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An In-Silico Reverse Vaccinology Approach in Predicting Vaccine Candidate Against *Mycobacterium tuberculosis* CCDC5180

Kunal Gharat^{1,2}, Vikas Jha^{1*}, Joshua Koli¹, Ashish Jhangiani¹, Vrushali Dhamapurkar¹, Diksha Poojari¹, Omkar Parulekar³, Simeen Rumani¹, Dhanashree Kulabkar⁴ and Bhakti Salekar⁴

¹National Facility for Biopharmaceuticals, Guru Nanak Khalsa College of Arts, Science & Commerce, Mumbai-19, Maharashtra, India

²Department of Biochemistry and Molecular biology, Faculty of Biology and Chemistry, University of Bremen, Bibliothekstraße 1, 28359 Bremen, Germany

³Department of Microbiology, St. Xavier's College, 5, Mahapalika Marg, Dhobi Talao, Chhatrapati Shivaji Terminus Area, Fort, Mumbai, Maharashtra, India

⁴Department of Five Years Integrated Course in Bioanalytical Sciences, GNIRD, G.N. Khalsa College, Matunga-19, Mumbai, Maharashtra, India

ABSTRACT

A quarter of the world's population is thought to be latently infected with *Mycobacterium tuberculosis*, creating a vast pool of possible future illnesses. Combined with the rising prevalence of multi-drug resistant *Mycobacterium tuberculosis* (Mtb) strains, TB poses one of the biggest challenges for world health, and the TB epidemic can only be eradicated with a new and more effective vaccine strategy. Since the Bacille Calmette-Guérin (BCG) vaccine is the only one used to treat tuberculosis (TB), Reverse vaccine technology aims to accelerate the development of subunit vaccines by identifying specific proteins in a pathogenic bacterial proteome that may be protective antigens. This approach was employed on four extracellular proteins namely Secreted fibronectin-binding protein C antigen 85-C, PE-PGRS family protein, Invasion protein RipA, and Invasion protein RipB. The shortlisted proteins were subjected to B-cell, CTL, and HTL epitope prediction. To further identify the most promising epitopes, specialized filtering techniques were employed to narrow down CTL epitopes that were non-allergenic, non-toxic, and antigenic as well as B-cell epitopes that produced antibodies. Similarly, HTL epitopes that generate IFN- γ but not IL-10 were selected. The analysis revealed invasion protein RipA as a potential candidate for *Mycobacterium tuberculosis* CCDC5180 immunization.

*Corresponding author

Vikas Jha, National Facility for Biopharmaceuticals, Guru Nanak Khalsa college, Mumbai 400019 India. E-mail: vikasjjha7@gmail.com

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Abbreviations: Tuberculosis (TB), *Mycobacterium tuberculosis* (*M. tb*), Antimicrobial resistance (AMR), Multidrug-resistant (MDR), Extensively Drug-resistant (XDR), Database of essential genes (DEG), Toll-like receptors (TLR), Inner membrane proteins (IMPs), Trans-membrane helices hidden Markov model (TMHMM), Interferon-gamma (IFN- γ), Helper T-lymphocytes (HTL), Cytotoxic T Lymphocytes (CTL), Heparin-binding hemagglutinin (HBHA), Major histocompatibility complex (MHC), Antigen-presenting cells (APCs), Interleukin-10 (IL-10), Cytokine synthesis inhibitory factor (CSIF), Reverse vaccinology (RV).

Introduction

Tuberculosis, Malaria and Acquired immune deficiency syndrome (AIDS) are the 3 major infectious diseases that cause a significant disease burden with high rates of morbidity and mortality [1]. There were an estimated 8.6 million incident cases of TB worldwide in 2012, which is equal to 122 cases per 100,000 individuals [2,3].

TB typically manifests as a pulmonary disease transmitted by droplet inhalation, with symptoms including a persistent cough, fever, and night sweats [4]. Individuals with healthy immune systems can often suppress the disease, remaining asymptomatic but infected. Immunocompromised patients who are unable to mount an immune response sufficient to suppress symptoms face difficulties [5]. The slow growth rate of *M. tuberculosis*, its complex pathogenesis, and its ability to remain dormant all pose significant challenges to the development of effective anti-TB treatments [6]. As a result, TB vaccination remains a top priority. The finding of essential mycobacterial virulence genes has been extremely beneficial. Extensive research on virulence genes, made possible by the use of transposon mutant libraries and a range of in vivo screening approaches, has shed light on the ability of the bacilli to survive to live and persist in their hosts [7, 8].

According to WHO survey data from around the world, 0.6 million cases of multidrug-resistant tuberculosis (MDR-TB) were reported, with approximately 0.24 million deaths [9]. MDR-TB strain growth leads to insufficient or irregular antibiotic intake and limited TB treatment. Individuals with low immunity are more resistant to infection and unable to respond to the immune system

effectively. As a result, more efforts are needed to advance the development of new TB vaccines [10]. Cell immunity (CD4+ and CD8+ T cells) accounts for the majority of the immune response to *Mtb*. Once activated, both CD4+ and CD8+ T cells secrete cytokines that cause an immune response. CD8+ cells also mediate cytotoxicity and lysis of infected cells. An Effective T cell responses are required for tuberculosis elimination. Complex pathogenesis, slow *Mtb* growth, and dormant ability are critical tasks in developing effective TB therapies.

Researchers have used subtractive genomics and reverse vaccinology to discover promising therapeutic targets and vaccine candidates against a variety of pathogenic microbes. Reverse vaccine technology seeks to accelerate the development of subunit vaccines by determining which proteins in a pathogenic bacterial proteome are possible protective antigens [11]. It was initially proposed in 2000, based on the discovery of new meningococcal vaccine candidates from a *Neisseria meningitidis* serogroup B strain's genomic sequence. Over the last decade, the use of reverse vaccinology has revolutionized how vaccine development is conducted, and this new age of vaccine research has been labelled a renaissance of vaccinology [12]. The fundamental benefit of reverse vaccinology over conventional vaccinology is the time and cost savings associated with identifying prospective vaccine candidates, as there is no need to culture the bacteria [13]. Furthermore, reverse vaccinology can find all potential protective protein antigens for a bacterial species as opposed to merely the most abundant antigens that can be identified from bacterial cultures using a conventional method. The accessibility of whole-genome sequencing has transformed the approach to vaccine development and led us to adopt fresh perspectives.

