Novel Multimer Chimeric Vaccine Candidate for Tuberculosis: A Comprehensive In-silico Study with Ag85 Protein Complex on Indonesian population

Background

Tuberculosis (TB) continues to devastate global health, new reports state 8.2 million new infections by 2023 and claiming more than 1.3 million lives. This marks the highest number recorded since the WHO initiated global TB monitoring in 1995, reflecting a dangerous rise. This persistent threat has compelled the World Health Organization (WHO) to set an ambitious target: a 90% reduction in TB-related deaths by 2030. Alarmingly, Indonesia is at the forefront of this crisis, with 845,000 cases reported in the same year and 13,947 preventable deaths (Iskandar et al., 2023). The gravity of these figures calls for immediate and innovative action to curb the TB epidemic, especially in high-burden countries like Indonesia.

The limitations of the current Bacille Calmette-Guerin (BCG) vaccine exacerbate the urgency for novel solutions. Despite being the global standard for TB prevention, the BCG vaccine offers inconsistent and often inadequate protection in adults, particularly in regions near the equator, including Indonesia (Dos Santos et al., 2024; Machlaurin et al., 2020). Furthermore, as its efficacy has diminished significantly over the last four decades, the need for a booster vaccine to enhance and sustain immunity has become increasingly critical (Machlaurin et al., 2020). These shortcomings leave millions vulnerable and underscore the critical need for an improved and reliable vaccine.

Even though it is known that around 12 vaccine candidates have entered clinical trials, none have yet firmly replaced the aging BCG vaccine (Machlaurin et al., 2020). Meanwhile, the threat of multidrug-resistant (MDR) TB keeps growing large, complicating treatment and amplifying the risks of unchecked transmission and mortality. According to (Kendall et al., 2017) Indonesia sees around 8,900 new cases of multidrug-resistant tuberculosis (MDR-TB) annually, representing roughly 2% of all new tuberculosis cases in the nation. Indonesia, as a high-burden country, faces a particularly dire challenge in managing MDR-TB cases, further straining its healthcare system.

Our study, focusing on the design of a multimer chimeric vaccine targeting the Ag85 protein complex, offers a huge opportunity to address these urgent challenges. Through the application of advanced in-silico techniques, this study seeks to develop a vaccine candidate specifically designed to address the distinct needs of Indonesia's population. Such innovation is not only a scientific imperative but a moral one, holding the potential to save countless lives, strengthen global TB control efforts, and pave the way for a future free from the devastating grip of tuberculosis.

Methods

1. MSA and Conservancy Analysis

Multiple sequence alignment (MSA) was performed using the Clustalw software (https://www.genome.jp/tools-bin/clustalw) with the aim of placing homologous sequences from different sources in the same column. The results of this process can then be further analyzed using the analysis of conserved sequences (Chatzou *et al*., 2015).

Conserved sequences were extracted from proteins resulting from multiple sequence alignments performed using MEGA (Molecular Evolutionary Genetics Analysis) software (www.megasoftware.net). By aligning the protein sequences, conserved sequences were identified in various *Mycobacterium tuberculosis* protein sequence samples (Bui *et al*., 2006).

2. Retrieval HLA of Indonesian Population as The Bases

The HLA allele types of Indonesian were retrieved from The Allele Frequency Net Database (http://www.allelefrequencies.net). The data of Indonesian allele types came from one study conducted in the Javanese and Sundanese Javanese population, two of the largest ethnic populations, accounting for 40% and 15,5% respectively (Gustiananda *et al*., 2021).

3. Epitope Generation

The protein sequences were obtained for quality control checks to remove non-relevant regions, such as signal peptides and transmembrane domains. This ensured the accurate selection of regions for epitope prediction.

2.1 Prediction of MHC Class I Epitopes

MHC class I epitopes were predicted using NetMHCpan-4.1 (https://services.healthtech.dtu.dk/services/NetMHCpan-4.1/). Peptides were set to a length of 9 amino acids, as required for MHC class I binding. Binding affinity was assessed for Indonesian HLA alleles, with strong and weak binding is defined by the percentile rank thresholds of 0.5% and 2%, respectively, and was used to identify strong binders. Predictions also incorporated immunogenicity analysis to prioritize epitopes with the potential to activate CD8+ T cells (Bui *et al*., 2006).

