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In Silico Identification of Immunogenic ERv 53-63 Epitope; A Potential Candidate for Tuberculosis Vaccine Design

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ABSTRACT

Background and Aim: Tuberculosis (TB), caused by *Mycobacterium (M.) tuberculosis* (MTB), remains a major global health burden and the Bacillus Calmette–Guérin (BCG) vaccine shows limited efficacy, particularly in adults. This highlights the urgent need for novel vaccine strategies. In silico approaches offer efficient, cost-effective tools for predicting immunogenic epitopes, thereby accelerating peptide-based vaccine development. This study aimed to identify and characterize the ERv 53–63 epitope, a novel candidate compared to previously studied sequences, as a potential B-cell-targeting epitope for TB vaccine design.

Materials and Methods: Epitope selection was based on antigenic regions within the ERv protein sequence. ERv 53–63 was chosen due to higher predicted antigenicity and favorable binding potential. VaxiJen was used for antigenicity assessment, AllerTOP v2.0 for allergenicity, and ToxinPred for toxicity evaluation. Structural docking simulations were conducted using PyMOL, with a human B-cell receptor (PDB ID: 5DRW) as docking target to evaluate epitope–receptor interaction.

Results: ERv 53-63 demonstrated high antigenicity (VaxiJen score: 0.9599). It was predicted as non-allergenic and non-toxic, and exhibited strong binding affinity with B-cell receptor (interaction energy: –877.8 kcal/mol), indicating stable complex formation.

Conclusion: These findings support ERv 53–63 as a novel and promising *in silico*-derived B-cell epitope, outperforming prior candidates such as ERv 105–118. It holds strong potential for peptide-based TB vaccine development. Further *in vitro* and *in vivo* studies are recommended to validate its immunogenicity and safety.

Keywords: B-cell Receptor, ERv 53–63 Epitope, *In silico*, Peptide-based Vaccine, Tuberculosis, Vaccine Design

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1. Introduction

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium (M.) tuberculosis*, which enters human body through airborne contact **(1)**. The body's immune systems, both innate and adaptive, work together to eliminate the bacteria or limit its replication **(2, 3)**. However, the disease can become severe if it spreads to other organs or body parts, complicating treatment and making it harder to cure **(4, 5)**. Global TB incidence has risen in recent years, reaching 10.8 million cases in 2023.

This resurgence has been partly attributed to disruptions in healthcare systems and diagnostic services during the COVID-19 pandemic, which affected TB detection and treatment continuity. The highest prevalence remains in the African, Southeast Asian, and Western Pacific regions, accounting for 87% of global cases, with India, Indonesia, China, the Philippines, and Pakistan bearing the greatest burden **(6, 7)**.

An ideal TB vaccine should trigger a strong cellular immune response by activating CD4+ and CD8+ T cells, which produce cytokines essential for macrophage activation **(8)**. Additionally, the vaccine should promote long-term tissue-resident memory T cells for immediate protection **(9)**. It must also stimulate innate immunity by activating macrophages, dendritic cells, and pattern recognition receptors. Humoral immunity plays a key role in opsonization and preventing bacterial entry into the host cells **(10)**. Furthermore, vaccine should control the infection without causing tissue damage **(11)**.

TB treatment methods include drugs, therapy, and vaccination **(12)**. However, their success depends on factors such as patient compliance, effectiveness of the treatment, side effects, and the chosen treatment method **(13)**. Vaccines effectively improve patients' chances of recovery and increase cure rates by strengthening the immune system and reducing infection rates. However, despite availability of vaccines, the number of TB cases has not decreased significantly. Challenges in TB vaccine development include ensuring long-term protection and addressing the need for effective vaccines across diverse age groups, including infants, the elderly, and immunocompromised individuals. Additionally, the effectiveness of the BCG vaccine is lower in tropical and subtropical climates **(14)**.

One major concern in TB vaccine development is the ineffectiveness caused by genetic mutations and strain variations **(15)**. Multi-epitope-based vaccines, such as the H56:IC31 vaccine, show promise in stimulating stronger and broader immune responses that are more resistant to mutations. These vaccines target multiple antigenic epitopes of *M. tuberculosis* **(16)**, triggering a more specific and long-lasting immune response. Ongoing research is exploring other protein pair candidates to improve the effectiveness of multi-epitope vaccines **(17)**. Despite this, challenges persist, such as low immunogenicity, potential degradation, and difficulties in ensuring proper immune recognition in populations with high genetic diversity **(18)**. For instance, a protein strain analysis conducted in a recent study **(19)** identified additional potential epitopes for an anti-TB vaccine, such as ERv 57-64. This epitope has the second-highest binding affinity after the ERv 105-118 epitope, making it a viable alternative to ERv 105-118.