Ali et al. applied a subtractive genomics approach to discover drug targets and vaccine candidates against *Borrelia burgdorferi* B31, *Ehrlichia chaffeensis* [14]. Arkansas et al. used a reverse vaccinology approach for designing vaccines against *Pseudomonas aeruginosa* [15]. In addition, they developed a chimeric vaccine against *Acinetobacter baumannii* using a combination of subtractive genomics and reverse vaccinology technique. Arshad Dar et al. developed a multi-epitope vaccine against *Klebsiella pneumoniae* using immunoinformatics tools [16]. Furthermore, they performed molecular docking studies using Toll-like receptors (TLR) to validate the vaccine design, demonstrating a strong contact between their design and TLR. Bibi et al. recently used a reverse vaccinology technique to find promising *M. tb* H37Rv vaccine candidates. They developed a multi-epitope vaccine using four antigens: Rv2608, Rv2684, Rv3804c, and Rv0125. The construct was further evaluated by docking it with TLR3 using four online servers: ClusPro, HADDOCK, FireDock, and HawkDock, with a score ranging from -30 to -42 kcal/mol [17]. The objective of this study was to identify potential vaccine candidates from the whole proteome of *M. tb* CCDC5180 by using online databases to uncover extracellular crucial proteins by subtractive proteomics approach. The transmembrane helices and the antigenicity of the protein were identified. Both epitope mapping and the construction of a multi-epitope vaccine was achieved. Further, the physicochemical characterization of these multi-epitope vaccines was carried out.

Materials & Methods

Protein Selection

The essential proteins shortlisted for the study were selected from the DEG database (<http://origin.tubic.org/deg/public/index.php>). The proteins were chosen based on their importance and location within the cell. These proteins were discovered and used as a

resource to find potential vaccine candidates.

Antigenicity and Signal Peptide Prediction

Identifying the protein's antigenic properties is a vital stage in the vaccine development process. Determining the antigenic nature of a protein is a time-consuming and expensive procedure, but the proteomics revolution in bioinformatics has made this determination incredibly simple and affordable. The antigenic nature of the shortlisted extracellular proteins was computed using VaxiJen 2.0 web server (<http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html>) [18]. A threshold was set at 0.4 and proteins above this threshold were considered to be potential antigens.

Secretory proteins can play a significant role in enhancing bacterial pathogenicity, by enabling adhesion to host cells and impairing their defense mechanism [19]. The secretory nature of the antigenic extracellular proteins was identified using the Signal-BLAST online server (<http://sigpep.services.came.sbg.ac.at/signalblast.html>). Antigenic proteins were labelled as signal peptides based on four criteria: sensitivity, prediction, specificity and cleavage location [20].

Estimation of Trans-Membrane Helices and Molecular Weight

The trans-membrane integral inner membrane proteins (IMPs) span the plasma membrane and contain α -helices. This is helpful in predicting potential vaccine candidates as proteins with less than two membrane helices are easy to clone and purify [21]. TMHMM 2.0 (Trans-membrane helices hidden Markov model for topology prediction) (<http://www.cbs.dtu.dk/services/TMHMM/>) was used to calculate the number of transmembrane helices. TMHMM is a membrane protein topology prediction method based on a hidden Markov model that accurately predicts transmembrane helices and distinguishes between membrane and soluble proteins. Similarly, proteins with molecular weight less than 100 kDa were preferred for the ease of purification. The ProtParam tool (<https://web.expasy.org/protparam/>) in the ExPASy server was used to estimate the molecular weight of these extracellular proteins [22].

Linear B-Cell Epitope Prediction

Continuous/linear and discontinuous epitopes are the two existing forms of B cell epitopes. The linear B-cell epitopes are 16-amino acid sequences with a short antigenic length that can cause the development of particular antibodies. Antigenic proteins having a molecular weight of less than 100 kDa and no more than two transmembrane helices are subjected to a further round of epitope prediction in linear-B cells. Using an artificial neural network, the ABCpred server predicts linear B cell epitope areas in an antigen sequence. This server aids in identifying epitope regions that are helpful in choosing candidates for synthetic vaccines, disease diagnosis and allergy studies. ABCpred (<http://crdd.osdd.net/raghava/abcpred/>) server was used to predict the B-cell epitopes [23]. Epitopes having a value higher than 0.8 were taken into consideration. Furthermore, several types of antibodies, including IgG, IgE, and IgA should be induced by the B-cell epitopes. This antibody-inducing epitope prediction was achieved using IgPred (<http://crdd.osdd.net/raghava/igpred/>) online tool [24].

Prediction of Cytotoxic T Lymphocytes (CTL) Epitope

Epitope mapping identifies the most promising B-cell and T-cell epitopes for the development of an epitope-based vaccine. T-cell epitopes range in length from 8 to 20 amino acids and can elicit a strong T-cell response. It is critical to determine whether epitopes are immunoprotective before developing a synthetic vaccine peptide [25]. The CTL epitopes are predicted using NetCTL 1.2 servers (<http://www.cbs.dtu.dk/services/NetCTL/>). The following

alleles were chosen to predict the CTL epitopes: HLA-A*0101 (A1), HLA-A*0201 (A2), HLA-A*0301 (A3), HLA-A*2402 (A24), HLA-A*2601 (A26), HLA-B*0702 (B7), HLA-B*0801 (B8), HLA-B*2705 (B27), HLA-B*3901 (B39), HLA-B*4001 (B44), HLA-B*5801 (B58), HLA-B*1501 (B62). The threshold for epitope identification was adjusted at 0.75. A potential CTL epitope is described as one that is non-allergenic, antigenic, and non-toxic. As a result, the predicted CTL epitope's allergenicity, antigenicity, and toxicity were investigated. The allergenicity of each CTL epitope was determined using Allergen FP v1.0 tool (<https://ddg-pharmfac.net/AllergenFP/>), while the antigenic nature was predicted using VaxiJen 2.0 web server (<http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html>) [26]. Lastly, the toxicity was detected using ToxinPred tool (<http://crdd.osdd.net/raghava/toxinpred/>). For further analysis, only the non-allergenic, non-toxic, and antigenic CTL epitopes were selected [25].