2.2. Prediction of MHC Class II Epitopes

For MHC class II epitope prediction, the NetMHCIIpan-4.1 (https://services.healthtech.dtu.dk/services/NetMHCIIpan-4.1/) was employed. Peptides of 15 amino acids in length were analyzed for their binding affinity to the HLA-DR Indonesian population. Strong and weak binding is defined by the percentile rank thresholds of 1% and 5%, respectively, and was used to identify strong binders. The cytokine-inducing potential of the predicted peptides was also evaluated to identify candidates with immunomodulatory properties (Bui *et al*., 2006).

2.3 Prediction of B-cell Epitopes

B-cell epitope prediction was conducted using ElliPro (http://tools.iedb.org/ellipro/), a tool designed for both linear and conformational epitope prediction. ElliPro identified surface-accessible regions and calculated antigenicity scores to rank potential B-cell epitopes (Bui *et al*., 2006). B-cell epitope also predicted with ABCPred (https://webs.iiitd.edu.in/raghava/abcpred/), a tool designed for linear epitope prediction (Usmani *et al*., 2018).

4. Immunogenicity Analysis of Epitopes

We used the Vaxijen tool (https://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) to predict antigenicity. In addition, we analyzed allergenicity with AllerCatPro v2.0 (https://allercatpro.bii.a-star.edu.sg/). Lastly, toxicity for each epitope was predicted using ToxinPred (http://crdd.osdd.net/raghava/toxinpred/). Only the epitopes that have the properties of antigen, non-allergen, and non-toxic were further screened. Next, the Indonesia population coverage for predicted CTL and HTL epitopes was assessed using the IEDB population coverage tool, which estimates the proportion of individuals likely to respond to a given set of epitopes based on recognized MHC restrictions (Bui *et al*., 2006).

5. Vaccine Candidate Construction

The vaccine was designed by joining each epitope into a polypeptide chain using the Swiss Model (https://swissmodel.expasy.org/). Large ribosomal subunit protein bL12 was used as an adjuvant. EAAAK, GPGP, AAY, GGGS, and KK are used as a linker for adjuvant, HTL

epitope, CTL epitope, and B cell epitope respectively. Furthermore, vaccine candidates were analyzed for the probability of immunogenicity and the physicochemical characteristics.

6. Immunogenicity Analysis of Vaccine Candidate

The immunogenicity of the vaccine construct was evaluated using a series of bioinformatic tools to assess antigenicity, allergenicity, and toxicity. Antigenicity prediction was performed using the VaxiJen v2.0 tool, with a threshold set to distinguish probable antigens. The AllerCatPro v2.0 tool was utilized to determine the allergenic potential of the construct, while ToxinPred predicted toxicity. These evaluations provided a comprehensive understanding of the vaccine construct's potential as a safe and effective immunogen (Bui *et al*., 2006).

7. Physicochemical Characteristics

The physicochemical properties of the vaccine construct were determined using the ExPASy ProtParam tool (https://web.expasy.org/protparam/). The parameters evaluated included molecular weight, theoretical isoelectric point (pI), instability index, aliphatic index, and the grand average of hydropathicity (GRAVY). Additionally, the estimated half-life of the protein in mammalian reticulocytes (in vitro), yeast (in vivo), and *Escherichia coli* (in vivo) were calculated to predict stability and longevity (Bui *et al*., 2006).

8. Homology with Human Peptides and Gut Microbiome

Homology analysis of peptides used for vaccine construction with human peptides (taxid: 9606) was conducted using the BlastP feature on the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi) with parameters algorithm consisting of expect threshold 30,000 and word size 2; scoring parameters matrix PAM30, gap cost existence 9 and extension 1; non-compositional adjustment and the low complexity regions filter was disabled (Gustiananda *et al*., 2021). The homology of the vaccine constructed with the human gut microbiome was also analyzed to validate that the constructed vaccine does not disrupt the gut immune homeostasis mechanism. Non-homology analysis was performed by submitting vaccine construct sequences to the Pipeline Builder for Identification of drug Targets server (http://www.pbit.bicnirrh.res.in/index.php) with non-homology considered when E-value < 0.005 or identical percentage < 50% (Shende *et al*., 2016).

