, the subunit vaccine needs intensive study to design an effective vaccine that could elicit a robust immune system and memory cells.[7,8] This obstacle is currently simplified through bioinformatics and immunoinformatic studies.[9] Epitope analysis by immunoinformatic analytical tools and servers relies on curated data repositories relevant to immune reactions and specific pathogens.[9]

Several immunoinformatic tools have been introduced for epitope prediction and factors associated with vaccine candidate potency.[9] There are RANKPEP, NHL aired, SVRMHC, SYFPEITHI, and NetCTL, and recently, the most frequently used is The Immune Epitope Database and Analysis Resource (IEDB), whose database is drawn from peer-reviewed scientific literature and data submitted by previous studies. [10–15] Moreover, to develop an effective and efficient vaccine candidate, it is required to evaluate its antigenicity, allergenicity, and toxicity.[14,15] Antigenicity data can be accessed on the Vaxijen server, allergenicity on the Allertop server, and toxicity can be viewed on the Toxinpred server.[14,15]

SARS-CoV-2 is composed of 4 main proteins: Spike (S) glycoprotein, Envelope (E) glycoprotein, Membrane (M) glycoprotein and Nucleocapsid (N) protein.[16] WHO vaccine tracker 2023 reports show that spike protein is the most developed SARS-CoV-2 protein subunit vaccine candidate.[1] Regardless, a few multi-epitope vaccine candidates have been developed recently.[1] Studies have referred to non-spike CD8+ T cell epitopes such as N, M, and ORFs, offering possible candidates to elicit a robust immune system against SARS-CoV-2.[4,17–21] The SARS-CoV-2 subunit vaccine developed mostly in the mammalian protein expression system (CHO cell line).[1] However, subunit protein vaccines can be produced in a bacterial expression system, for example, *Escherichia coli.*[1,27]

Escherichia coli (*E. coli*) is often used as a host for recombinant DNA technology to express proteins because it is easy to use, can be grown quickly and cheaply at high densities, has well-known genetics, and has a wider range of compatible molecular tools. [22] Limitations of *E. coli* as a host for protein expression are the inability of *E. coli* as a Prokaryotic to carry out Eukaryotic posttranslational modifications, limited ability to carry out extensive disulphide bond formation; some proteins are kept in an insoluble form due to protein misfolding, aggregation, and intracellular accumulation as inclusion bodies. The protein degradation or insufficient translation occurs due to obstruction of the translation process. The codon sequence for specific amino acids in eukaryotes differs from prokaryotes such as *E. coli*. [22] This is known as "codon bias" which can inhibit protein synthesis and gene expression in *E. coli*. [22] Codon optimisation and ribosomal binding analysis aim to obtain high expression levels in new hosts.[23,24] Codon optimisation is done by converting the foreign DNA sequences into sequences the new host easily recognises.[24,25] *E. coli* commonly uses a plasmid as the expression vector.[26]

The plasmids used in protein expression today resulted from various combinations of replicons, promoters, selection markers, multiple cloning sites, and fusion protein/fusion protein removal strategies.[27] The pET15b vector was used in this research because it has a T7 promoter. It is widely used for recombinant protein production because of its simple genetic operation, high expression levels, and tightly regulated targeted gene expression.[25,28] Furthermore, this plasmid encodes an N-terminal His6-tag that expresses His-tagged proteins that will benefit the purification process.[29] It can be purified and detected easily due to the histidine residues binding to several immobilised metal ions, including nickel, cobalt, and copper, under specific buffer conditions.[29]

This study aims to assess strategies for developing subunit vaccines to stimulate immune responses of CD4 and CD8 T cells in silico and protein expressed in bacterial protein expression system. Vaccine development evaluated conservancy, antigenicity, safety, population coverage, and optimised codon in bacterial vector.

