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Research

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In silico prediction of multi-epitope vaccine candidates against *Mycobacterium leprae*

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Abstract

Background Leprosy, also known as Hansen's disease, is an infectious disease caused by *Mycobacterium leprae*. Despite ongoing efforts to control the disease, leprosy remains a global health concern, with Indonesia ranking third in the world for the highest number of cases.

Objective This study aims to identify epitopes that can induce T and B cell immune responses through an in silico approach, to design a multi-epitope vaccine candidate against *Mycobacterium leprae*.

Methods The study used an in silico vaccine design approach utilizing ESAT6, Ag85B, ML2028, ML2380, and ML2055 proteins from *Mycobacterium leprae*. The process involved sequence alignment, T cell (CTL and HTL) and B cell epitopes identification, and antigenicity, allergenicity, and toxicity assessment. Selected epitopes were constructed into a multi-epitope vaccine candidate using linkers. The tertiary structure of the vaccine was modeled with AlphaFold and evaluated via Prosa-web. The stability and interaction between the vaccine candidate and TLR4 were analyzed using molecular docking.

Results The vaccine candidate demonstrated stable interactions with TLR4, with a binding free energy of -13.9 kcal/mol. The vaccine candidate was also predicted to be stable, antigenic, non-allergenic, non-toxic, and hydrophilic.

Conclusion This in silico design of a multi-epitope vaccine candidate shows potential for development as a vaccine against leprosy.

Keywords in silico, leprosy, multi-epitope, *Mycobacterium leprae*, vaccine candidate

Introduction

Leprosy, caused by *Mycobacterium leprae*, is a chronic infectious disease affecting the skin, peripheral nerves, and other tissues. Without early treatment, it leads to permanent disabilities (Hernani *et al.*, 2007). Leprosy remains a global public health issue, affecting over 120 countries, with more than 10,000 new cases reported annually. Indonesia ranks third globally in terms of new leprosy cases, following India and Brazil, with 12,443 cases reported in 2022 (WHO, 2023).

The Indonesian Ministry of Health (Kemenkes RI) has set to eliminate leprosy. Current treatment relies on multi-drug therapy (MDT), combining antibiotics administered over 6 to 18 months (Kemenkes, 2022). However, prolonged treatment duration and drug side effects lead to non-compliance, increasing the risk of MDT-resistant strains. As new cases persist, more effective interventions, such as vaccination, are urgently needed to support elimination efforts. Vaccination aligns with Indonesia's health transformation agenda, including developing pharmaceutical products, vaccines, and medical devices to strengthen national health resilience (Kemenkes, 2024).

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Systematic reviews have found that the Bacillus Calmette-Guérin (BCG) vaccine, primarily used for tuberculosis (TB), provides partial protection against leprosy (Coppola *et al.*, 2018). The *Mycobacterium indicus pranii* (MIP) vaccine, developed in India, demonstrated only 39% efficacy in clinical trials (Reed *et al.*, 2016). Another promising candidate, LepVax, is currently in preclinical trials, but its safety profile remains uncertain (Duthie *et al.*, 2018).

Several proteins expressed by *M. leprae*, including ML2028, ML2380, and ML2055, are secretory proteins involved in the pathogenesis and immune response during infection. These proteins elicit specific cellular and humoral responses in leprosy patients (Sampaio *et al.*, 2011). Geluk *et al.* (2002) identified L-ESAT-6, a homolog of *M. tuberculosis* ESAT-6 (T-ESAT-6), with a 63% homology between the two proteins. Efforts to develop new vaccines for TB have focused on Ag85B and ESAT-6 proteins. Preclinical studies in animal models, such as mice and rabbits, have shown that the Ag85B-ESAT6 fusion protein offers protective immunity against TB, and this cross-reactivity suggests potential protection against leprosy (Coppola *et al.*, 2018).