To address the challenges posed by genetic mutations and strain variations in pathogens such as *M. tuberculosis*, *in silico* methodologies have emerged as critical tools in contemporary vaccine and drug development. These computational approaches offer a cost-effective and time-efficient alternative to traditional laboratory-based methods, particularly in the design of multi-epitope-based vaccines. *In silico* techniques facilitate the prediction of key immunological properties—including antigenicity, allergenicity, and toxicity—thereby enabling the early identification and refinement of promising vaccine candidates without immediate need for the wet-lab validation **(20-22)**. Moreover, bioinformatics tools can be employed to optimize epitope selection, model molecular interactions, and simulate three-dimensional (3D) binding conformations between epitopes and host immune receptors. These capabilities enhance the precision of vaccine and drug design by identifying molecular targets involved in pathogen-host interaction pathways **(23)**. Molecular docking and dynamics simulations further contribute by assessing the binding affinity and structural stability of epitope-receptor complexes, which are essential for eliciting a robust immune response. Collectively, *in silico* approaches accelerate the early stages of biomedical research, improve candidate prioritization, and reduce the cost and duration of preclinical development **(22)**.

This study focuses on identification and evaluation of ERv 53–63, a peptide epitope derived from the ERv protein of *M. tuberculosis*. This region is highly conserved across major MTB lineages, increasing its potential as a broadly protective immunogen. It is important to note that ERv 53–63 demonstrates higher predicted antigenicity and stronger binding affinity in preliminary analyses, justifying its selection for further investigation. The study applies a comprehensive *in silico* approach to evaluate ERv 53–63 immunological characteristics and interaction with B-cell receptors. The goal was to assess its suitability as a component of future peptide-based or multi-epitope TB vaccines.

2. Materials and Methods

2.1 Sample Preparation for Epitope Prediction

The samples for testing were obtained from the NCBI website (https://www.ncbi.nlm.nih.gov/protein/WP_003899110.1/) as FASTA files. The FASTA file was submitted to the IEDB webserver (http://tools.iedb.org/ellipro/),

and the epitope prediction software BepiPred 2.0 was employed.

2.2 Antigenicity, Allergenicity, and Toxicity Testing of Vaccine Candidates

Protein antigenicity was evaluated using the Vaxijen v2.0 website (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.htm) with a threshold of 0.4. Allergenicity was assessed with the AllerTop v2.0 webpage (https://www.ddg-pharmfac.net/AllerTOP/), while toxicity was evaluated through the ToxinPred web server employing protein scanning tools (https://webs.iiitd.edu.in/raghava/toxinpred/protein.php).

2.3 Epitope Selection and Naming Convention

Predicted epitopes were designated based on their amino acid position within the parent ERv protein. Epitope selection was guided by the structural accessibility, antigenicity, and the absence of allergenic or toxic residues. Conservation analysis confirmed that the ERv 53–63 region is highly conserved across major *M. tuberculosis* lineages, supporting its suitability as a vaccine target.

2.4 Epitope Modeling

3D structural models of selected epitopes were generated using the SWISS-MODEL webserver (https://swissmodel.expasy.org/interactive). Templates with the highest sequence identity and coverage were chosen for model construction. Where possible, model quality was confirmed using GMQE and QMEAN scores. No additional energy minimization or molecular dynamics (MD) simulations were performed following model generation.

2.5 Analysis of Physicochemical Properties of Vaccine Candidates

The physicochemical properties of vaccine candidates were assessed utilizing the Expasy website with ProtParam tool (https://web.expasy.org/protparam/), which provided computed parameters, including molecular weight and theoretical isoelectric point (pI).

2.6 Ligand-Receptor Docking Analysis

To predict binding interactions between the selected epitope and host immune receptors, molecular docking was performed using the ClusPro 2.0 platform (https://cluspro.bu.edu). The receptor used was a crystallized human B-cell receptor Fab fragment with PDB ID: 5DRW, obtained from the Protein Data Bank (https://www.rcsb.org/structure/5DRW).