9. Immune Simulation

The immune response toward the vaccine model was simulated using the C-ImmSim server (https://kraken.iac.rm.cnr.it/C-IMMSIM/index.php?page=1). The simulation parameters were kept to the default except for the time steps in 1, 84, and 170, with 1050 total simulation phases. Hence, there were three injections at the interval of four weeks.

10. Docking TLR-4

The interaction between the vaccine & TLR4 was tested using molecular docking with HDOCK software (http://hdock.phys.hust.edu.cn/). HDOCK will provide information on the predicted binding affinity and 3d interaction of the vaccine & TLR4.

11. Plasmid construction

We utilized the SnapGene ver 3.2.1 to analyze codon optimization and cloning the plasmid. In this study, the expression vector pET-21(+) was used for cloning, and its sequences were obtained from the Addgene vector database.

Results

1. MSA and Conservancy Analysis

Result for Multiple Sequences Alignment (Supplementary folder)

Table 1. Conserved Peptide Sequences from Ag85A, Ag85B, & Ag85C.

Protein	Sequence

Ag85A	MQLVDRVGAVTGMSRRLVVGAVGAALVSGLVGAVGTTAGAFSRPGLPVEYL QSMGRDIKVQFQSGGANSPAYLLDGLRADDFGWINTPAFEWQSSVMPVGG QSSFSDWYQPACGKAGCTYKWETFLSELPGWLQAHKPTGSAVVGLSMSSA LTLAIYHPQQFYAGASGLLDSQAMGPTLILAGDAGGYKDMWGPKEDPWQR NDPLLNVGKLIANNTRWYCGNGKPLGGNLPAKFLEGFVRTSNKFQDAYNA GGGNGVFDFPDSGTHSWEYWGAQLNAKPDLQRLGATPNTGPQG
Ag85B	MTDVSRKIRAWGRRLMITAAAVLPGLVGLAGGAATAGAFSGLPVYLQPSMG RDIVQFQSGNNSPAVYLLDGLRAQDDYNGWITPAFEWYQSGLSMPVGGQSS FYSDWYSPACGKAGCTYKWELSLQWLANAVKPTGSAAGSMAGSSAMILA AYHPQQFIYGSLSLLDSQGMPSLIGLAMGDAGYKADMWGPSSPAWERNDPT QQIPLANRLYCGNGPNELGGNPAEFLENFRSSNLKFQDYNGGHNAVFNFPPN GTSWEYWAQLNAKGLSSLGG
Ag85C	MFFEQVRRLRSAATTLPRLAIAAMGAVLVYGLVGGGPATAAFSRPGLPVEYL QVPSASMGRDIVQFQGGGHAVYLLDGRAQDDYNGWITAFEYQSGLSVIMP VGGQSSFYTDWYQPSGQNYTYKWEFLTREPAWLQANKVPTGAAVGLSMSG GSALILAAYYPQFPYAALSGFLNPSEGWWPTLIGLANGGYNANSMWGPSDP AWKRNDPVQIPLVANTIVCGNGTPSDLGGDNPAKLGTLRTNQFRDTYADGG RNGFNFPPNGTHSWPYWEQLVAMKADIQVLNGATPPAA

Conserved peptide sequences were collected from MEGA software and used as bases for Epitope Generation.

2. Retrieval HLA of Indonesian Population as The Bases

We use the most predominant HLA Class I alleles in Indonesian population such as HLA-A*24:07 (20.7%), HLA-A*33:03 (16.9%), HLA-A*11:01 (16.4%), HLA-A*24:02 (14.4%), HLAB*15:13 (11.0%), and HLA-B*15:02 (10.7%). Meanwhile, the most predominant HLA Class II alleles of Indonesian population were HLA-DRB1*12:02 (36.8%), HLA-DRB1*15:02 (24.1%), and HLA-DRB1*07:01 (13.7%).