Prediction of Helper T-lymphocytes (HTL) Epitope

HTL (CD4), CTL, regulatory T-cells, and memory T-cells are the four kinds of T-cell lymphocytes that contribute to the T-cell-mediated immune reaction. HTL play an important role in aiding B-cells and CTL in producing antibodies and destroying pathogens, respectively. In addition, they interact with innate immune cells and assist cytotoxic CD8 T cells in expanding and sustaining memory [27]. Considering the importance of HTL in preventing *M. tb* infection, HTL epitopes were identified using NetMHCII 4.0 (<http://www.cbs.dtu.dk/services/NetMHCIIpan/>) online tool [28]. There are 34 distinct types of Human Leukocyte Antigen (HLA) on this server. The study was only able to examine four alleles, as suggested by Pradhan et al. as the HLA-DR supertype is significant: HLA-DRB1*0101, HLA-DRB3*0101, HLA-DRB4*0101, and HLA-DRB5*0101 [29]. The threshold for strong interactors and weak interactors was set at 2% and 5% respectively [30].

Interferon-Gamma (Ifn- γ) And IL-10 Inducing Epitope Prediction

A crucial cytokine in the regulation of *M. tb* infection is IFN- γ , a Type II interferon [31]. Presentation by inducing the production of MHC class I and II molecules and boosting CD4 T during infection, CD4 and CD8 T cells both generate this cytokine. Its crucial function is to activate macrophages, allowing them to carry out bactericidal functions. It also improves antigen cell maturation into T-helper type 1 cells. In order to prove that not all MHC class II binders produce the same type of cytokines, the chosen HTL epitopes were exposed to IFN- IL-10 prediction [32]. The IFN- γ inducing epitope was predicted using IFN epitope online tool (<http://crdd.osdd.net/raghava/ifnepitope/index.php>) while the IL-10 inducing 34 epitopes were predicted using IL-10Pred (<https://webs.iitd.edu.in/raghava/il10pred/algo.php>) online tool [33]. IFN epitope is an online prediction tool that identifies and creates peptides from protein sequences that can cause CD4+ T lymphocytes to produce IFN-gamma. The IL-10Pred tool can create, locate and map peptides that could activate IL-10.

Population Coverage Analysis

MHC molecules have a wide range of diversity. More than 225 HLA Class I and 986 HLA Class II allelic variants have been found in humans so far. Furthermore, distinct MHC variations occur at varying rates in various populations, complicating the development of epitope-based vaccines. The identified epitopes trigger a significant immunological response by binding to multiple HLA supertype alleles for maximum population coverage, which is one of the key ideal qualities for multi-epitope vaccine candidates. Finding the best HLA allele collections with the greatest coverage

for various populations would be a worthy pursuit in this situation [34]. The shortlisted CTL and HTL epitopes with their respective HLA allele types were submitted to the IEDB population coverage analysis tool (<http://tools.iedb.org/population/>) [35]. Calculations for MHC class I and MHC class II were done separately. This research focused on the following populations: World, Europe, East Asia, Northeast Asia, South Asia, Southeast Asia, and Southwest Asia.

Construction of Multi-Epitope Vaccine

The next step in this investigation will be to create a multi-epitope vaccine using the shortlisted B-cell, CTL, and HTL epitopes. An adjuvant is a chemical or biological compound with inherent immunostimulatory properties that considerably boosts the host's immune response in comparison to reactions set off by the epitope alone. In this study, the Heparin-binding hemagglutinin (HBHA) protein of *M. avium subsp. paratuberculosis* (Accession no. AGV15514.1) was used as an adjuvant. The fact that this protein efficiently induces potent immune responses and functions as a TLR4 activator is the main justification for evaluating it as an adjuvant. In order to achieve prolonged conformation (flexibility), protein folding, and functional domain separation, linkers are necessary. These factors increase the stability of the protein structure [36]. B-cell, CTL, and HTL epitopes were linked with the GGS linker whereas the adjuvant was linked with the EAAAK linker.

Physicochemical, Allergenicity and Antigenicity Analysis of Vaccine Constructs

The ProtParam tool (<https://web.expasy.org/protparam/>) was used to assess the physicochemical properties such as molecular weight, isoelectric pH, aliphatic index, hydropathicity, instability index, GRAVY values and estimated half-life of both multi-epitope vaccine constructs. Further, the nature of the allergenicity of the constructs was identified using Allergen FP tool (<https://ddg-pharmfac.net/AllergenFP/>), while the antigenic nature was predicted using VaxiJen 2.0 web server (<http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html>) [26].

