Chimeric vaccine against multi-drug resistant *Mycobacterium tuberculosis* using *in silico* reverse vaccinology approach

Arpita Batta¹², Vineeta Singh¹, Bhartendu Nath Mishra¹*, Tapankumar N. Dhole², Prahlad Kishore Seth³

¹Department of Biotechnology, Institute of Engineering and Technology, Dr. A. P. J. Abdul Kalam Technical University, Lucknow, India.
²Department of Microbiology, Sanjay Gandhi Post Graduate Institute of Medical Sciences, Lucknow, India.
³Biotech Park, Lucknow, India.

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**ABSTRACT**

The aim of this study was to predict promiscuous vaccine candidates against *Mycobacterium tuberculosis* (MTb) using *in silico* reverse vaccinology. Antigenic peptides from selected MTb strain LJ319 (4,025 proteins) were analyzed by various immunoinformatics tools; from which 165 outer membrane proteins (OMPs) suitable for vaccine designing were predicted. Further antigenicity, allergenicity, transmembrane α-helices, and solubility filters refine this number to 16 OMPs common in other members of Tb complex. By further analysis, T-cell and B-cell epitopes were predicted and subjected to characterization studies. After characterization, 26 promiscuous Epitopic peptides (MHC I: 4, MHC II: 7, and B cell: 15) were screened and joined to form 3 possible vaccine constructs (VC1, VC2, and VC3). To enhance immunomodulating effect of these constructs adjuvants (Accession No. WP_003403353.1, WP_031737436.1, and WP_094028633.1), and PADRE sequence (AKV AAWTLKAAAC) were added. The physiochemical characterization and molecular docking studies of vaccine constructs with HLA genes revealed VC1 can be further studied to control host and Tb interactions as it had the highest binding score was also a safe and immunogenic construct. Further studies are needed to ensure the expression and translation efficiency of the potential vaccine construct.

**INTRODUCTION**

The worldwide escalation of mycobacterial resistance (Dookie *et al.*, 2018; Nguyen *et al.*, 2019) [pulmonary and extrapulmonary tuberculosis (Tb)] to conventional vaccines and antibiotics poses a serious concern to modern medicine (Castan *et al.*, 2014). In 2019, the World Health Organization’s Global Tuberculosis Report estimates the occurrence of 10 million Tb cases globally. Besides this, 484,000 new cases of resistance to rifampin were also reported in a year, from which 78% of cases had multiple drug-resistant (MDR-Tb) (WHO, 2020a). It decreases the effectiveness of current treatments and causes thousands of deaths. Therefore, the need to brainstorm for this disease and its remedies still persist.

Tuberculous meningitis (TbM) is severe form of extrapulmonary Tb which is associated with high mortality of around 13% to 57% even after 12 months of anti-tubercular treatment (Donovan *et al.*, 2019; Rohlwink *et al.*, 2019; Soria *et al.*, 2019; Thwaites *et al.*, 2013). *Mycobacterium tuberculosis* (MTb) causing TbM in human is creating serious condition globally including in India as the estimated mortality is 627,000 annually (WHO, 2020b). Neuro-inflammation is a key pathological process that eventually forms millary TbM, increasing endovascular pressure, cranial nerve infarction, and obstruction in hydrocephalus that can be observed in computed tomography scan or magnetic resonance imaging (Donovan *et al.*, 2019). Other clinical signs and symptoms of TbM include recurrent periods of chills and fever, headache, abdominal pain, vomiting, altered cautiousness, nausea, hepatomegaly, and hypertension (Rohlwink *et al.*, 2019). Various host genetic factors regulating immunological pattern recognition molecules, such as Toll-like receptors polymorphisms were found to render susceptibility to TbM (Faksri *et al.*, 2018; Gagneux *et al.*, 2006; Thuong *et al.*, 2007).

*Corresponding Author
Bhartendu Nath Mishra, Department of Biotechnology, Institute of Engineering and Technology, Dr. A. P. J. Abdul Kalam Technical University, Lucknow, India. E-mail: prof.bnmishra.iet@gmail.com