3. Epitope Generation and Immunogenicity Analysis

Table 2. Chosen Epitopes for Vaccine Construction

Peptide	Immunogenicity			Indonesia HLA Bind to Peptides
	Allergenicity	Toxicity	Antigenicity	
B-Cell Epitopes				
GDAGGYKDMWGPKEDP	Non-Allergen	Non-Toxin	Antigen	-
GLAGGAATAGAFSGLP	Non-Allergen	Non-Toxin	Antigen	-
GGHAVYLLDGRAQDDY	Non-Allergen	Non-Toxin	Antigen	-
HTL Epitopes				
PVEYLQSMGRDIKVQ	Non-Allergen	Non-Toxin	Antigen	DRB1_1502, DRB1_0701
GLPVEYLQVPSASMG	Non-Allergen	Non-Toxin	Antigen	DRB1_1202, DRB1_1502,

				DRB1_0701
PGLPVEYLQVPSASM	Non-Allergen	Non-Toxin	Antigen	DRB1_1202, DRB1_0701
CTL Epitopes				
GWITPAFEW	Non-Allergen	Non-Toxin	Antigen	HLA-A24:07, HLA-A24:02, HLA-B15:13
NERSSNLKE	Non-Allergen	Non-Toxin	Antigen	HLA-A24:07, HLA-A24:02, HLA-B15:02
YKWELSLQW	Non-Allergen	Non-Toxin	Antigen	HLA-A24:07, HLA-A24:02, HLA-B15:13

Epitope was chosen with criteria non-allergen, non-toxin, antigen, and minimal exist on 2 or 3 alleles of the population. The coverage population will be analyzed to estimate the coverage of vaccine candidate. Vaccine candidate must have coverage above 60% of the population.

4. Vaccine Candidate Construction

The vaccine (Figure 1) was constructed by joining 3 B-cell epitopes, 3 HTL epitopes, 3 CTL epitopes, and large ribosomal subunit protein bL12 from *Mycobacterium* using linkers such as GPGPG, AAY, GGGS, KK, and EAAAK respectively. Large ribosomal subunit protein bL12 was designed as an adjuvant and joined in construction at the N-terminal to increase the antigenicity of the vaccine candidate.

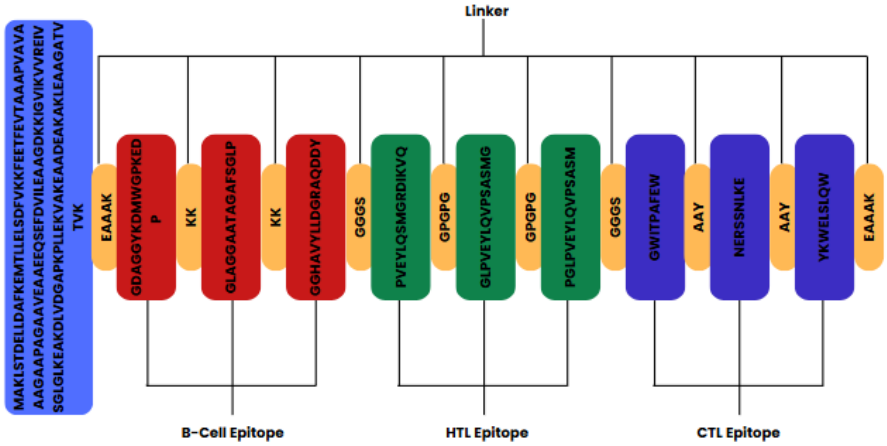


Figure 1. Vaccine Candidate Construction

5. Immunogenicity Analysis of Vaccine Candidate

The vaccine construct's immunogenic profile is summarized in the table below. The results indicate high antigenicity, non-allergenicity, and non-toxicity, making it a suitable candidate for vaccine development.

Table 3. Immunogenicity Analysis of Vaccine Candidate

Vaccine Construct	MAKLSTDELLDAFKEMTLLELSDFVKKFEETFEVTAAAPVAVAAA GAAPAGAAVEAAEEQSEFDVILEAAGDKKIGVIKVVREIVSGLGL KEAKDLVDGAPKPLLEKVAKEAADEAKAKLEAAGATVTVKEAA AKGDAGGYKDMWGPKEDPKKGLAGGAATAGAFSGLPKKGGHA

	VYLLDGRAQDDYGGGSPVEYLQSMGRDIKVQGPGPGGLPVEYL QVPSASMGGPGPGPGLPVEYLQVPSASMGGGSGWITPAFEWAYY NERSSNLKEAYYYKWELSLQWEAAAK
Parameters	**Results**
Antigenicity	Probable ANTIGEN (Overall prediction score: 0.4122)
Allergenicity	Probable NON-ALLERGEN
Allergen Similarity	Armadillo repeat-containing protein 5 (ARMC5), human protein classified NON-ALLERGEN
Toxicity	Non-Toxin

6. Population Coverage

The estimated population coverage of CTL and HTL epitopes was done with the IEDB tool, specifically targeting the Indonesian population. The list of coverage is shown in Table 4. (Supplementary text, below) CTL epitopes cover 75.50% of the population, while HTL epitopes cover 90.23%. Combined, the CTL and HTL epitopes provide 97.61% population coverage.