Results and Discussion

Protein selection

The proteins were selected on the basis of their importance in the DEG database and their location in the cell's extracellular area. The localization of previously identified 425 essential proteins was done using PSORTb 3.0 server. According to this tool, only four proteins were found to be extracellular. These four proteins are secreted fibronectin-binding protein C antigen 85-C (Ag85C) (AHJ53223.1), PE-PGRS family protein (AHJ53877.1), invasion protein RipA (AHJ54662.1) and invasion protein RipB (AHJ54663.1). The *M. tb* Ag85 complex comprises Ag85A, Ag85B, and secreted fibronectin-binding protein C antigen 85-C (Ag85C). Interestingly, this complex is ubiquitous in all *Mycobacteria* species and has gained a lot of attention due to its significance in *M. tb* pathogenicity. This Ag85 complex is a major secretory antigen that has been found to be highly immunogenic in both in-vivo and in-vitro analyses. This complex evades the host immunological response by binding to the fibronectin, mycolyltransferase and inhibiting phagosome maturation [37-39]. Therefore, this complex has numerous applications, such as diagnostics and vaccine candidates. Moreover, Kumar et al. observed that Ag85C had the maximum accuracy of 92% out of all the antigens utilized in ELISA for antibody detection [40]. On the other hand, Ag85 A and B are exploited in the development of vaccine candidates [41]. The following extracellular protein belongs to the PE-PGRS family, which are surface antigens. It

is a subgroup of the PE family proteins and has a domain with multiple repeats of Gly-Ala residue. PE-PGRS genes are expressed during the infection, according to Delogu et al. animal model research [42]. Furthermore, PE-PGRS33 is the only PE-PGRS protein that has been thoroughly investigated so far. Lastly, the two invasion proteins RipA and RipB are involved in the invasion of host macrophages. The deletion of RipA makes the pathogen avirulent and increases antibiotic susceptibility. A similar observation was seen upon deletion of RipA in *M. smegmatis* [43]. Similar to PE-PGRS, the two invasion proteins have limited data available. Therefore, further studies are required to understand the role of these proteins.

Antigenicity and Signal Peptide Prediction

Determining the antigenic nature of the protein, which was formerly a time-consuming and expensive process, is now exceedingly simple and inexpensive owing to the bioinformatics revolution of proteomics. VaxiJen is one of the most prevailing computational tools for determining protein antigenicity [43]. In the present analysis, VaxiJen 2.0 was used to predict the antigenic nature of the extracellular proteins. A threshold of 0.4 was set to eliminate non-antigenic proteins. As shown in (Table 1), all proteins were determined to be antigenic since their probability values were more than 0.4.

Table 1: List of antigenic proteins predicted using VaxiJen 2.0 server

Sr no.	Accession Id	Protein name	Antigenicity	Probability Scores
1	AHJ53223.1	Secreted fibronectin-binding protein C antigen 85-C	Antigen	0.4402
2	AHJ53877.1	PE-PGRS family protein	Antigen	0.6446
3	AHJ54662.1	Invasion protein RipA	Antigen	0.6576
4	AHJ54663.1	Invasion protein RipB	Antigen	0.612

Pathogens use a variety of strategies to infect their hosts. One pivotal mechanism of infecting the host is by secreting protein through phospholipid membranes. Furthermore, these secreted proteins contribute significantly in the virulence of the pathogen [44]. Signal peptide proteins have been demonstrated to target the secretory system exhibiting high immunogenicity and sensitivity, making them promising vaccine candidates [44]. In the present analysis, Signal-BLAST was used to predict the secretory nature of the protein. On the basis of four parameters, namely sensitivity, prediction, specificity, and cleavage site, antigenic proteins were classified as signal peptides. According to Table 2, only three antigenic proteins possess a signal peptide.

Table 2: List of Signal Peptide Proteins Predicted Using Signal-Blast

Sr no.	Accession Id	Protein name	Sensitivity	Prediction	Specificity	Cleavage Site
1	AHJ53223.1	Secreted fibronectin-binding protein C antigen 85-C	Yes	Yes	Yes	Yes
2	AHJ53877.1	PE-PGRS family protein	No	No	No	No
3	AHJ54662.1	Invasion protein RipA	Yes	Yes	Yes	Yes
4	AHJ54663.1	Invasion protein RipB	Yes	Yes	Yes	Yes

Estimation of Trans-Membrane Helices and Molecular Weight

A transmembrane helix is a 17-25 amino acid protein sequence that forms an α helix structure that spans the cell membrane. The transmembrane helices interfere with the protein purification. As a result, the fewer the transmembrane helix, easier is the protein purification. TMHMM 2.0 (Trans-membrane helices hidden Markov model for topology prediction) was used to predict the number of transmembrane helices in this study. Only proteins with no more than two transmembrane helices were chosen. All the shortlisted proteins had a molecular weight of less than 100 kDa, making them easier to clone and purify. The list of shortlisted proteins with the total number of transmembrane helices and the molecular weight is given in Table 3.

Table 3: List of shortlisted proteins on the basis of Transmembrane helices and Molecular weight (kDa)

Sr no.	Accession Id	Protein name	Transmembrane helices	Molecular weight kDa
1	AHJ53223.1	Secreted fibronectin-binding protein C antigen 85-C	1	36.77
2	AHJ54662.1	Invasion protein RipA	0	49.8
3	AHJ54663.1	Invasion protein RipB	0	24.62

Linear B-Cell Epitope Prediction

B cells play a central role in the host humoral immunity by producing antigen-specific antibodies. These antibodies have the capability to counteract a wide range of antigens, including those found in viruses, bacteria, and other foreign agents. Further, these antibodies are classified into five major classes: IgG, IgA, IgM, IgD, and IgE [45,46]. The most prevalent and extensively researched class of immunoglobulins is IgG. IgG antibodies play a crucial role in the immune response, including the eradication of viruses and pathogens. IgA protects the mucosal surface from allergens, viruses, and bacteria by neutralizing or preventing them from adhering to it. Moreover, intracellular IgA helps to prevent bacterial infection. IgM antibodies are the first to develop in response to infection, and they are critical for infection prevention in the early stages. IgE, a very powerful immunoglobulin, is involved in allergic responses [47].