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Vaccine and antibiotics currently used in the treatment are also facing their limitations such as increase in the probability of the emergence of MDR Mycobacterium strains which is due to long treatment duration and improper administration of drugs (Cresswell et al., 2019; Singh et al., 2019). The main drawbacks of current conventional anti-tubercular agents are the hepatotoxicity, various adverse side effects and development of MDR (Singh et al., 2019). Drug-resistant bacteria require higher doses of antibiotics that often cause intolerable toxicity (Dookie et al., 2018). Similarly, a vaccine that is currently used to cure Tb is bacillus Calmette–Guérin (BCG) produced from the live, attenuated Mycobacterium bovis. It induces some immune-activating factors and prevents TbM in children, but provide a limited contribution to the cure of patient suffering from pulmonary and latent Tb (Barry et al., 2009). Thus, to overcome the limitations of BCG and to reduce the Tb infection at initial stages, more efficient vaccines are required (Andersen and Doherty, 2005; Darrah et al., 2019; Nguipdop et al., 2016; Nieuwenhuizen and Kaufmann, 2018). The advent of reverse vaccine technology has reduced the time duration and cost of vaccine production over conventional methods. Although many vaccines including whole-cell derived vaccines, recombinant BCGs (Honda et al., 2008; WHO, 2017), recombinant viral vectors, mycobacterial extracts, protein-adjuvant combinations, and reverse vaccine-derived epitope vaccines are produced but they are still in pre-clinical phase or different phases of clinical trials (Sable et al., 2020). Two anti-Tb agent’s bedaquiline (class: diarylquinoline) and delamanid (class: nitroimidazoles) have been introduced to the market (Evans et al., 2016; Grzelak et al., 2019), but soon during the retrospective study on 24 cases of MTb in Iraq, Ghajavand et al. (2019) reported their resistant strain (Polsfuss et al., 2019; Veziris et al., 2017). Therefore, there remains an urgent need to discover new anti-Tb drugs that can shorten the treatment period and overcome the growing problem of drug resistance (Young et al., 2019).

Recently, the epitope-based vaccine designing technique has successfully used in finding the control of infectious diseases like shigellosis (Pahl et al., 2017). In this way, epitope-based vaccine designing is becoming a powerful tool in the stimulation of cellular and humoral immunity against infectious diseases (Majid and Andleeb, 2019).

Outer membrane and secreted proteins of MTb are required for membrane integrity, protection from toxins and are also necessary for pathogenicity and virulence. These proteins help in nutrition uptake as well as guide the bacterial multidrug-efflux pump to extrude the therapeutic drugs thus, enabling resistance in MTb strain when it is inside the host macrophage (Young et al., 2019). Goldberg et al. (2012) in their study discussed that the virulence decreased in peptidoglycan [outer membrane protein (OMP)] mutated strains. In another study by Stamm et al. (2019) they depicted that exoprotoome consisting of membrane as well as secreted proteins of MTb that interacts with the eukaryotic membrane to induce host dependent interaction with the Tb bacterium. Therefore, it was hypothesized that OMPs prove to be efficient target for vaccine designing.

In the present study, comparative proteome analysis and reverse-vaccine based techniques have been applied to design a chimeric multi-epitope vaccine against drug-resistant MTb.

MATERIALS AND METHODS

The complete protocol of the study is summarized as a flow chart in Figure 1.

**Proteome selection**

The genome of drug-sensitive and multidrug-resistant clinical strains of MTb along with reference strains of pathogenic (MTB H37rv), as well as of non-pathogenic (MTB H37ra) and BCG Bovine were retrieved from NCBI. To select a suitable genome sequence, an online database Public database for
molecular typing and microbial genome diversity (PubMLST) (Zeng et al., 2017) was used. The proteome of selected strain was retrieved from NCBI and in-silico reverse vaccinology techniques were applied in identifying the potential vaccine targets.

Prediction of novel antigenic proteins and their localization

Vaxign server (He et al., 2010) (http://www.violinet.org/vaxign/index.php) [genome and proteome-based vaccine prediction server using filters like transmembrane regions (TM), subcellular localization, adhesion properties] was used for predicting the consensus vaccine candidates (antigenic proteins). The complete proteome of the selected strain in the FASTA format was subjected and the experimental threshold value was assigned as 0.51. All the proteins with values ≥0.51 were considered to possess good adhesion property and selected as consensus antigens. To reduce any cross-reactivity between the developed vaccine and human cell, only non-homologous proteins were considered as vaccine candidates. For this, BLASTp (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) analysis was carried out and the sequences having an expectation value (E-value) ≤ 10^-4 were considered as homologous sequences and were excluded from the study. Furthermore, the localization of the non-homologous above-identified proteins was sorted as extracellular, periplasmic, OMPrs (Laal and Zolla-Pazner, 2010), inner membrane, or cytoplasmic using PSORTb 3.0.2 (database for subcellular location of proteins) (Yu et al., 2010), and CELLO (predictive software determining the protein cellular location of bacteria based on support vector machine based on n-peptide composition) (Yu et al., 2006) servers.