7. Physicochemical Characteristics of Vaccine Candidate

The physicochemical analysis provided insights into the stability and behavior of the vaccine candidate, as summarized in the below table.

Table 5. Physicochemical Characteristics of Vaccine Candidate

Protein	Molecular Weight (kDa)	isoelectric point (pI)	Estimated Half-Life	Instability Index	Aliphatic Index	GRAVY
Vaccine Construct	29,897.86	4.78	30 hours (mammalian); >20 hours (yeast); >10 hours (*E. coli*)	33.38 (Stable)	79.44	-0.192

The molecular weight indicates an appropriately sized protein for vaccine development, as it is within the range that supports effective immune recognition. The isoelectric point (pI) of 4.78 suggests that the protein has an acidic nature, which can enhance solubility and stability under physiological conditions. The estimated half-life across various systems confirms the vaccine's robustness and suitability for experimental applications, ensuring that it remains functional in mammalian, yeast, and bacterial cells.

The instability index also classifies the vaccine construct as stable, which is crucial for maintaining its structural integrity during production and storage. The high aliphatic index of 79.44 implies strong thermostability, ensuring the vaccine can withstand varying temperatures, a critical factor for distribution and storage. Lastly, the GRAVY score shown in the table reflects the hydrophilic nature of the construct, which supports solubility in aqueous environments and facilitates its formulation into injectable solutions.

8. Homology with Human Peptides and Gut Microbiome

Based on the homology analysis of HTL, CTL, and B-cell epitopes with human peptides, it is known that there are no 9-mers HTL, CTL, and B-cell epitopes that are 100% homologous with 9-mers of human peptides. Similarly, homology analysis of vaccine construction with the human gut microbiome showed that the constructed vaccine sequences had no homology with the human gut microbiome (E-value < 0.005).

9. Immune Simulation

The immune responses from candidate vaccines were built upon the initial injection, with the second and tertiary injections having a greater increase in immunoglobulin and immunocomplexes (Figure 2a), B cell population (Figure 2b), and T helper cell populations (Figure 2c). Immune simulations also indicate the involvement of a possible innate and cytokine-based regulation, with macrophages being active and an increasing IFN-γ level for a certain time period (Figure 2e, c).

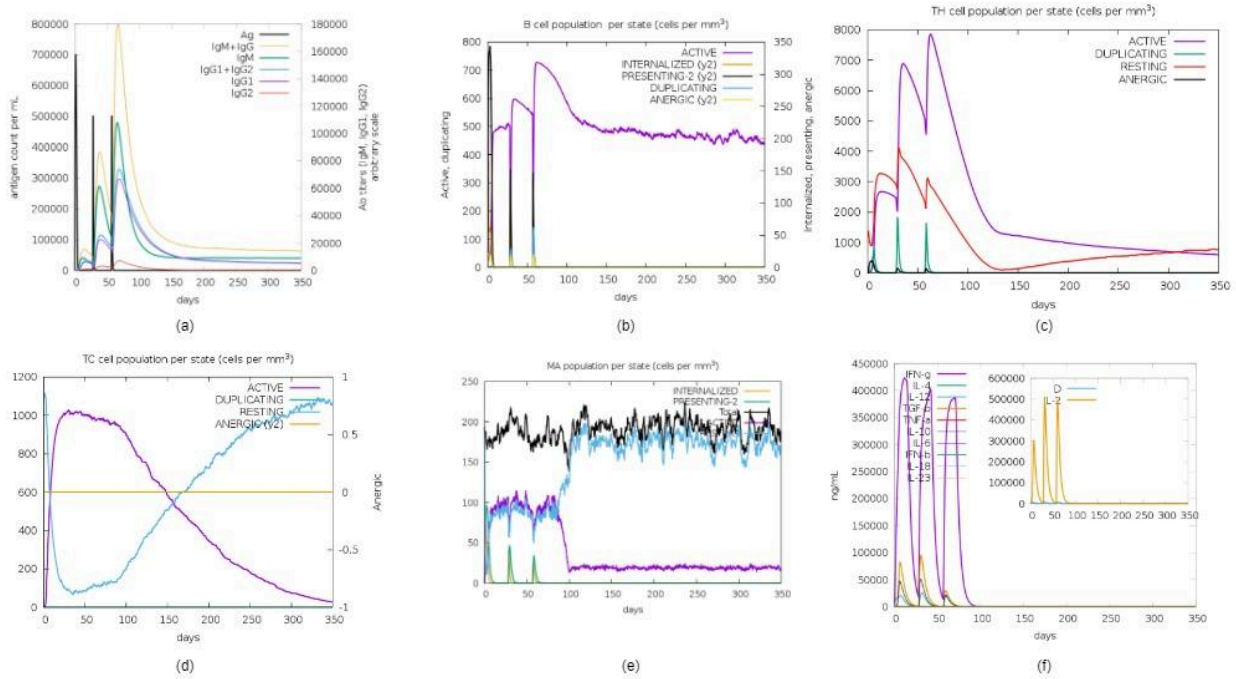
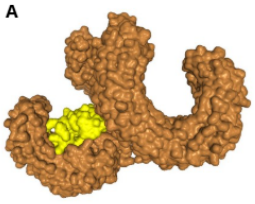