Immunoinformatics has transformed the role of immunology in the development of diagnostic and vaccine candidates against a broad spectrum of pathogens. Furthermore, it has eased the prediction of B-cell and T-cell epitopes [46]. Linear B-cell epitopes are short antigenic 16-amino-acid sequences that might cause the development of particular antibodies [48]. The ABCpred server was utilized to detect B-cell epitopes in this study. The results revealed that secreted fibronectin-binding protein C antigen 85-C (Ag85C) and invasion protein RipB had 10 B-cell epitopes, whereas invasion protein RipA had 24 B-cell epitopes. As a result, the IgPred server was utilized to eliminate such non-inducing antibody epitopes. According to this tool, there was a significant decrease in epitopes. There were ten epitopes in secreted fibronectin-binding protein C antigen 85-C (Ag85C); however, only three could trigger antibody production. Additionally, invasion proteins RipA and RipB possessed 24 and 10 epitopes, respectively, which were reduced to 5 antibody-inducing epitopes. Detailed information about the antibody-inducing epitopes is given in **Table 4**.

Table 4: List of Antibodies Inducing B-Cell Epitopes

Protein	Rank	Sequence	Start position	Score	Antibody
Secreted fibronectin-binding protein C antigen 85-C	1	NSMWGPSSDPAWKRRND	221	0.9	IgG
	2	GQSSFYTDWYQPSQSN	118	0.88	IgG
	5	LRAQDDYNGWDINTPA	86	0.85	IgG
Invasion protein RipA	4	FRMWDPGSGPAGGRAW	248	0.87	IgG
	6	AVMANLQRARTERVNT	157	0.85	IgG
	7	SEGGQGAPPFRMWDPG	239	0.84	IgA
	7	ARTERVNTESAARLAK	165	0.84	IgG
	8	QVTANMGRKFLEQLGI	299	0.83	IgA
Invasion protein RipB	2	LVSAGAPGDPLAVANA	43	42	IgG
	3	GDLIFYGPGGGQHVTLL	189	0.92	IgG
	4	AEPGQWDPTLPALVSA	31	0.9	IgG
	5	GSLQGPKSGVDSGANT	132	0.88	IgG
	8	APSAATTGASRIPRAN	92	0.81	IgG

Prediction of Cytotoxic T Lymphocytes (CTL) Epitope

The adaptive immune system is categorized into humoral immunity and cell-mediated immunity. B-cells are involved in humoral immunity, whereas T-cells are involved in cell-mediated immunity. There are two types of T-cells: cytotoxic T lymphocytes (CD8) and helper T lymphocytes (CD4) [49,50]. The immunological response of T cells works in the following way: Antigens are first detected by antigen-presenting cells (APCs), which then deliver these antigens to T-cells using major histocompatibility complex (MHC) molecules. Epitope mapping is an emerging area of Immunoinformatics that emphasizes on identifying the most promising B-cell and T-cell epitopes for the development of an epitope-based vaccine. T-cell epitopes range in length from 8 to 20 amino acids and can elicit a strong T-cell response. Furthermore, to manage diseases including Rickettsia, Salmonella, Listeria, Mycobacteria, and Chlamydia, a powerful immunogenic response is necessary [51].

In the present study, T-cell epitopes were predicted based on 12 different HLA alleles: HLA-A*0101 (A1), HLA-A*0201 (A2), HLA-A*0301 (A3), HLA-A*2402 (A24), HLA-A*2601 (A26), HLA-B*0702 (B7), HLA-B*0801 (B8), HLA-B*2705 (B27), HLA-B*3901 (B39), HLA-B*4001 (B44), HLA-B*5801 (B58) and HLA-B*1501 (B62). There were 77 CTL epitopes predicted for the secreted fibronectin-binding protein C antigen 85-C (Ag85C) protein58 and 70 for the invasion proteins RipA and RipB, respectively. Refer to (**Table. 5**)

Table 5: List of Ctl Epitopes on the Basis of Allergenicity, Antigenicity, and Toxicity

Protein	Subtype	Position	Epitope	Allergenicity	Antigenicity	Toxic
Secreted fibronectin-	HLA-A*0101	129	PSQSNGQNY	Non- Allergen	Antigen	Non-toxic
	HLA-A*0201	137	YTYKWETFL	Non- Allergen	Antigen	Non-toxic
		170	SMSGGSALI	Non- Allergen	Antigen	Non-toxic
binding protein C antigen 85-C	HLA-A*0301	277	TLRTNQTFR	Non- Allergen	Antigen	Non-toxic
	HLA-A*2402	181	AYYPQQFPY	Non- Allergen	Antigen	Non-toxic
	HLA-B*0702	49	RPGLPVEYL	Non- Allergen	Antigen	Non-toxic
		114	MPVGGQSSF	Non- Allergen	Antigen	Non-toxic
		268	IPAKFLEGL	Non- Allergen	Antigen	Non-toxic
	HLA-B*1501	73	QGGGPHAVY	Non- Allergen	Antigen	Non-toxic
	HLA-A*0201	265	GLWDPTLPM	Non- Allergen	Antigen	Non-toxic
	HLA-A*0301	177	RLAKQKADK	Non- Allergen	Antigen	Non-toxic
		389	YSFAGVGIK	Non- Allergen	Antigen	Non-toxic
	HLA-A*2402	400	HYSGSQYNL	Non- Allergen	Antigen	Non-toxic
	HLA-B*0702	271	LPMIPSANI	Non- Allergen	Antigen	Non-toxic
	HLA-B*2705	340	RQASEYVIR	Non- Allergen	Antigen	Non-toxic
		457	VRTAGMTPY	Non- Allergen	Antigen	Non-toxic
Invasion protein RipB	HLA-B*3901	221	ERDAAQARL	Non- Allergen	Antigen	Non-toxic
	HLA-A*0101	34	GQWDPTLPA	Non- Allergen	Antigen	Non-toxic
	HLA-B*0702	63	TAQATQTTL	Non- Allergen	Antigen	Non-toxic
		224	SPVRKAGMT	Non- Allergen	Antigen	Non-toxic
	HLA-B*2705	117	RRAGSQMGV	Non- Allergen	Antigen	Non-toxic