This receptor was selected based on the relevance to humoral immune activation and suitability for docking with peptide ligands. The epitope–receptor docking models were assessed based on the cluster scores and lowest energy values. Complexes were visualized using PyMOL (v2.5), allowing for the structural analysis of key binding interactions such as hydrogen bonding and hydrophobic contacts. No post-docking MD simulations or structural refinements were applied **(24)**. A schematic summarizing the study pipeline is provided to facilitate a clearer understanding of the overall methodology (Figure 1).

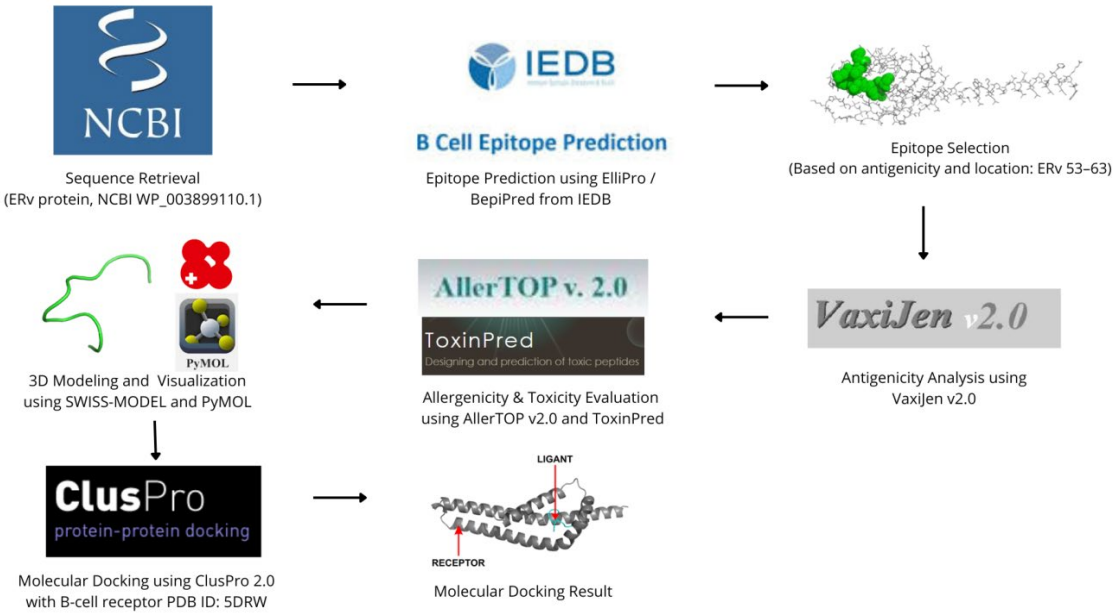

Figure 1. Schematic study pipeline **(Designed by Authors, 2025)**.

3. Results

3.1 Sample Preparation for Epitope Prediction

The following results represent the predicted epitopes identified through *in silico* analysis using epitope prediction tools (Figure 2).

ElliPro scores provide quantitative measures of epitope antigenicity based on protrusion index and residue clustering. Higher ElliPro scores indicate stronger potential for immune recognition. For instance, residues in the ERv 53-63 define as epitope (E) showed scores ranging from approximately 0.506 to 0.554, reflecting moderate to high antigenic propensity, which supports their selection as vaccine candidates (Table 1).

3.2 Antigenicity, Allergenicity, and Toxicity Tests of Vaccine Candidates

The predicted vaccine candidates were further evaluated for their antigenicity, allergenicity, and toxicity. The results showed that all selected epitopes exhibited high antigenicity scores, were classified as non-allergenic, and showed no predicted toxicity, indicating their suitability for further vaccine development (Table 2).

3.3 Epitope Modeling

The primary structure of the epitope consists of 11 sequences with the peptide sequence LLSYRPDTVQH. Epitope modeling was performed using PyMOL software (Figure 3). The modeling results showed that ERv 53-63 epitopes are loop-shaped.

3.4 Physicochemical Properties of Vaccine Candidates

Table 3 shows the results for physicochemical properties and stability of vaccine candidate.