In silico epitope-based and multi-epitope vaccine design, utilizing immunoinformatic and bioinformatics, offers a promising approach for identifying potential vaccine candidates. Reverse vaccinology, combined with bioinformatics tools, enables the prediction of epitopes and the identification of promising peptide candidates for vaccine development (Goodswen *et al.*, 2023). While in silico models cannot guarantee vaccine efficacy, preliminary studies using these methods can expedite the identification of promising targets for further in vitro and in vivo validation (Awad-Elkareem & Salih, 2017). For example, Ysrafil *et al.* (2022) successfully designed a multi-epitope vaccine from HABA and L7/L12 proteins, which activated both cellular and humoral immune responses in silico. This methodology could be similarly applied in developing a multi-epitope vaccine for *M. leprae*. In this study, we focused on predicting epitopes from *Mycobacterium leprae* proteins to develop a multi-epitope vaccine candidate using an in silico approach.

Methods

Study location, duration, and design

The study was conducted at the Department of Medical Laboratory Technology, Politeknik Kesehatan Kemenkes Bandung, from September to October 2023. This research employed a quasi-experimental design performed in silico, using data and tools from various web servers. The study population consisted of *Mycobacterium leprae* protein sequences, with the specific samples being the partial sequences of proteins ESAT6 (OAR19522.1, OAR19682.1, OAR20282.1, OAX70072.1, OAX70580.1, OAX71810.1), Ag85B (P46842.2, P31951.2), ML2028 (P31951.2), ML2380 (Q9CB68, CAC31896.1), and ML2055 (P46842.2, CAC31010.1).

Protein sequence retrieval

Protein sequences of *Mycobacterium leprae* were retrieved from GenBank at the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/) and UniProt (https://www.uniprot.org/) databases (NCBI Resource Coordinators, 2018; Magrane & UniProt Consortium, 2011). These sequences were aligned using the MAFFT server (https://mafft.cbrc.jp/alignment/server/) to identify sequence homology (Kuraku *et al.*, 2013; Katoh *et al.*, 2019). The consensus sequence was then generated using the EMBOSS Cons web server (https://www.ebi.ac.uk/Tools/msa/emboss_cons/).

T-cell (CTL and HTL) epitope prediction

Cytotoxic T lymphocyte (CTL) epitopes were predicted from the consensus sequence using the NetCTL 1.2 server (https://services.healthtech.dtu.dk/service.php?NetCTL-1.2), which predicts 9-mer epitopes across 12 MHC class I supertypes (Larsen *et al.*, 2007). Epitopes were selected with a threshold score of 0.75. For helper T lymphocyte (HTL) epitopes, predictions were made using NetMHCII 2.3 (https://services.healthtech.dtu.dk/service.php?NetMHCII-2.3), targeting HLA-DR, HLA-DQ, and HLA-DP alleles with strong (2%) and weak (10%) binding affinities (Jensen *et al.*, 2018).

Antigenicity, allergenicity, and toxicity prediction

To further validate the immunogenic potential of predicted CTL and HTL epitopes, antigenicity was predicted using VaxiJen v2.0 (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) with a bacterial target threshold of 0.4 (Doytchinova & Flower, 2007). Allergenicity was evaluated using AllerTop (https://www.ddg-pharmfac.net/AllerTOP/) (Dimitrov *et al.*, 2014), and toxicity predictions were made using ToxinPred (https://webs.iitd.edu.in/raghava/toxinpred/protein.php), which leverages SVM-based algorithms (Gupta *et al.*, 2013).

B-cell epitope prediction

B-cell epitopes were predicted using the Immune Epitope Database (IEDB) server (http://tools.iedb.org/bcell/) with the Bepipred Linear Epitope Prediction method and a threshold of 0.5 (Jespersen *et al.*, 2017).

Multi-epitope vaccine construction

The selected CTL, HTL, and B-cell epitopes were assembled into a multi-epitope vaccine construct. The CTL epitopes were linked using AAY linkers (Bhatnager *et al.*, 2020), HTL epitopes with GPGPG linkers (Livingston *et al.*, 2002), and B-cell epitopes using KK linkers (Nain *et al.*, 2020). A 6xHis tag was added at the C-terminus for purification purposes (Ayyagari *et al.*, 2022).