Prediction of Helper T-lymphocytes (HTL) Epitope

HTL (CD4), CTL, regulatory T-cells, and memory T-cells are the four kinds of T-cell lymphocytes that contribute to the T-cell-mediated immune reaction. In addition, they interact with innate immune cells and assist cytotoxic CD8 T cells in expanding and sustaining memory [52]. Although the role of CD4 is uncertain, evidence suggests that it is essential for the establishment of an anti-*M. tb* immune response. Its absence can lead to the pathogen's reactivation. MHC-II-restricted anti-*M. tb* responses are studied in depth, and several *M. tb* antigens targeting CD4 have been discovered so far. The HTL activation requires the presentation of antigens by the MHC-II molecules [50]. Considering the importance of HTL in preventing *M. tb* infection, the HTL epitopes were predicted using NetMHC-II 4.0 web server. The following alleles were chosen owing to significance in humans: HLA-DRB1*0101, HLA-DRB3*0101, HLA-DRB4*0101, and HLA-DRB5*0101. According to the findings, there were 26 epitopes predicted for the secreted fibronectin-binding protein C antigen 85-C (Ag85C). While 33 HTL epitopes were predicted for the invasion protein RipA, none were predicted for the HLA-DRB3*0101 allele. Similarly, 35 HTL epitopes were predicted for the invasion protein RipB [53].

Interferon-Gamma (IFN- γ) and IL-10 Inducing Epitope Prediction

Cytokines are the signalling molecules produced by different cell types that principally facilitate intercellular communication. A crucial cytokine in the regulation of *M. tb* infection is IFN- γ , a Type II interferon and CD4 and CD8 T cells both produce this cytokine during infection [54]. The cytokine's main function is to activate macrophages, allowing them to carry out bactericidal activity. It also improves antigen presentation by inducing the production of MHC class I and II molecules and boosting CD4 T cell maturation into T-helper type 1 cells. In recent studies, patients with lower levels of IFN- γ were found to be more vulnerable to Mycobacterial infections [55,56]. Therefore, to increase the efficacy of the vaccine against *M. tb*, the HTL epitopes that induce IFN- γ were shortlisted. According to the findings, eight, nine, and ten epitopes from secreted fibronectin-binding protein C antigen 85-C (Ag85C), invasion protein RipA, and invasion protein RipB, respectively, triggering IFN- γ were shortlisted. Interleukin-10 (IL-10) is another type of cytokine produced by macrophages and T-lymphocytes during *M. tb* infection. It is also known as a cytokine synthesis inhibitory factor (CSIF) since it prevents T-cells from producing cytokines. It is responsible for macrophage deactivation during *M. tb* infection, including suppression of IL-12 production, which eventually reduces IFN- γ production. Consequently, the pathogen has a better chance of surviving in this environment [57]. Therefore, it is critical to identify and eliminate the epitopes that activate IL-10 production to control the infection. This prediction was done using the IL-10 Pred server. Only one epitope out of eight IFN-inducing epitopes in secreted fibronectin-binding protein C antigen 85-C was discovered to induce IL-10 production. Similarly, four epitopes in invasion protein RipA were predicted to promote IL-10 production, whereas five epitopes in invasion protein RipB triggered IL-10 production. The list of HTL epitopes that induce IFN- γ and not IL-10 is given in **Table 6**.

Table 6: List of shortlisted HTL epitopes on the basis of IFN- γ and IL-10

Protein	Subtype	Position	Epitope	IFN- γ	IL-10	Allergenicity
Secreted fi-bronectin-binding protein C antigen 85-C	HLA- DRB1*0101	52	LPVEYLQVPSASMGR	Positive	Negative	Non- Allergen
		53	PVEYLQVPSASMGRD	Positive	Negative	Non- Allergen
	HLA- DRB4*0101	65	GRDIKVFQGGGPHA	Positive	Negative	Non- Allergen
		81	YLLDGLRAQDDYNGW	Positive	Negative	Non- Allergen
		82	LLDGLRAQDDYNGWD	Positive	Negative	Non- Allergen
		83	LDGLRAQDDYNGWDI	Positive	Negative	Non- Allergen
		84	DGLRAQDDYNGWDIN	Positive	Negative	Non- Allergen
Invasion protein RipA	HLA- DRB1*0101	175	AARLAKQKADKAAAD	Positive	Negative	Non- Allergen
		176	ARLAKQKADKAAADA	Positive	Negative	Non- Allergen
	HLA- DRB4*0101	177	RLAKQKADKAAADAK	Positive	Negative	Non- Allergen
		283	PIAVVNQVLGISATS	Positive	Negative	Non- Allergen
	HLA- DRB5*0101	447	DVGLKVRVAPVRTAG	Positive	Negative	Non- Allergen
	HLA- DRB1*0101	200	QHVTLYLGNGQMLEA	Positive	Negative	Non- Allergen
Invasion protein RipB	HLA- DRB3*0101	143	SGANTVGFDCSGLVR	Positive	Negative	Non- Allergen
		144	GANTVGFDCSGLVRY	Positive	Negative	Non- Allergen
		145	ANTVGFDCSGLVRYA	Positive	Negative	Non- Allergen
		146	NTVGFDCSGLVRYAF	Positive	Negative	Non- Allergen

Population Coverage Analysis

Different populations experience diverse MHC variations at varying rates, complicating the development of epitope-based vaccines. The identified epitopes trigger a significant immunological response by binding to multiple HLA supertype alleles for maximum population coverage, which is one of the ideal qualities for multi-epitope vaccine candidates [58,59].