Figure 2. Vaccine immune simulation using C-ImmSim server. (a) Antigen and immunoglobulins, (b) B cell population (c) TH cell population, (d) TC cell population, (e) macrophage population, production of cytokine and interleukins with Simpson index (f) production of cytokine and interleukins with Simpson index.

10. Docking of Vaccine Candidate to TLR-4



B

Rank	1
Docking Score	-275.60
Confidence Score	0.9250
Ligand rmsd (Å)	63.54
Interface residues	model_1

Figure 3. Molecular docking of Vaccine Candidate with TLR-4. (a) bind between VC and TLR-4, (b) Docking score between VC with TLR-4.

The molecular docking results indicate a possible interaction between the vaccine with TLR4. This is indicated by the binding affinity value which is quite strong (-275.60 kcal in model I vaccine & TLR4 (Figure 3b). The 3d structure shows the possibility of a good interaction between the vaccine and TLR4, which is characterized by the visualization of the bond between the vaccine and the binding site of TLR4 (Figure 3a).

11. Plasmid construction

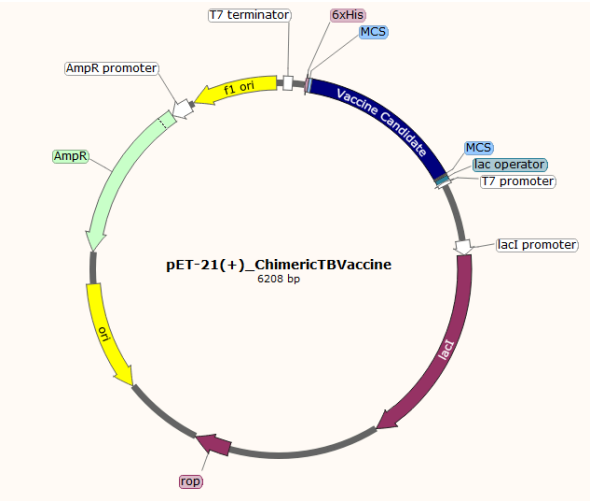


Fig 4. Vaccine Construction in Plasmid pET-21(+)

The plasmid was constructed with SnapGene Ver 3.2.1 using plasmid pET-21(+). The sequence was codon optimized to *Escherichia coli* B and inserted on a plasmid with the T7 RNA polymerase expression system. The total length of the plasmid with sequences is 6208 bp.