3.5 Ligand-Receptor Interaction Test

The ERv 53–63 epitope was docked with a B-cell receptor using ClusPro 2.0, which applies FFT-based rigid-body docking and energy scoring functions combining van der Waals, electrostatic, and desolvation energies. Clustering was performed with a 9.0 Å RMSD radius. Of 9 resulting clusters, the one with the lowest energy score (–877.8) was selected, indicating a stable epitope–receptor interaction (Table 4). The B-cell receptor model was obtained from the Protein Data Bank (PDB ID: 5DRW), chosen for its high-resolution crystallographic structure and biological relevance in antibody–antigen interactions. This model provided a robust structural framework for simulating the binding process and ensuring the biological relevance of the docking analysis.

Table 1. Epitope prediction score results using the Elipro website.

Position	Residue	Score	Assignment
53	L (Leucine)	0,506	E
54	L (Leucine)	0,509	E
55	S (Serine)	0,537	E
56	Y (Tyrosine)	0,54	E
57	R (Arginine)	0,533	E
58	P (Proline)	0,554	E
59	D (Aspartate)	0,549	E
60	T (Threonine)	0,538	E
61	V (Valine)	0,524	E
62	Q (Glutamine)	0,521	E
63	H (Histidine)	0,517	E

Total number of negatively charged residues (Asp + Glu): 1

Total number of positively charged residues (Arg + Lys): 1

Table 2. Antigenicity, allergenicity and toxicity test results of vaccine candidates.

Peptide	Antigenicity	Allergenicity	Toxicity
LLSYRPDTVQH	0.9599 (Antigen)	Non-allergen	Non-toxin

Table 3. Test results for the physicochemical properties and stability of vaccine candidate.

Peptida	MW (Da)	Th. pI	Alph. Idx	GRAVY	Stb. Idx
LLSYRPDTVQH	1.191,49	6,74	97,27	-0,667	-9,29

MW: Molecular weight; Th. pI: Theoretical pI; Alph. Idx: Aliphatic index; GRAVY: Grand average of hydropathicity; Stb. Idx: Instability index.

Table 4. Ligand-Receptor Interaction Test Results.

Cluster	Members	Representative	Weighted Score
0	423	Center	-734.4
	423	Lowest Energy	-877.8
1	220	Center	-742.3
	220	Lowest Energy	-837.5
2	109	Center	-749
	109	Lowest Energy	-812.1
3	100	Center	-729.6
	100	Lowest Energy	-855.9
4	54	Center	-767.5
	54	Lowest Energy	-810.1
5	38	Center	-749.3
	38	Lowest Energy	-766.3
6	22	Center	-736.6
	22	Lowest Energy	-781.3
7	16	Center	-762.9
	16	Lowest Energy	-762.9
8	9	Center	-730.4
	9	Lowest Energy	-764.3
9	7	Center	-745
	7	Lowest Energy	-745

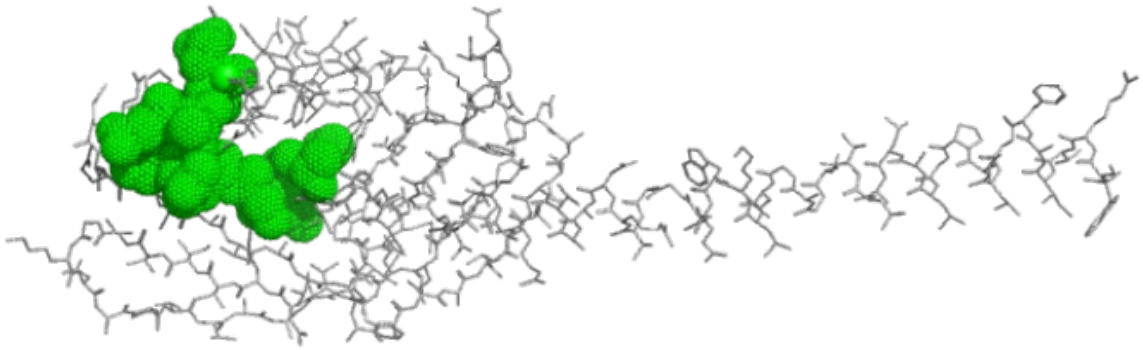

Figure 2. Results of epitope prediction analysis. The green color in the protein structure indicates the position of the linear epitope ERv 53–63 **(Desined by Authors, 2025)**.