In the present study, population coverage analysis of the shortlisted CTL and HTL was performed using the IEDB population coverage analysis tool. The following population was considered in this study: East Asia, Europe, Northeast Asia, South Asia, Southeast Asia, Southwest Asia, and World. East Asia covers Japan, Japan Oriental, Korea, South Korea, South Oriental, Mongolia, and Mongolia Oriental. Europe covers Turkey, Italy, Czech Republic, Scotland, France, Georgia, Slovakia, Ireland South, Wales, Norway, Belarus, Slovenia, Germany, Belgium, Spain, Ukraine, Ireland Northern, Netherlands, Denmark, Poland, Finland, Macedonia, Sweden, Croatia, Switzerland, Russia, Bulgaria, Romania, England, Portugal, Serbia, United Kingdom, Austria, and Greece. Northeast Asia covers China and Hong Kong while South Asia covers Sri Lanka, Pakistan, and India. Southeast Asia covers Indonesia, Singapore, Philippines, Borneo, Malaysia, Vietnam, Thailand, and Taiwan whereas Southwest Asia covers Israel, Iran, Oman, Lebanon, Saudi Arabia, Jordan, and UAE.

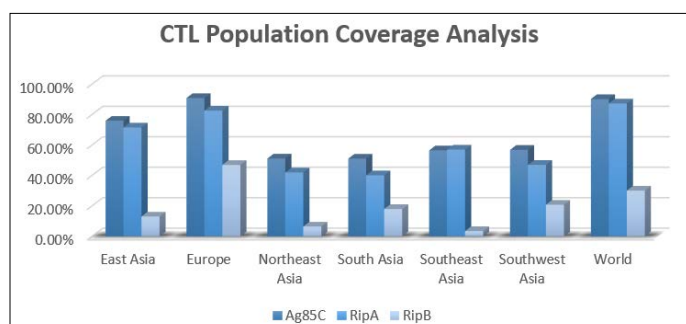


Figure 1: Graphical Representation of the Population Coverage Data of CTL Epitopes

From **Figure 1**, it is evident that Europe had the highest population coverage for CTL epitopes of Ag85C (90.80%) and RipA (82.55%), whereas RipB had the lowest population coverage (46.88%). Ag85C had a population coverage of 90.15% worldwide, whereas RipA had a population coverage of 87.28%. On the contrary, RipB had a population coverage of only 30% worldwide, which is extremely low. Further, the population coverage of Ag85C, RipA and RipB in East Asia was found to be 75.90%, 71.49%, and 12.99%, respectively. Ag85C had a population coverage of 50-51%, whereas RipA had a 40-41% population coverage in Northeast and South Asia. In comparison, RipB had a population coverage of 6.42% and 17.93% in Northeast and South Asia. In Southeast and Southwest Asia, the population coverage of Ag85C was 56.46 % and 56.80%, respectively. Similarly, the population coverage of RipA was 56.97% and 46.91% in Southeast and Southwest Asia, respectively. On the other hand, the population coverage of RipB was extremely low for all the populations taken into consideration. This indicates that the predicted CTL epitopes for the selected protein do not cover these populations. Hence, it cannot be taken further for vaccine construction.

Figure 2 shows that the HTL epitopes of Ag85C (88.90%) and RipA (89.45%) covered the maximum population of the world, whereas RipB covered only 24.10% population of the world. The population coverage in Europe for Ag85C, RipA and RipB was 85.60%, 80.67%, and 50.34%, respectively. Ag85C covered 72.80% of the East Asian population, whereas RipA covered 78.36%, and RipB covered 20.45% of the East Asian population. Ag85C had 62.45% and 55.19% population coverage in Northeast and Southeast Asia, respectively, whereas RipA had 54.85% and 57.21%. RipB, on the other hand, barely covered 10.04 percent and 12.80 percent of the population in Northeast and Southeast Asia, respectively. In South and Southwest Asia, the population coverage of Ag85C was 52.76% and 54.90%, respectively. Similarly, the population coverage of RipA was 51.72% and 56.09% in South and Southwest Asia, respectively. Thus, it was observed that the population coverage of RipB CTL and HTL epitopes covered a small proportion of the population, making it a less effective vaccine candidate when used by such a population. Therefore,

further research should be conducted to determine whether it covers the rest of the population listed on the server in order to be used as a viable vaccination candidate.

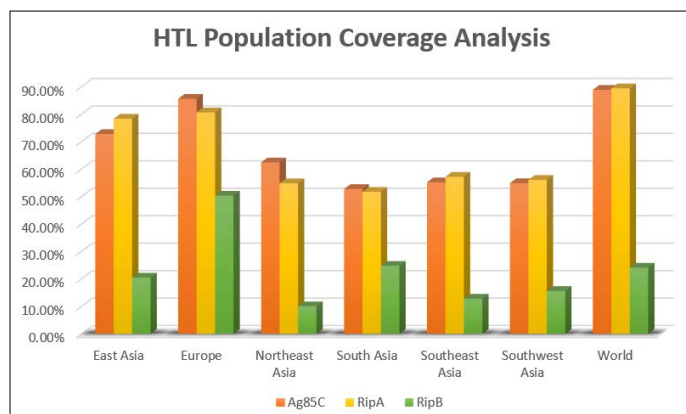


Figure 2: Graphical Representation of the Population Coverage Data of HTL Epitopes

Construction of Multi-Epitope Vaccine

The next step in this analysis was to construct the multi-epitope vaccine using the shortlisted B-cell, CTL, and HTL epitopes. In this study, the Heparin-binding hemagglutinin (HBHA) protein of *M. avium subsp. paratuberculosis* (Accession no. AGV15514.1) was used as an adjuvant. The fact that this protein efficiently induces potent immune responses and functions as a TLR4 activator is the main justification for evaluating it as an adjuvant [60]. Linkers are essential for achieving prolonged conformation (flexibility), protein folding, and functional domain separation, hence making the protein structure more stable. The adjuvant was joined with the EAAAK linker, whereas B-cell, CTL, and HTL epitopes were joined with the GGS linker. Construct 1 refers to the vaccine developed from epitopes (Figure 3), whereas Construct 2 refers to the vaccine constructed from RipA epitopes (Figure 4).