Discussion

The Ag85 complex represents a 30-32 kDa family of three proteins (Ag85A, Ag85B, and Ag85C), each of which has enzymatic activity mycolyl-transferase activity involved in the incorporation of mycolic acid to cell wall arabinogalactan and in the biogenesis of cord factor. These proteins are also known for their ability to bind the extracellular matrix protein fibronectin (Huygen, 2014). The rationale behind the selection of the ag85 complex as a target protein in vaccine epitope determination in this paper is due to its ability to induce a strong th1-type immune response and by virtue of its role in cell wall integrity and cord factor synthesis, this abundantly expressed protein has long been considered a virulence factor (Romano, *et al*., 2006). In this current study, we employed large ribosomal subunit protein bL12 as an adjuvant to enhance the immunogenicity of the formulated vaccine (Sethi, *et al*., 2024).

HLA molecules determine the repertoire of peptides presented to T-cells, influencing immune responses. Due to the polymorphic nature of HLA, different alleles are prevalent in different populations, affecting vaccine efficacy (Bui *et al*., 2006). By analyzing HLA allele frequencies, vaccines can be designed to include epitopes that bind to the most common HLA alleles in a target population, thereby maximizing the number of individuals who can retrieve an effective immune response (Liu *et al*., 2020). Therefore, in this study, we aimed to identify the T-cell epitopes from Mtb to be used in the vaccine formulation considering the predominant HLA alleles in Indonesia. The most predominant HLA alleles in the Indonesian population were HLA-A24:07, HLA-A33:03, HLA-A11:01, HLA-A24:02, HLA-B15:13, HLA-B15:02 from Class I and HLA-DRB1_1202, HLA-DRB1_1502, HLA-DRB1_0701 from Class II. The selected epitopes from these alleles were then analyzed for population coverage to determine the percentage of individuals in a population whose HLA alleles can present the epitopes of the selected antigens in the vaccine. Selectivity and population coverage are the principal advantages of this vaccine because it can be used to design specific vaccines and create herd immunity against Mtb in certain target populations. The population coverage expected to create herd immunity in a population is about 60-90% (Plans-Rubió, 2022). Based on the results obtained, the 3 epitopes selected from Class I had a population coverage of 75.50% and 3 epitopes from Class II of 90.23% with a total combination of both at 97.61% This shows that the selected

epitopes are able to cover almost the entire population and are expected to create herd immunity in the Indonesian population, making them excellent candidates for the Tb vaccine construction. Moreover, each of the selected epitopes was evaluated for its characteristics as antigens to stimulate an immune response. In this study, we further tested the allergenicity, toxicity, and antigenicity characteristics for screening peptides as vaccine construction candidates. The results obtained indicate that the nine selected proteins are non-allergenic and non-toxic to vaccine recipients while having high antigenicity.

Physicochemical characteristics of vaccine constructs are critical in determining vaccine immunogenicity and efficacy. Further analysis was conducted to determine the physicochemical characteristics of the constructed vaccines, which included molecular weight (Da), isoelectric point (pI), estimated half-life, instability index, aliphatic index, and GRAVY score. Vaccine particle size can significantly affect their uptake, processing, and presentation by antigen-presenting cells, and correlates to the strength of the resulting immune response. Smaller particle sizes are generally more efficiently absorbed by cells (Benne *et al.*, 2016). The candidate vaccine construct has a molecular weight of 29,897.86 kDa. This indicates an appropriate protein size for vaccine development, as it is within the range that supports effective immune recognition. Isoelectric points close to the pH of blood and body fluids tend to favor the ability of vaccines designed for delivery by injection (Negahdaripour *et al*., 2018). This vaccine construct has a pI of 4.78 which tends not to be too far from the pH of blood and body fluids. Its acidic properties also tend to increase solubility and stability under physiological conditions. The estimated half-life in various systems confirms the robustness and suitability of the vaccine for experimental applications, confirming that the vaccine remains functional in mammalian, yeast, and bacterial cells. It also indicates that vaccine constructs can be synthesized using these cell systems (Gustiananda *et al*., 2021). The stability index classifies vaccine constructs as stable, which is critical for maintaining their structural integrity during production and storage. The high aliphatic index of 79.44 implies strong thermostability, ensuring the vaccine can withstand varying temperatures, which is an important factor for distribution and storage. Gravy is a measure of the hydrophobicity or hydrophilicity of a molecule. A negative result on the Gravy score indicates that the vaccine construct exhibits properties that are hydrophilic in nature which favors solubility in aqueous environments and facilitates its formulation into injectable solutions (Gasteiger *et al*., 2005).