Evaluation and characterization of physicochemical properties

The physicochemical properties of the constructed multi-epitope vaccine were evaluated using ProtParam (https://web.expasy.org/protparam/) to predict molecular weight, theoretical pI, instability index, aliphatic index, and GRAVY score (Wilkins *et al.*, 1999). The solubility of the protein in water was predicted using SOLpro (https://scratch.proteomics.ics.uci.edu/).

Secondary and tertiary structure prediction

The secondary structure of the vaccine construct was predicted using SOPMA (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma), while the tertiary structure was predicted using AlphaFold (https://colab.research.google.com/github/deepmind/alphafold/blob/main/notebooks/AlphaFold.ipynb#scrollTo=XUo6foMQxwS2) or I-TASSER (https://seq2fun.dcmb.med.umich.edu/I-TASSER/) (Jumper *et al.*, 2021; Roy *et al.*, 2010). The accuracy of the predicted 3D structure was evaluated using the C-score and validated using ProSa (https://prosa.services.came.sbg.ac.at/prosa.php) (Wiederstein & Sippl, 2007).

Molecular docking

Molecular docking was performed using HADDOCK2.4 (https://wenmr.science.uu.nl/haddock2.4) to evaluate interactions between the multi-epitope vaccine and the TLR4 receptor (PDB ID: 4G8A). The best docking models were selected based on the lowest HADDOCK score, indicating the most favorable receptor-ligand interaction. Binding energies were calculated to determine the spontaneity of the interactions.

Recombinant plasmid construction

The nucleotide sequence for the vaccine was optimized using J.Cat (http://www.jcat.de/) to ensure effective expression in an expression vector (Grote *et al.*, 2005). The optimized sequence, codon adaptation index (CAI), and GC content were generated. Recombinant plasmid construction was designed using SnapGene.

Results

Protein sequence retrieval

Protein sequences of ESAT6 (OAR19522.1, OAR19682.1, OAR20282.1, OAX70072.1, OAX70580.1, OAX71810.1), Ag85B (P46842.2, P31951.2), ML2380 (Q9CB68, CAC31896.1), ML2055 (P46842.2, CAC31010.1), and ML2028 (P31951.2) were retrieved from the NCBI and UniProt databases. The sequences were aligned using the EMBOSS web server, generating a consensus sequence (**Table 1**). The consensus sequence was utilized for subsequent epitope prediction.

T cell epitope (CTL and HTL) prediction

Four CTL and four HTL epitopes were selected based on the highest antigenicity scores, non-allergenicity, and non-toxicity predictions (**Table 2**). These predicted epitopes were used for constructing the multi-epitope vaccine.

B Cell Epitope Prediction

B cell epitopes were predicted which epitope can stimulate a robust humoral immune response. Four B cell epitopes were identified based on their antigenicity scores (**Table 3**).