Material and Methods

SARS-CoV-2 epitope analysis. All sequences of the Spike (S) [ref.seq: P0DTC2], Nucleocapsid (N) [ref.seq:

P03466], ORF3a [ref.seq: P0DJZ8], and ORF1ab [ref.seq: P0C6YO] SARS-CoV-2 protein were retrieved from the UniProt database (https://www.uniprot.org) and covered all the wild type and the mutant gene that enlisted in variance of concern by WHO. All the sequences later analysed the consensus sequence utilised UGENE software to obtain conserved genes for the epitopes candidate. Epitope prediction was performed using T-cell Epitope Prediction Tools (http://tools. iedb.org/main/ tcell/) equipped by The Immune Epitope Database and Analysis Resource (IEDB). The immunogenicity of the MHC class I epitope candidate was selected by choosing the predicted epitopes above the generalised linear model (GLM) score.[30] MHC class II was analysing epitope candidates' ability to induce IFN-γ's secreting with IFNepitope (http://crdd.osdd.net/raghava/ifnepitope /scan.php). An epitope that met the criteria was tested for binding to MHC using a molecular docking Cluspro server (https://cluspro.bu.edu/home.php) and followed by an analysis of the RMSD score using Pymol. The PDB molecular docking result from Cluspro also analysed the protein-ligand interaction by screening the hydrogen bond, non-bounded bond, and salt-bridges bond in the PDBsum server (https://www.ebi.ac. uk/thornton-srv/databases/pdbsum/Generate .html).

Codon optimization. *E. coli* BL21-competent cells will synthesize the recombinant proteins S, N, ORF3a, and ORF1ab through protein expression. The linker sequence that was used to link each of the epitope peptide candidates is the flexible linker sequence (GGGGS).[31] The codon was optimised using the Optimizer codon optimization tools (http:// genomes.urv.es/ OPTI MIZER/) following rare codon analysis tools by Genscripts (https:// www.genscript. com/tools/ rare-codon-analysis). The parameters analysed were negative CIS elements (NCISE), CFD, CAI, and GC content.

Plasmid design. Synthetic recombinant DNA of multi-epitope SARS-CoV-2 vaccine candidates that have been codon optimised cloned to the pET-15b plasmid. The restriction sites were determined with SnapGene 6.1 by selecting the sites that cannot cleave the ORF section but are available at the multiple cloning site (MCS) of the pET-15b plasmid.

Ribosomal binding site analysis. RBS exposition analysis was started by analyzing the secondary structure of DNA sequences with RNA structure 6.4 (http://rna.urmc.rochester.edu/ RNAstructureWeb/Servers /Predict1/Predict1.html). This step aims to see the best arrangement so as not to form hairpin structures around the RBS region and start codons.[32]

Transformation All utilised *E. coli* competent cells in this research were the inventories of Pusat Riset Virologi dan Kanker Patobiologi (PRVKP), Faculty of Medicine, Universitas Indonesia. The gene target was inserted into competent cells of *E. coli* DH5α for gene cloning and BL21 for protein expression. The transformation was performed by the heat-shocked method. The Heat shock was carried out by moving competent cells into a water bath at 38ºC for 90 seconds. The cells were then transferred back into the ice-filled container for 60 seconds. The medium SOC was added to competent cells which were then incubated for 1 hour at 37ºC with an agitation speed of 200rpm. Incubation results from SOC medium were then inoculated into LB agar medium supplemented with 100 µg/L antibiotic ampicillin and incubated overnight at 37ºC. After incubation, calculate the transformation efficiency (best efficiency if the number of colonies is 10^6-10^8/ug of plasmid added). The transformation efficiency was determined by this formula:

$$\textbf{Transformant (cfu)} = \frac{\text{the total of transformant colony x dilution factor x the total volume of culture}}{\text{The volume of culture that was transformed}}$$

$$\textbf{The transformation efficiency} = \frac{\text{Transformant (cfu)}}{\text{Vector concentration}}$$

Gene confirmation Gene sequencing was used to confirm the presence of the insert in *E. coli* transformants. Previously, the transformed pET-15b plasmid containing the target gene was incubated overnight in an LB agar medium supplemented with the antibiotic ampicillin for gene cloning. Overnight colonies were harvested and isolated using the Qiaspin miniprep kit [Qiagen]. The concentration of the isolated plasmid DNA was then measured using a nanodrop tool. The isolated genes were first confirmed by the success of the transformation using gel electrophoresis before being sent to the Macrogen vendor for gene sequencing.

Protein expression The plasmid vector containing the target gene from gene cloning was then transformed into *E. coli* BL21 for protein expression. The protein expression was done by incubated the *E.coli* BL21 contain target gene culture with TB broth medium supplemented with 100 µg/L ampicillin antibiotics at 37ºC (200 rpm) and inducted with IPTG for 4 hours. The protein expression yield was run in SDS polyacrylamide gel electrophoresis (SDS-PAGE). The molecular weight was used to determine the protein target. The GelAnalyzer 19.1 was utilised to determine the protein band.