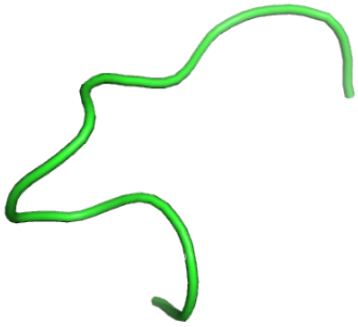

Figure 3. ERv 53-63 Visualization Using PyMOL Software **(Desined by Authors, 2025)**.

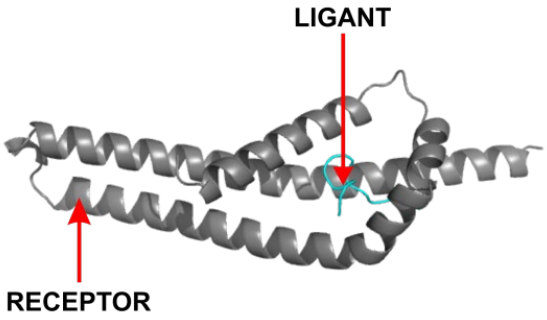

Figure 4. Docking visualization. Hydrogen bonding and hydrophobic contacts between the Homo sapiens B-cell receptor (gray color) and the ERv 53–63 ligand (turquoise color) **(Desined by Authors, 2025)**.

4. Discussion

The study analyzed 161 amino acid sequences from *Mycobacterium tuberculosis* membrane proteins using FASTA format data with the accession code WP_003899110.1. Epitope prediction through the ElliPro web server identified 11 residues as potential epitopes (Figure 2), which are biologically relevant and advantageous for the vaccine development. Longer epitopes offer significant benefits for interactions with B-cell receptors **(25)**, as they influence the strength and specificity of antigen-receptor interactions. This can reduce the likelihood of cross-reactivity and undesired immune responses **(19)**.

Epitopes longer than 10 residues are often recommended for vaccine candidates due to their ability to elicit more specific immune responses **(26)**, reducing the risk of non-specific interactions that might trigger autoimmune reactions **(27)**. Among the predicted epitopes, the sequence spanning residues 53-63, consisting of 11 amino acids, met the criteria for a potential vaccine candidate. Longer epitopes also

have the advantage of forming additional hydrophobic or hydrogen bonds, enhancing the stability of the antigen-antibody complex **(28)**.

Residue 58, with the highest predicted score of 0.554 (Table 1), demonstrated a strong potential for recognition by B cells and for triggering an immune response **(19)**. This suggests that residue 58 could be an effective target for a tuberculosis vaccine. Further analysis of charged residues using the Expasy website revealed a balance between negatively charged (1 Asp + Glu) and positively charged (1 Arg + Lys) residues (Table 1), indicating a well-balanced protein structure **(29)**.

The predicted epitopes were assessed for antigenicity using the VaxiJen v2.0 web server, with a protective antigen threshold value set at 0.4 **(30)**. The candidate vaccine epitope scored 0.9599, well above this threshold, indicating high antigenic properties (Table 2). This high antigenicity reflects the epitope ability to stimulate a robust adaptive immune response. It ensures that the immune system can recognize the antigen specifically and effectively **(31)**, offering high efficacy in preventing reinfection through long-term immunity.

High antigenicity plays a crucial role in vaccines by enabling the immune system to recognize pathogens and build immunity **(32-34)**. This occurs because the immune system can bind specifically to epitope residues, enhancing the immune response to infections **(35, 36)**. Highly antigenic antigens stimulate B cells to produce specific antibodies and activate T cells through antigen presentation via MHC molecules **(37)**. Furthermore, high antigenicity allows the immune system to recognize and respond quickly to the pathogens during future infections **(38)**. Vaccines protect by generating long-term immunity through memory cell formation and improving antibody efficiency in neutralizing pathogens **(39)**. The immune response is triggered when epitope antigenicity enables specific binding to immune response products **(40)**. In this process, B cells, assisted by T cells, differentiate into plasma cells that produce targeted antibodies **(41)**. These neutralizing antibodies block viruses from entering host cells, effectively limiting infection, providing strong protection, and preventing reinfection **(42)**.