Prediction of signal peptide antigens

Signaling nature [classical, non-classical secreted proteins as well as proteins with GPI (Glycosylphosphatidylinositol)-anchor] of the above-selected proteins were predicted by SignalP 4.1, SecretomeP 2.0, and PredGPI (Angala et al., 2014; Pierleoni et al., 2008), respectively. Based on the Sec-dependent pathway, SignalP 4.1 (Henrik, 2017) server was applied for the prediction of the classical group of secretory proteins. The positional limit for prokaryote organisms was set as 70 residues truncation and for remaining parameters, default values were considered. SecretomeP version 2.0 (Bendtsen et al., 2004, 2005) was used for the prediction of non-classical groups of secretory proteins by selecting the default values/options and all the proteins having N–N score ≥ 0.5 were considered as non-classical secreted proteins. Similarly, PredGPI (Angala et al., 2014; Pierleoni et al., 2008) was also used to predict both the presence of the GPI-anchor and the position of the ω-site using default values.

Antigenicity and allergenicity prediction of the screened proteins

After getting insight into the signaling nature of the above-selected proteins, VaxJen and AntigenPro web servers were used for the screening of consensus antigenic proteins (Doytchinova and Flower, 2007; Magnan et al., 2010) using the default parameter (threshold value > 0.7). Proteins predicted as positive by both the tools were considered as consensus antigens and were subjected to Allergen FP v.1.0 (Dimitrov et al., 2014) tool to investigate the allergic nature of the selected proteins employing the default parameters.

Characterization of physicochemical properties of proteins

TM of the non-allergic proteins were checked using the Transmembrane hidden Markov model (TMHMM) method (Krogh et al., 2001). ABTGP PRO server (Cheng et al., 2005) (http://scratch.proteomics.ics.uci.edu/) was used to characterize whether a selected protein sequence belongs to transmembrane protein or not. This server also described the probabilities of TM as an alpha-helical or a beta barrel transmembrane protein. As the protein should be soluble in the cytoplasm during over expression in Escherichia coli during large scale vaccine production; therefore, the SOLPRO (Magnan et al., 2009) tool was used to depict the solubility of the selected proteins. Finally, the sequence similarity between the selected OMPs of MTb and its intraspecies, i.e., MTb complex (MTbC) was also observed by the Ortho MCL database (Chen, 2006).

T-cell (MHC-I, MHC-II) and B-cell epitopes prediction

For potent epitopes identification, T-cell epitope analysis was performed using four servers (i) Immune Epitope Database (IEDB) Major Histocompatibility Complex (MHC-I) prediction server, (ii) Peptides Naturally Processed by Major Histocompatibility Complex (MHC-NP), (iii) NetCTLPan1.1, and (iv) NetMHCpan 3.0. The IEDB (http://tools.immuneepitope.org/processing/) MHC-I prediction server with default parameters was used to identify the epitopes having the possibility to interact with MHC-I proteins. MHC elution-pattern-based server MHC-NP (http://tools.immuneepitope.org/mhcnp/) was used to predict the probability of a selected peptide can be processed naturally or not. Similarly, the NetCTLPan1.1 server (http://www.cbs.dtu.dk/services/NetCTLPan/) was used to predict the cytotoxic lymphocyte epitopes of proteins. Finally, NetMHCpan3.0 server (http://www.cbs.dtu.dk/services/NetMHCpan/) was used to predict the ability of peptide-MHC class I binding.

Consensus T-cell epitopes having the binding ability to MHC class II molecules were identified by four prediction servers like IEDB, MHC Class-II (http://tools.iedb.org/mhcii/), Propred (https://webs.iiitd.edu.in/raghava/propred/index.html), and NetMHC-II (http://tools.iedb.org/services/NetMHCIId-2.2/) under default parameter conditions.

Similarly, B-cell epitopes prediction was done by ABCPred (https://webs.iiitd.edu.in/raghava/abcpred/ABCsubmission.html), the BCpred (https://webs.iiitd.edu.in/raghava/bcpred/bcpred_submission.html), and IEDB server (http://tools.iedb.org/bcell/) with cut-off score value >0.8. Common epitopes in all three servers were considered for further studies.