Result and Discussion

SARS-CoV-2 epitope analysis. The peptide candidate that was successfully analysed is shown in Table 1.

Table 1. Epitope candidate

Protein	Peptide	Human Alele	RMSD (Å)	Hydrogen bond	non-bounded bond	*salt-bridge* bond
S	*S11*	A*02:01	3.770	13	121	*
N	*N11*	B*07:02	2.586	9	158	*
	N21	DRB1 *01:01	5.113	2	59	-
ORF1ab	*OAB11*	B*39:01	2.286	9	138	*
	OAB12	B*08:01	2.508	4	132	*
	OAB13	B*07:02	3.900	9	164	*
	OAB21	DRB1*01:01	6.307	4	33	-
ORF3a	*OA11*	A*24:02	3.397	4	108	*
	OA12	B*57:01	2.961	5	115	*
	OA21	DRB1*01:01	4.902	3	62	1
	OAB22	DRB1*01:01	3.607	5	72	1

* the bold text indicates candidate MHC-II epitopes, while italics font indicates candidate MHC class I epitopes; (*) data not available.

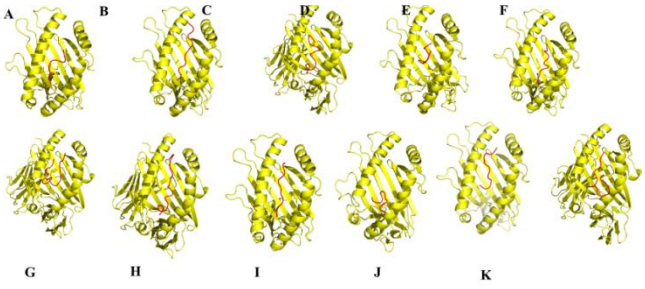

Figure 1. The model of Cluspro docking result visualised with Pymol. A) S11-A*02:01; B) N11-B*07:02; C) N21-DRB1 *01:01; D) OA11-A*24:02; E) OA12-B*57:01; F) OA21-DRB1*01:01; G) OA22-DRB1 *01:01;H) OAB11-B*39:01; I) OAB12-B*08:01; J) OAB13 (B*07:02); K) OAB21-DRB1*01:01. It is showed that the ligand successfully binds with the receptor. The receptor is human HLA (yellow), and the ligand is The peptide of the epitope of the vaccine candidate (red).

Codon optimisation. The recombinant vaccine candidate sequence showed the ideal value of codon optimisation, which has an ideal CAI score in the range of 0.8-1.0, GC content in the range of 30-70%, CFD below 30%, and no negative CIS elements were found. The result of codon optimisation and rare codon analysis is presented in Table 2.

Table 2. Codon optimisation result

Status	CAI	%GC	%AT	NCISE	CI
Q	0.8	60.9	39.1	-	-
O	1.00	52.7	47.3	0	0

*Q=query;O=Optimised.

Plasmid design. The epitope candidate sequences were successfully inserted in the pET-15b plasmid. The plasmid size was 6230 bp. The result is presented in Figure 1.

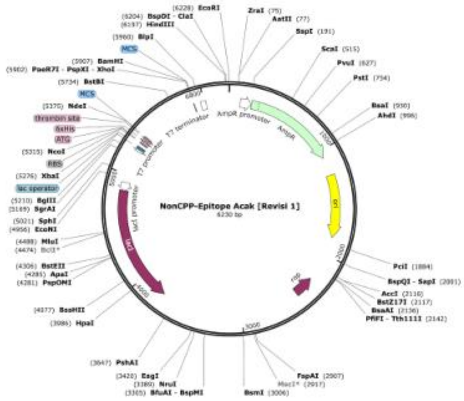

Figure 2. The plasmid design result.

Ribosomal binding site analysis. Based on the RBS analysis, it showed that the RBS and ATG 2D structures are still open in all probability structures. Structure 1 of the secondary structure analysis server result was chosen because it has a structure with the lowest free energy level, which indicates the folding stability of the protein structure.