Table 1 Consensus sequences of aligned protein data

Protein	GenBank ID	Consensus sequences
ESAT6	OAR19522.1 OAR19682.1 OAR20282.1 OAX70072.1 OAX70580.1 OAX71810.1	>EMBOSS0001 MTAAHFMTDPQAMRDMARKFDMHAQNVRDESHKMFMSSMDIAGAGWSGTAQLTSHDTMGQINQAFRHI VTLLQDVRDQLGTAADRYEHQEENSRKILSGS
Ag85B &ML2028	P46842.2 P31951.2	>EMBOSS0001 MNQVDLDSTHRKWGLWAILAIAVVASASAFTMPLPAAANADPAPLPPSTATAAPSPAQEIITPLPGAPVSSEAQ PGDPNAPSLDPNAPYPLAVDPNAGRITNAVFEWYYQGGFSFVLPAGWVESEASHLDYGSVLLSKAGCTTYK WETFLTIEQPPVLGQPTVVATDTRIVLGRSMAGLDQKLYASAEADNIKAAVRLGSDMGEFYLPYPGTRINQET IPLHANGIAGSASYYEVKDMFSDPNKPIGQICTSVVGSPAASTPDVGPSQRWFVVWLGTSNNPVDKGAAKEL AESIRSFVHGSNLKFQDAYNGAGGHNAVFNLNADGTHSWEYWGAQLNEMAPDIPASVSAPAPVG
ML2380	Q9CB68 CAC31896.1	>EMBOSS0001 MSRLSTSLCKGAVFLVFGIIPVAFPTTAVADGSTEDFPIPRRQIATTCDAEQYLAAVRDTSPIYYQRYMIDMHNK PTDIQQAAVNRIHWFYSLSPTDRRQYSEDTATNVYYEQMATHWGNWAKIFFNNKGVVAKATEVCNQYQAG DMSVWNWP
ML2055	P46842.2 CAC31010.1	>EMBOSS0001 MNQVDLDSTHRKGLWAILAIAVVASASAFTMPLPAAANADPAPLPPSTATAAPSPAQEIITPLPGAPVSSEAQPG DPNAPSLDPNAPYPLAVDPNAGRITNAVGGFSFVLPAGWVESEASHLDYGSVLLSKAIEQPPVLGQPTVVATD TRIVLGRLDQKLYASAEADNIKAAVRLGSDMGEFYLPYPGTRINQETIPLHANGIAGSASYYEVKFSDPNKPIG QICTSVVGSPAASTPDVGPSQRWFVVWLGTSNNPVDKGAAKELAESIRSEMAPIPASVSAPAPVG

Construction of the multi-epitope vaccine protein

The selected CTL, HTL, and B cell epitopes were assembled into a single vaccine construct. The CTL epitopes were linked using the AAY linker, HTL epitopes with the GPGPG linker, and B cell epitopes with the KK linker. A 6xHis tag was added to the C-terminus to facilitate purification (**Figure 1**). The final construct represents a candidate multi-epitope vaccine.

Physicochemical evaluation and characterization of the multi-epitope vaccine

The antigenicity of the constructed multi-epitope vaccine was re-evaluated using the VaxiJen v.2.0 server, yielding an antigenicity score of 0.8242, indicating strong antigenic properties. Allergenicity was re-assessed using AllerTop, confirming the vaccine is non-allergenic. The result of the physicochemical evaluation of the multi-epitope vaccine is shown in **Table 4**. Solubility was analyzed using the SOLpro web server, which predicted that the protein is soluble with a probability of 0.9642.

Secondary and tertiary structure prediction of the vaccine protein

The secondary structure prediction revealed that the vaccine protein consists of 16.67% beta strands, 22.9% alpha helices, and 60.42% coils (**Figure 2**). The tertiary

Table 2 Prediction of T cell epitopes (CTL and HTL)

T Cell Epitope	Protein	Epitope	Antigenicity	Allergenicity	Toxicity
CTL	ESAT6	QLGTAADRY	1,0606 (Antigen)	Non-allergen	Non-toxin
	Ag85B	GAGGHNAVF	1,6272 (Antigen)	Non-allergen	Non-toxin
	ML2380	SEDTATNVY	1,3050 (Antigen)	Non-allergen	Non-toxin
	ML2055	GRLDQKLYA	1,2273 (Antigen)	Non-allergen	Non-toxin
HTL	ESAT6	SSMDIAGAGWSGTAQ	1,1433 (Antigen)	Non-allergen	Non-toxin
	Ag85B	VFEWYYQGGFSFVLP	1,2235 (Antigen)	Non-allergen	Non-toxin
	ML2380	TDRRQYSEDTATNVY	1,0664 (Antigen)	Non-allergen	Non-toxin
	ML2055	ANGIAGSASYYEVKF	1,1568 (Antigen)	Non-allergen	Non-toxin