The allergenicity of the epitope was evaluated using AllerTOP v2.0, which assesses the potential for allergic reactions **(43-45)**. A safe vaccine must be non-allergenic to ensure its safety for the population, avoiding harmful reactions **(46, 47)**. Non-allergenic epitopes help ensure immunological tolerance and minimize the risk of adverse immune reactions **(48)**. This is particularly important for populations with allergies, as it allows for safe administration without serious side effects **(43)**. Predicting allergenicity is crucial to prevent undetected contraindications and chronic allergic reactions **(49)**.

Toxicity testing of the candidate epitope was conducted using the ToxinPred web server, which confirmed that the candidate epitope does not exhibit toxic properties **(50)** (Table 2). This non-toxic nature is essential for reducing the risk of excessive immune reactions, such as hypersensitivity, which could harm vaccine recipients. Furthermore, the lack of toxicity promotes better immunological tolerance and reduces the risk of autoimmunity or tissue damage **(51)**. Therefore, the tested protein epitope is safe for stimulating the body's immune response **(52)**.

Both allergenicity and toxicity tests support the conclusion that the candidate epitope, LLSYRPDTVQH, is non-allergenic and non-toxic **(53)**, a key indicator for developing a safe vaccine for the general population. Immunological tolerance is crucial for effective and safe vaccination, especially for vulnerable groups such as children, the elderly, and individuals with specific health conditions. Ensuring that candidate epitopes have a low risk of allergenicity and are vital to minimize the likelihood of hypersensitivity reactions, whether local or systemic.

The ERv 53-63 epitope, consisting of 11 peptide sequences (Figure 3), has a loop structure that plays a critical role in the epitope functionality. Loop structures, typically found on the flexible surface of proteins, enable specific interactions with target molecules. These structures improve the binding stability by forming hydrogen bonds, enhancing specificity **(54, 55)**. Additionally, loop structures prevent binding with incompatible proteins by pushing them away, allowing only those with a strong affinity to bind after weaker peptides are displaced **(56)**. A study showed that 3 out of 5 loop structures had high antigenicity, improving vaccine effectiveness **(57)**.

The physicochemical properties of the protein were analyzed using ToxinPred (Table 3), revealing a molecular weight of 1,191.49 Da, which aids in determining the correct vaccine dosage **(15)**. The protein isoelectric point (pI) is 6.74, with a neutral pH and a high hydrophobicity index of 97.27 **(58, 59)**. The protein stability index is -9.29, suggesting its stability, as proteins with an index below 40 are considered stable **(29)**. The protein GRAVY index is also -0.667, indicating hydrophilicity **(60)**. Hydrophilic proteins with negative GRAVY values have a polar surface and interact well with water molecules, facilitating dissolution and transport. Hydrophilic epitopes function effectively within the body and are easily recognized by the immune system, potentially

enhanced by conformational interactions with water-based vaccine adjuvants **(61, 62)**.

Ligand-receptor interaction testing for the ERv 53-63 epitope was conducted using docking simulations via the ClusPro web server (Figure 4). The docking results revealed nine binding clusters between the ligand and receptor. The lowest binding energy, -877.8, was observed in cluster 0 (Table 4). A low binding energy indicates that the interaction between the epitope and receptor is stable, suggesting that the epitope-receptor complex is likely to form more easily and remain stable **(63)**. This stability is important because molecules with high binding affinity are more likely to induce a strong immune response. The stability of the epitope-receptor interaction, indicated by the low binding score, enhances the ability of the epitope to be recognized and processed by the immune system **(64)**. These docking results serve as a preliminary validation of the epitope potential to stimulate an adaptive immune response through interactions with B-cell receptors **(65)**.

ERv 53–63 demonstrates superior vaccine potential compared to the previously studied ERv 105–118 epitope, offering optimal length for B-cell engagement, higher antigenicity (0.9599), and enhanced safety with confirmed non-toxicity and non-allergenicity **(50)**. Its favorable loop structure and balanced charge improve immune recognition and structural stability **(54)**. Loop regions are often surface-exposed and flexible, enabling them to form specific and stable interactions with immune receptors through hydrogen bonding and hydrophobic contacts **(55)**. Studies have demonstrated that loop epitopes in viral vaccines, such as influenza hemagglutinin and HIV gp120 proteins, effectively elicit strong neutralizing antibody responses due to their accessibility and conformational stability **(66)**. Similarly, bacterial vaccine research targeting loop epitopes in surface proteins of pathogens like *Neisseria meningitidis* has shown enhanced immunogenicity and protective efficacy in animal models **(67)**. These findings underline the advantage of targeting loop structures in vaccine design, as they improve antigen stability and promote effective immune activation, supporting the potential of ERv 53–63 as a promising epitope for tuberculosis vaccine development.