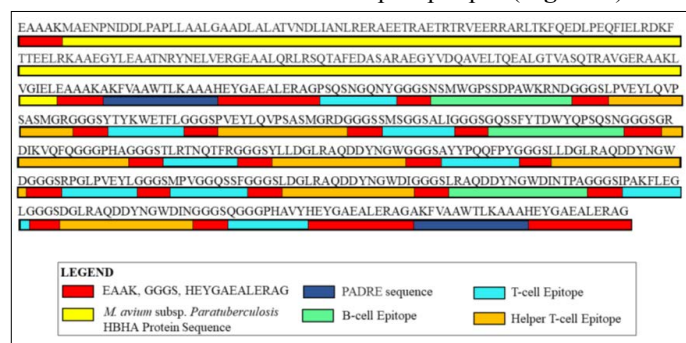


Figure 3: Pictorial representation of multi-epitope Construct 1

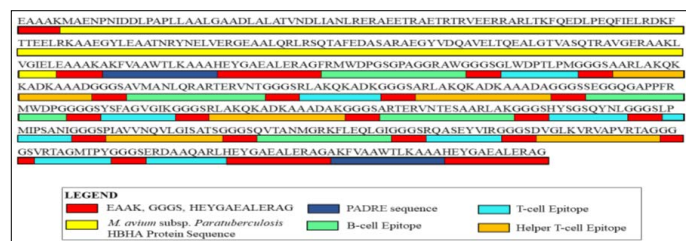


Figure 4: Pictorial Representation of Multi-Epitope Construct 2

Physicochemical, Allergenicity and Antigenicity Analysis of Vaccine Constructs

ExPASy ProtParam Server was used to evaluate the physicochemical parameters, including molecular weight, theoretical isoelectric

point (pI), aliphatic index, instability index, and GRAVY value of the two vaccine constructs. Construct 1 and 2 have a molecular weight of 56.09 and 53.83 kDa, respectively, which is less than 100 kDa, indicating that they would be quick and easy to purify. The isoelectric point (pI) is where the net charge on amino acid is zero as it produces an equal amount of positive and negative ions. A high pI indicates basic amino acid, whereas acidic amino acid has a low pI value. Construct 1 has a pI of 4.72, while Construct 2 has a pI of 9.27. This indicates that Construct 1 is acidic in nature, whereas Construct 2 is basic.

Table 7: Physicochemical Characterization and Allergenicity and Antigenicity Prediction of The Multi-Epitope Vaccine Constructs

	MW kDa	Isoelectric Point	Aliphatic Index	Instability Index	GRAVY	Allergenicity	Antigenicity
Construct 1	56.09	4.72	62.51	47.59	-0.58	Non- allergen	Antigen
Construct 2	53.83	9.27	69.87	35.77	-0.425	Non- allergen	Antigen

Another physiochemical property is the instability index, which approximates the stability of a protein. If the instability index of a protein is more than 40, it is considered unstable. The instability index of Construct 1 was predicted to be 47.59, which is greater than 40, indicating that this construct is unstable. On the contrary, the instability index of Construct 2 was estimated to be 35.77, indicating that it is stable. The aliphatic index is used to define how much volume of a protein is taken up by aliphatic side chains (valine, leucine, isoleucine, and alanine). It is a measurement of the thermostability of a protein. A high aliphatic index protein is more thermally stable. The aliphatic index of both constructs was in the range of 60-70, suggesting that they are thermally stable. Lastly, the grand average of hydropathicity (GRAVY) value is used to calculate the Hydropathicity average. A protein with a positive GRAVY value is said to be hydrophobic and non-polar, while one with a negative GRAVY value is said to be hydrophilic, polar, and better at interacting with water. The GRAVY value of Constructs 1 and 2 was found to be -0.58 and -0.425, respectively. This suggests that both proteins are hydrophilic in nature. Further, the allergenicity and antigenicity of both the constructs were evaluated using AllergenFP and VaxiJen server. Both constructs were shown to be non-allergenic and antigenic, implying that they can elicit a powerful immunogenic response without causing allergy responses in the host. To summarise, the results indicate that the construct 2 is physiochemically suitable for vaccine production, whereas construct 1 requires certain modifications before it can be considered a vaccine candidate. The details of the physiochemical properties, allergenicity and antigenicity nature of the two constructs is given in Table 7.

Conclusion

The emergence of MDR strains has aggravated the problem of tuberculosis making it a major public health concern worldwide. The computational approaches presented in this paper have the potential to provide better understanding *M. tb* vaccine antigens and vaccine candidates that cannot be obtained through pre-clinical, in-vitro, or animal studies. An immuno-informatics tool was used in this study to define a tuberculosis novel multi-epitope subunit vaccine that is highly immunogenic and has the properties of a carrier vaccine and for which the whole proteome of *M. tb* CCDC5180 was analyzed. Using the subtractive proteomics method, four extracellular proteins such as RipA, RipB, PE-PGRS and Ag85C protein were discovered and used in the vaccine

candidate analysis. Each of these proteins was examined for the presence of signaling peptides and antigenicity. Multiple B-cell and T-cell epitopes were analyzed using epitope prediction to improve the vaccine's immunogenicity. Similarly, HTL epitopes that trigger IFN- γ but not IL-10 were selected. In addition, the CTL and HTL epitopes of the two proteins Ag85C and RipA were chosen since they covered the maximum population. Finally, multi-epitope vaccines were constructed using the shortlisted epitopes. The vaccine's antigenicity, allergenicity, solubility and, physiochemical properties were all found to be very satisfactory for Rip A protein. However, in-vitro and in-vivo experiment validation is required to confirm the construct's potency. The long-term goal of the study is to predict the tertiary structure of the identified vaccine targets. Another aim could be to predict more HTL epitopes based on the remaining HLA alleles available on the NetMHCII server. The vaccine can then be docked with human TLR receptors to validate the RipA vaccine design using molecular dynamics simulation tests.

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