Table 3 Prediction of B cell epitopes

Protein	B Cell Epitope
ML2055	AANADPAPLPPSTATAAPSPAQEIITPLPGAPVSSEAQPGDPNAPSLDPNAPYPLAVDPNAGRITNAV
ML2380	LSPTDRRQYSEDTATNVYYEQMATH
Ag85B & ML2028	SEASHLDYGSVLLSKAGCTTYK
ESAT6	IAGAGWSGTAQLTSHDT

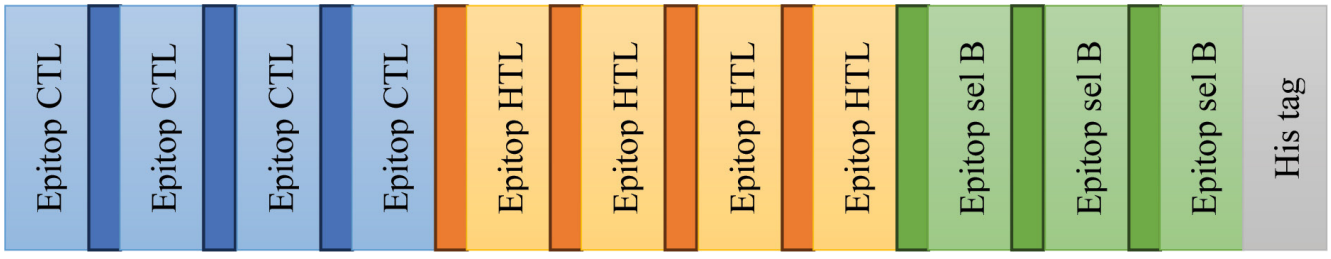

Figure 1 Structural arrangement of the multi-epitope vaccine candidate protein. This figure illustrates the design and arrangement of epitopes and linkers in the multi-epitope vaccine construct. *Linker* AAY (■), *linker* GPGPG (■), dan *linker* KK (■).

Table 4 Physicochemical properties of the vaccine candidate

Parameter	Value
Number of amino acids	240
Molecular weight	24,726.12 Da
Theoretical pI	6,12
Half-life	30 hours (mammalian reticulocytes, *in vitro*), >20 hours (*yeast, in vivo*), and >10 hours (*E. coli, in vivo*).
Instability index	32.79 (Stable protein)
Aliphatic index	57.58
GRAVY (Hydropathy index)	-0.470

structure was validated using the ProSA web server, which returned a z-score of -2.36, indicating good overall model quality (**Figure 3**).

Molecular Docking

Molecular docking was performed to evaluate the interaction between the candidate vaccine protein and the TLR4 receptor, which plays a crucial role in the innate immune response. The molecular docking analysis of the vaccine and TLR4 are shown in **Figure 4** with a binding affinity value of -13.9 kcal/mol, indicating strong binding potential (**Table 5**). This interaction suggests that the multi-epitope vaccine could potentially elicit an effective immune response via TLR4 signaling.

Table 5 Binding free energy and dissociation constant (Kd) prediction

Parameter	Value
ΔG (kcal/mol^{-1})	-13.9
Kd (M) at °C	6.9e^{-11}
ICS charged-charged	22
ICS charged-polar	30
ICS charged-apolar	18
ICS polar-polar	13
ICS polar-apolar	28
ICS apolar-apolar	5
NIS charged	23.03
NIS apolar	35.3

Figure 2 Secondary structure of the vaccine candidate. In the secondary structure visualization, red denotes beta strands, blue denotes alpha helices, and yellow denotes coils.