Epitope characterization

Above predicted epitopes were compared and the common antigenic epitopes were subjected to the IEDB server to identify the epitopes having immunogenic property. The epitopes showing a positive immunogenicity score were shortlisted for antigenic analysis using VaxJen version 2.0 (Doytchinova and Flower, 2007). According to the criteria described by Khan et al. (2019), peptides showing score value ≥1.0 were selected for the toxicity prediction by the ToxinPred tool.
For the chimeric vaccine, epitopes should be hydrophilic (present on the surface), otherwise they will not be able to initiate the immune reaction in the host cell. The epitope hydropathy was analyzed through the grand average of hydropathy (GRAVY) score analysis through the ProtParam tool. The GRAVY value of epitope was calculated by using the following calculation:

\[ G = \frac{\varepsilon}{N} \]

\( G = \) grand average of hydropathy value; \( \varepsilon = \) hydropathy values of amino acids; \( N = \) number of amino acid residues in a given protein

A positive value of GRAVY score indicates the hydrophobic nature and a negative value suggests the hydrophilic nature of proteins.

“MHC restricted allele prediction tool” of the IEDB server was used to identify the MHC class I and II-restricted epitopes. Identified epitopes were further crosschecked by the MHCellucrator 2.0 server to confirm the above prediction (Thomsen et al., 2013).

Construction of chimeric vaccine

The chimeric vaccine sequences were designed manually using the results of epitopes analysis. Overlapping sequences of epitopes were merged and three chimeric vaccine candidates (VC1, VC2, and VC3) were constructed by the protocol described by Solanki et al. (2019). Briefly, all the selected epitopes were joined using universal amino acid linker sequences (HEYGAEALERAG and GGGG). Further to enhance the Immunogenicity of constructs distinct adjuvant were added using “EAAAK” linkers at both the termini (N and C). The adjuvant used for VC1, VC2 and VC3 were 50s ribosomal L7/L12 protein (Lee et al., 2014), beta-defensin and HBHA respectively. Further to enhance the vaccine competence, a sequence of 13 amino acid universal epitope (AKVAAWTLKAAC) also known as non-natural pan-DR (PADRE) (Alexander et al., 2000) was used.

Characterization of vaccine constructs

The above three vaccine constructs were analyzed according to antigenicity, allergenicity, and solubility prediction. For the prediction of allergenic nature, the AlgPred server (Marti et al., 2007) was used, whereas the antigenicity of the constructs was predicted using ANTIGENpro (Magnan et al., 2010) and VaxiJen 2.0 server. Solubility and corresponding probability (>=0.5) of the vaccine constructs were predicted by the SOLpro server (Magnan et al., 2009).

Physiochemical properties [amino acids count, Isoelectric Point (PI) values, their molecular weight, hydropathicity GRAVY score, aliphatic, and instability index] of the vaccine constructs were characterized using the Expsys ProtParam server (Gasteiger et al., 2005). The 2nd structure of all three vaccine constructs was predicted by PSIPRED v3.3 program (McGuffin et al., 2000). Furthermore, the tertiary structures of the vaccine constructs (VC1, VC2, and VC3) were predicted by the Phyre2 (Kelley et al., 2015) online tool. The structures were saved in .pdb file format.

Molecular docking study

Interaction studies of vaccine constructs (VC1, VC2, and VC3) with 10 different HLA alleles (Axelsson et al., 2015) [(HLA-A*02:01(6EQA), HLA-A*24:02 (4F7M), HLA-B*15:01 (1XR8), HLA-B*35:01 (1A1N), HLA-B*39:01 (4O2E), HLA B*44:02 (1N2R), HLA-B*58:01 (5IM7), HLA-DR2 (DRA*0101, DRB1*1501) (1BX2), HLA-DRA1*0101/DRB5*0101 (1H15) and HLA-DQ2.3 (DQA1*03:01/DQB1*02:01) (4D8P)] was performed using the PatchDock server. 3D structures of all the HLA alleles were obtained from the protein data bank Research Collaboratory for Structural Bioinformatics - Protein Data Bank (RCSB-PDB) and saved in the .pdb file format. The best 10 solutions to the PatchDock were further refined by FireDock.

Codon optimization and in-silico cloning of vaccine construct

Codon optimization was performed by Java Codon Adaptation Tool (JCAT) to enhance the production of heterologous protein (vaccine construct) in E. coli (Chauhan et al., 2019). During optimization, the rho-independent transcription terminators, prokaryotic ribosomal binding sites, and few restriction sites were kept constant. The expression of the vaccine construct was predicted by the Snapgene tool after cloning the gene sequence of a construct in E. coli pET28a vector (Solanki et al., 2019).