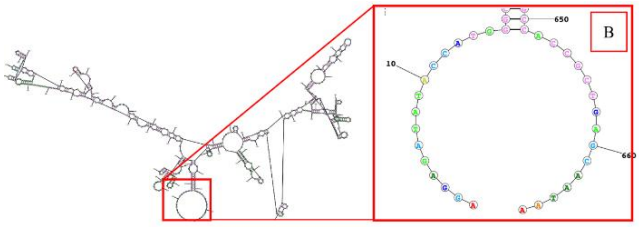

Figure 3. The RNA structure 6.4 server displays the RBS exposition analysis result. A. the whole mRNA secondary structure. B. magnified picture of the RBS and ATG region from the whole mRNA secondary structure The open sequence of RBS refers to the ability of the mRNA sequence to bind to the ribosomal binding site. An ATG sequence that was not formed in the hairpin structure can be held in translation.

Transformation. The transformation results of the pET-15b recombinant vaccine plasmid in *E. coli* DH5α showed an efficiency of 11.6×10^5 and 2×10^5 in *E. coli* BL21.

Gene confirmation. The gene sequencing confirmed that the cloning gene used in this study was the SARS-CoV-2

multi-epitope recombinant vaccine plasmid gene. There is no noise found in the gene sequencing result

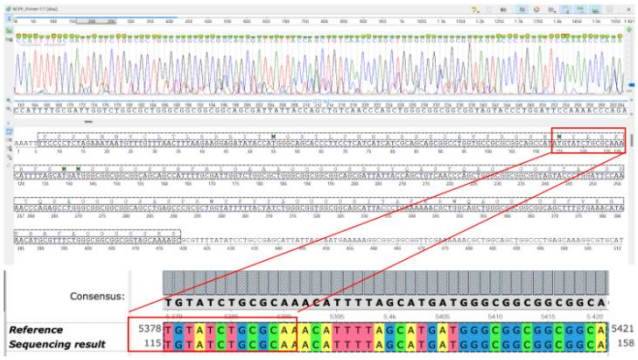

Figure 4. The sequencing results and multiple alignment analysis performed with MUSCLE on the recombinant SARS-CoV-2 vaccine gene produced from gene cloning performed by *E. coli* DH5α.

Protein expression The SDS-PAGE result shows that *E. coli* BL21 successfully expressed recombinant vaccine candidate protein.

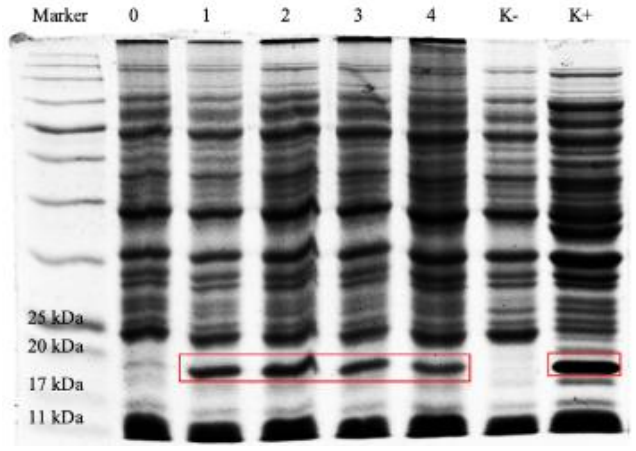

Figure 5. The SDS-PAGE result of recombinant vaccine candidate protein expression in *E. coli* BL21. The protein size is 17.9 kDa; 0,1,2,3,4 are IPTG induction time intervals (0: before IPTG induction, 1,2,3,4: 1st, 2sc, 3rd, 4th hours after IPTG induction); K- was a negative control in protein expression (*E.coli* BL21 which did not contain the target plasmid); K+ is a positive control in protein expression (The NDX protein with the molecular weight of 18.66 kDa).

Discussion

Predominantly immunisations focus on triggering antibodies against the SARS-CoV-2 spike protein, although it is questionable to obtain adequate protective immunity against SARS-CoV-2.[15,33] The emergence of various new variants of SARS-CoV-2 raises the question of whether it is sufficient to rely solely on neutralising antibodies as a vaccination target. [5] This question highlighted the importance of introducing CD4 and CD8 T cells with other protein of SARS-CoV-2 in the immune response after COVID-19 vaccination.[5] A study of important non-spike CD8+ T cell epitopes like N, M, and ORFs suggests possible candidates for designing a multi-epitope vaccine that will make the immune system work strongly against SARS-CoV-2.[4] This study selected the epitope candidates around 9-mer to 10-mer peptides for MHC class I due to the significantly lower affinity of 8-mer peptides than the selected peptides, and there is no difference in binding affinity between 9 to 11-mer peptides.[34] In this study, IEDB referenced 15-mer peptide length as an acceptable candidate for the MHC class II. This peptide length was recommended because the nine amino acids of the ligand peptides would interact with the binding groove of the receptor molecule, and the flank residues would interact with the outside of the receptor groove.[35]