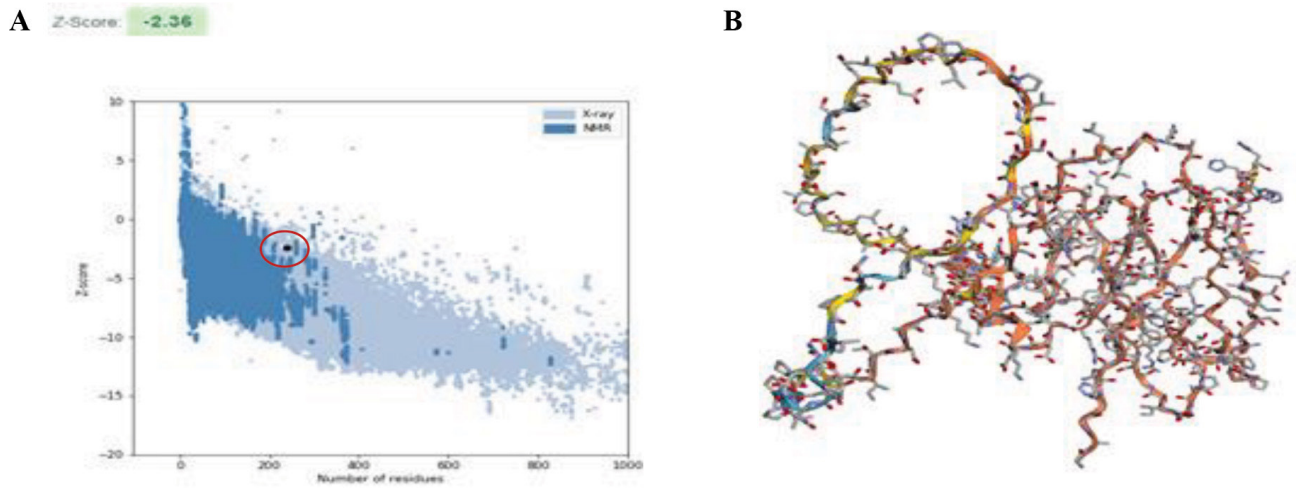

Figure 3 Tertiary structure of the vaccine candidate and z-score validation. (A) z-score of -2.36, indicating good model quality. (B) Tertiary structure of the multi-epitope vaccine candidate.

Recombinant Plasmid Construction

Codon optimization was performed using the J.Cat server to check the optimization of gene expression in *Escherichia coli*. The optimized nucleotide sequence exhibited a Codon Adaptation Index (CAI) of 1.0 and a GC content of 57.36%, indicating ideal conditions for bacterial expression. The pET-28a plasmid was selected for in silico cloning, facilitated by SnapGene software. EcoRI and XhoI restriction enzymes were used to insert the target gene into the multiple cloning site (MCS) of the plasmid (**Figure 5**).

Discussion

This study aimed to design a potential vaccine candidate against leprosy using a reverse vaccinology approach. Reverse vaccinology identifies specific epitopes that elicit cellular and humoral immune responses (Awad-Elkareem & Salih, 2017). The proteins selected for this study (ESAT6, Ag85B, ML2028, ML2380, and ML2055) are crucial for Mycobacterium leprae's pathogenesis and structural integrity.