Non-homologous testing in vaccine constructs is critical to ensure that vaccine components do not inadvertently mimic human peptides or microbiome elements, which could cause adverse immune reactions or reduce vaccine efficacy. Vaccine constructs were analyzed to ensure that HTL, CTL, and B-cell epitopes had no homology with human peptides and human gut microbiomes. A non-homologous assay was performed to find 9-mer peptide sequences in the human proteome that match the selected peptides. The 9-mer (residues 4-12 of a 15-mer) was used because it is the core component of the peptide whose side chain interacts with HLA molecules and interacts with T-cell receptors (Gustiananda *et al*., 2021; Sethi *et al.*, 2024). Based on the results, none of the HTL, CTL, and B-cell 9-mers had 100% sequence similarity to peptides from human peptides and the vaccine constructs had no significant similarity to the human gut microbiome as revealed by analysis using PBIT (E-value < 0.005). This means that the vaccine construct will not interfere with host immune homeostasis. Based on the physicochemical characteristics and homology of the vaccine construct from the assay, it can be assumed that the preliminary screening of the vaccine construct is judged to be safe, can induce a

sufficient immune response against Tb, and has the potential to be a promising Tb vaccine candidate.

All selected epitopes were joined using linkers (GPGPG, AAY, GGGS, KK, and EAAAK) to enhance protein folding and flexibility. Each linker held a specific function for the efficacy of the vaccine. The GPGPG linker minimizes junctional immunogenicity, restoring individual epitope activity, while EAAAK ensures proper spacing between adjuvants and epitopes, preserving their functionality (Chen *et al*., 2013). Protein-protein docking showed the vaccine's strong affinity for TLR-4, forming multiple H-bonds and non-polar interactions. In *M. tuberculosis* infection. Numerous studies have also shown a positive correlation between TLR-4 expression with protective function against Mtb infection (Agustin *et al*., 2023; Biyikli *et al*., 2016; Park *et al*., 2020). Immune simulations further confirmed the vaccine's ability to elicit robust B and T cell responses, and also cytokines production such as IFN-γ, which is the primary mediator that activates macrophages, driving the production of reactive nitrogen species, particularly nitric oxide (NO), to inhibit M. *tuberculosis* growth (Shanmuganathan *et al*., 2022).

We visualized the result of the in-silico cloning vaccine on plasmid pET-21(+) (Fig. 4). The plasmid was utilized for the construction of a protein and DNA expression vector incorporating a T7 RNA polymerase as an expression system using SnapGene Ver 3.2.1. The total length size of the construct is 6208 bp. In addition, the genes of interest are depicted in position after lac operator.

The vaccine construct in this study has the potential for application in controlling and preventing Tb. With 97.61% population coverage based on the prevalence of HLA alleles in the Indonesian population, this vaccine is capable of eliciting effective immune responses in the majority of the population. This makes it highly relevant for use in Indonesia, which has a high rate of Tb. The selectivity epitopes for the dominant HLA alleles in Indonesia increase the likelihood of successful antigen presentation to T-cells in the target population. This approach not only enhances the effectiveness of vaccines but also reduces the possibility of failure of immunization due to incompatibility with HLA.

The immunogenicity analysis of vaccine results explains if this vaccine is immunogenic and affirms this vaccine's safety and potential for broad implementation. From the physicochemical characteristics, the vaccine is suitable for distribution and storage. This statement is based on characteristics such as a high aliphatic index and is classified as stable with a negative GRAVY score. These characteristics ensure that the vaccine can withstand temperature variations typical of distribution chains and enable effective formulation in injectable solutions. So, it's ideal for large-scale immunization.

Conclusion

This study successfully designed a multi-mer vaccine construct targeting Tb that is tailored to the Indonesian population, achieving a high population coverage of 97.61% by incorporating dominant HLA alleles. This vaccine construct demonstrates the high immunogenicity, safety, and reducing the risk of adverse immune reactions. Furthermore, the proposed vaccine also has the potential for broad-scale immunization for the Indonesian population due to its characteristics. This vaccine holds promising potential to support global efforts in eradicating Tb cases through targeted immunization for specific populations with a high effective type of vaccine candidate. To ensure the applicability of the vaccine, further steps such as clinical trials and validation of safety and efficacy with wet-lab-based research will be essential to ensure its success on a large population scale.