The immunogenicity of the epitope candidate was investigated through its capability to elicit interferon-gamma (IFN-γ), a vital cytokine to differentiate CD8 T cells into full effector CTLs and memory CTLs.[36] Further, this study performed 3D molecular docking on the peptide of the epitope candidates to learn the binding of the ligand and its receptor. This study also focused on the safety of the vaccine candidate. All allergenic or toxic candidates were excluded. Additionally, this study tested the binding potential of the allele with the mouse HLA for further pre-clinical studies, which are critical in the vaccine development phase.

The molecular docking of this study aims to confirm the binding between antigens to the MHC receptor. This purpose was to expect the more stable the bond, the higher the success rate of releasing the cytokine IFNγ. IFNγ is an important cytokine for differentiating CD8 T cells into effector CTL and memory CTL. The 90% of molecular docking results in this study presented RMSD values above 2.5 Å. Only the binding between the OAB11 peptide epitope and HLA-B*39:01 shows an RMSD value below 2.5 Å, which indicates a strong binding affinity between the ligand and the protein. Even though the RMSD value is above 2.5 Å, molecular docking results still contain at least 2 hydrogen bonds and 1 salt-bridge bond in the interaction between the ligand and the protein. Hydrogen bonds

encourage high-affinity receptor-ligand interactions by reducing competitive interference between hydrogen bonds and water. [37]

Salt-bridge bonds between MHC class II receptor proteins and their ligand peptides strengthen the bonds' stability. [38]

Codon optimisation of the vaccine peptide candidate sequence that the GGGGS linker linked shows an ideal codon optimisation score, and no negative cis-elements (NCISE) were found. The cis-elements are regions of noncoding DNA that regulate the transcription of nearby genes.[39] NCISE is an element that inhibits the cis-elements action in a gene sequence's promoter, thereby inhibiting translation.[40] Therefore, the absence of NCISE indicates that the recombinant protein candidate in this study is predicted to be able to be translated well.

The sequence of plasmid design results, followed by ribosomal binding site (RBS) exposition analysis, shows the probability of the RBS sequence being opened and the hairpin not forming from the start codon sequence (ATG) in the 2D structure. Closing off the RBS sequence and making hairpins in the gene start codon sequence will stop translation. The closure of the RBS sequence and the formation of hairpins in the gene start codon sequence will inhibit translation. [23]

The transformation results of the pET-15b recombinant vaccine plasmid in Escherichia coli DH5α, for gene cloning purposes, had a transformation efficiency of $11.6x10^5$ cfu, while in Escherichia *coli* BL21, which aims for protein expression, it was $2x10^5$. The transformation efficiency of this study was considered adequate for cloning applications.[41] The recombinant protein vaccine candidate expressed by *E.coli* BL21 based on the results of SDS-PAGE analysis using GelAnalyzer 19.0 showed that the vaccine candidate was successfully expressed after IPTG induction, with the vaccine candidate's molecular size being 17.9 kDa. The calibration curve and linearity measure (R^2) from the GelAnalyzer 19.0 application shows a value of 0.997, which conforms with the standard calibration curve above 0.97.[42] This study obtained multi-epitope candidates that were successfully expressed in *E. coli* BL21. The vaccine candidates can be studied in vitro and in vivo.

Conclusion

The result of the study showed congruency of in-silico analysis and bacterial protein expression system. Seven epitope candidates for MHC Class I and four for MHC Class II have been selected from S, N, ORF1ab, and ORF3a SARS-CoV-2 protein. Epitope candidates are then combined into protein structures expressed successfully in *E. coli* BL21. The strategy of in-silico epitope selection and codon optimisation of peptide candidates could be used for any protein recombinant vaccine research. The strategy will benefit the development of more precise and efficient epitope selection followed by optimised expression in bacterial protein expression systems. Recombinant epitope candidates also have the potential to be used as SARS-CoV-2 candidates that can be tested in pre-clinical settings.

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