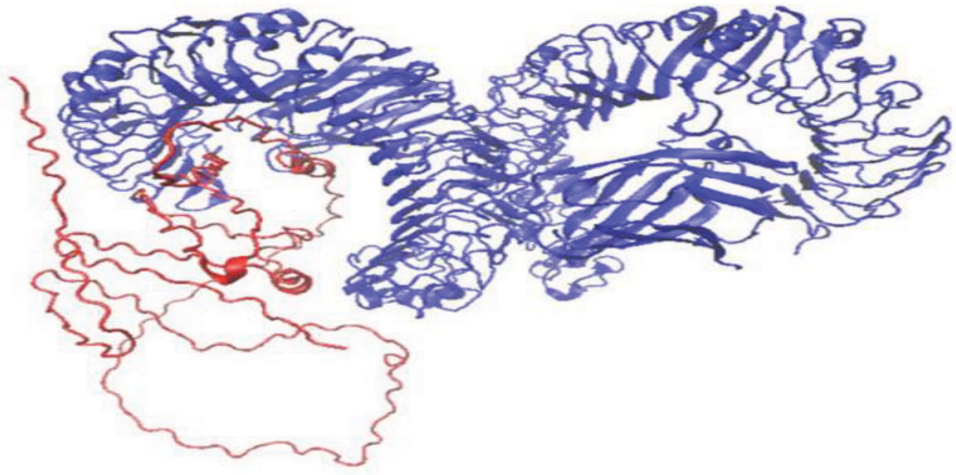

Figure 4 Molecular docking results of the vaccine candidate with TLR4 receptor. The vaccine is represented in red, and the TLR4 receptor in blue. The docking visualization uses a new cartoon representation highlighting secondary structures.

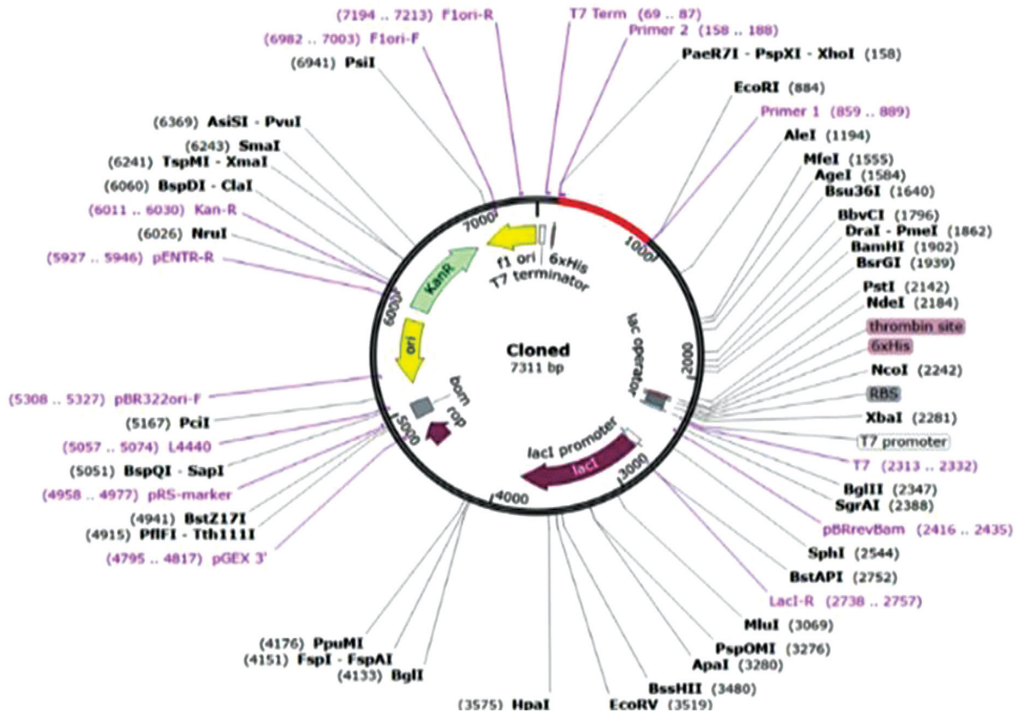

Figure 5 In silico cloning results of the vaccine candidate in *E. coli* (strain K-12). The vaccine component is indicated in red, cloned into the pET-28a expression vector, showing the successful insertion of the vaccine gene.

ESAT6 is an extracellularly secreted protein with a molecular weight of 6 kDa, playing a pivotal role in the pathogenicity of leprosy (Hadi & Kumalasari, 2017). ML2028, part of the Ag85 complex, is a crucial enzyme for trehalose monomycolate (TMM) and trehalose dimycolate (TDM) synthesis, essential components for the cell wall's biogenesis and integrity (Brennan & Spencer, 2019). ML2055 is a surface-associated protein that binds fibronectin, aiding bacterial adherence and internalization (Soares *et al.*, 2021). These proteins were selected due to their immunogenic properties and potential as vaccine targets.