Molecular docking reveals a stronger binding affinity (–877.8), indicating a more effective and stable interaction with immune receptors **(63, 64)**. This stable epitope-receptor complex is crucial for enhancing the activation signal that initiates the adaptive immune response **(66)**. Such interactions support B-cell activation and enable antigen-presenting cells (APCs) to activate T-helper cells **(68)**, which then promote B-cell differentiation into plasma cells that produce antibodies **(69)**. These antibodies aid macrophages in eliminating MTB within the phagosome, while cytokines like tumor necrosis factor-alpha (TNF-α) contribute to granuloma formation, a key mechanism in controlling TB infection **(70, 71)**. Overall, ERv 53–63 outperforms ERv 105–118 in critical immunological and structural aspects, making it a more promising candidate for tuberculosis vaccine development.

While ERv 53–63 exhibits strong antigenic and structural features *in silico*, it is important to consider immunogenetic variability among human populations, which can significantly influence vaccine efficacy **(72)**. Human leukocyte antigen (HLA) polymorphisms vary widely across ethnic and geographic groups, affecting how epitopes are presented to T cells and, thus, how the immune system responds **(73, 74)**. An epitope with high binding affinity in one population may be poorly presented in another due to differences in prevalent HLA alleles. This variation can impact the robustness and breadth of the immune response, potentially limiting the universal applicability of a single-epitope vaccine **(75)**. Therefore, while ERv 53–63 shows promise, its immunogenicity should be validated across diverse HLA types using both computational population coverage analysis and experimental assays.

The study demonstrates several strengths, including a comprehensive *in silico* evaluation using tools like AllerTOP v2.0, ToxinPred, and ClusPro, which support the epitope safety, immunogenicity, and binding stability. The confirmed non-allergenicity and non-toxicity indicate its suitability for vaccine development, while structural and physicochemical analyses highlight its stability and hydrophilicity, aiding immune recognition and formulation. The computational approach offers a cost-effective and time-efficient strategy compared to traditional methods, and molecular docking suggests a strong binding affinity to immune receptors, reinforcing its potential effectiveness.

However, the study is limited by its lack of experimental validation, with results relying solely on computational predictions. It also narrowly focuses on epitope-receptor interactions, neglecting broader immune responses such as T-cell dynamics and memory formation. Additionally, the models may not account for human genetic diversity, complex biological environments, or post-translational modifications. Finally, focusing on a single epitope could restrict immune protection, indicating that multi-epitope or whole-protein strategies may be more effective.

5. Conclusion

In silico methods, like docking analysis using ClusPro, can expedite the development of tuberculosis vaccines, reducing the time and cost of conventional methods. These methods allow for rapid identification and validation of epitope candidates, predicting their stability and immunogenic potential. This approach reduces the need for costly laboratory experiments, focusing resources on more promising candidates. This approach is particularly relevant for improving the availability and accessibility of effective vaccines in the context of tuberculosis. To validate and translate computational findings into effective tuberculosis vaccines, follow-up experimental studies are essential. These should begin with peptide synthesis and in vitro assays to confirm antigenicity and immune activation, followed by in vivo animal trials to assess immunogenicity, safety, and protection. Testing across genetically diverse models is also recommended to address population-wide variability and ensure broad efficacy.

6. Declarations

6.1 Acknowledgment

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6.2 Ethical Considerations

This study did not involve human participants or animal subjects, therefore ethical approval was not required.

6.3 Authors' Contributions

The authors confirm contribution to the paper as follows: study conception and design: MRD; data collection: ADW, TSA, NRH; analysis and interpretation of results: ADW, TSA, NRH; draft manuscript preparation: MRD, ADW, TSA, NRH. All authors reviewed the results and approved the final version of the manuscript.

6.4 Conflict of Interests

The authors have no conflicts of interest to declare.

6.5 Financial Support and Sponsorship

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6.6 Using Artificial Intelligence Tools (AI Tools)

AI tools were used only for language editing and proofreading, with all final revisions made by the authors.

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