Thirteen protein sequences were retrieved, and epitope prediction was conducted to identify epitopes capable of being recognized by B cells, cytotoxic T lymphocytes (CTLs), and helper T lymphocytes (HTLs). The recognizable by immune cells is crucial for generating a robust immune response involving both humoral and cellular immunity. The epitopes were selected for their ability to interact with MHC class I and MHC class II molecules, essential for effective antigen presentation and subsequent T cell activation.

Linker sequences, such as AAY, GPGPG, and KK, play a critical role in enhancing epitope presentation, vaccine stability, and overall antigenicity (Livingston *et al.*, 2002; Tahir ul Qamar *et al.*, 2020). An overlapping sequence, SEDTATNVY, was identified for both CTL and B cell epitopes, enabling dual induction of cellular and humoral responses. A HisTag was added to the C-terminus to facilitate the purification of the vaccine protein (Ayyagari *et al.*, 2022).

The physicochemical evaluation revealed that the designed vaccine has a molecular weight of 24,726.12 Da and an isoelectric point (pI) of 6.12, indicating that the protein is moderately acidic. Proteins with a molecular weight below 110 kDa are typically easy to purify, enhancing their applicability in vaccine development (Naz *et al.*, 2015). Additionally, the aliphatic index of 57.58 suggests that the vaccine protein is thermally stable, while the stability index of 32.79 classifies it as stable (Enany, 2014). The GRAVY score of -0.47 indicates that the vaccine is hydrophilic, facilitating interaction with the immune system in aqueous environments. The solubility prediction confirmed that the vaccine has excellent solubility with a probability of 0.964 (Magnan *et al.*, 2009).

Structural analysis indicated that the vaccine comprises 16.67% beta strands, 22.9% alpha helices, and 60.42% coils, reflecting a balanced secondary structure. The z-score of -2.36 from tertiary structure validation confirms that the vaccine's overall structure is highly quality, aligning well with native protein structures (Wiederstein & Sippl, 2007).

Molecular docking results showed a high binding affinity between the vaccine and TLR4, with a free energy (ΔG) of -13.4 kcal/mol. This strong interaction suggests the vaccine may effectively stimulate TLR4-mediated innate immune responses. The low dissociation constant (Kd of $6.9e^{-11}$) further supports the stability of the vaccine-TLR4 complex under physiological conditions (Tedjokusumo *et al.*, 2023).

Codon optimization for expression in E. coli yielded a CAI of 1.0 and a GC content of 57.36%, within the ideal range for efficient transcription and translation (Narula *et al.*, 2018). The gene encoding the vaccine was successfully inserted into the pET-28a plasmid using EcoRI and XhoI restriction sites, confirmed by in silico mapping using SnapGene (Puigbò *et al.*, 2008). These restriction enzymes were selected for their compatibility with the plasmid's multiple cloning site (MCS) and their positioning relative to the ribosome binding site (RBS), ensuring efficient translation (Shilling *et al.*, 2020).

Conclusion

The in silico design of the multi-epitope vaccine identified several promising epitopes that could serve as alternative candidates for a leprosy vaccine. The designed vaccine demonstrated stable, antigenic, non-allergenic, non-toxic, and hydrophilic properties, critical for inducing a robust immune response. Additionally, molecular docking analysis revealed strong binding affinity between the vaccine and TLR4, with a binding free energy of -13.4 kcal/mol, suggesting its potential efficacy in stimulating the innate immune system. These findings provide a solid foundation for further experimental validation, positioning the predicted multi-epitope construct as a promising candidate for leprosy vaccine development.

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Conflict of interest The author declares no conflict of interest in this research.

Author contribution AS and AINI conceptualized the study design. AS conducted the research and drafted the manuscript, while AINI supervised the research and validated the findings. SFR contributed to the methodology design, and FM assisted with analyzing the research results and the overall research process.

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