RESULTS AND DISCUSSION

Comparative subtractive proteomic approach to screen the MTb strains

The complete genome sequences of seventeen MTb strains were compared by PubMLST and the results are summarized in Table 1. Out of 17 strains, 6 having drug-resistance were found suitable for the study. Furthermore, among the six selected strains, only three clinical strains showing their isolation source from the cerebrospinal fluid sample having a greater possibility to possess MTb virulence were screened. The proteome of possible three clinical strains were further analyzed for similar proteins using multiple alignment tools (data not shown). Finally, to reduce redundancy and based on alignment, MLST values, proteome size the MTb strain LJ319 (NZ_CP026742.1) having 4,025 proteins was selected for the study (Hatolkar et al., 2018).

Prediction of novel antigens

To identify the potential proteins for vaccine construct, all the 4,025 proteins of reference proteome (LJ319) were filtered according to their subcellular localization using vaxign, CELLO and PSORTb tool. Out of 4,025 proteins, 982 different proteins having their localization either in the periplasmic or the extracellular or outer membrane of the bacterial cell were found suitable for the study (data not shown). The rest of the proteins that were present either in the cytoplasm or inner cytoplasmic membrane region were excluded from the study. Cellular localization of bacterial Possibly surface exposed (PSEs) and outer membrane plays an essential role in pathogenesis such as drug efflux pumps, permeability barrier; membrane protein also helps in integrity, active transport, and diffusions of nutrients (Angala et al., 2014). Sajjad et al. (2020) during the study of Acinetobacter nosocomialis also used the above tool for designing multi epitope vaccine which depicts the authenticity of the results obtained through the tools.

Above screened 982 proteins were examined for their adhesion nature through vaxign server, and only 165 OMPs were found to possess the adhesion property. Adhesion and signal
Table 1. Complete genome sequences of seventeen M. tuberculosis strains compared by PubMLST (Multi Locus Sequence Typing).

| S.No. | Accession No. | Strain Name | Isolated Location | Sample Type | Drug Resistants | Proteome | MLST |
|-------|---------------|-------------|-------------------|-------------|-----------------|----------|------|
| 1     | NC_000962.3   | H37Rv       | USA               | Sputum      | -               | 3906     | ST215 |
| 2     | NC_009525.1   | H37Ra       | USA               | Sputum      | -               | 4127     | ST215 |
| 3     | NC_008769.1   | M. bovis BCG Pasteur 1173P2 | France | Bovine | - | 3977 | ST268 |
| 4     | NC_017522.1   | CCDC5180    | China-Beijing Family Lineage | Sputum      | +               | 4048     | ST276 |
| 5     | NZ_AP018033.1 | HN-024      | Vietnam-East African-Indian Family Lineage | Sputum      | -               | 4062     | ST215 |
| 6     | NZ_CP028428.1 | CAS         | India             | CSF         | +               | 4014     | ST276 |
| 7     | NZ_CP026742.1 | LJ319       | India             | CSF         | +               | 4025     | ST276 |
| 8     | NZ_CP010968.1 | PR10        | Malaysia          | CSF         | +               | 4015     | ST215 |
| 9     | NZ_CP019612.1 | H107        | Hong Kong         | CSF         | -               | 4118     | ST215 |
| 10    | NZ_CP010895.1 | PR08        | Malaysia          | CSF         | -               | 3951     | ST215 |
| 11    | NZ_CP009186.1 | TRS2        | USA               | CSF         | -               | 4066     | ST215 |
| 12    | NZ_CP023170.1 | C3          | India             | CSF         | -               | 3922     | ST215 |
| 13    | NZ_CP029065.1 | TBMENG-03   | India             | Sputum/CSF  | -               | 4045     | ST215 |
| 14    | NZ_CP018778.1 | DK9897      | Denmark           | Sputum      | -               | 4098     | ST319 |
| 15    | NZ_CP029326.1 | LJ338       | India             | Sputum      | +               | 4023     | ST276 |
| 16    | NZ_CP023169.1 | S3          | India             | Sputum      | -               | 3980     | ST215 |
| 17    | NC_017524.1   | CTRI-2      | Russia            | Sputum      | +               | 4098     | ST279 |

Table 4. Results of SignalP, SecretomeP 2.0, and PredGPI for the study; summarized in Table 2.

Characterization of physiochemical properties of proteins

The identification of TM α-helices by TMHMM method suggests that all the 36 OMPs containing either 0 or 1 helix, confirming their presence in the outer membrane region (Supplementary Table 3). Hence, the results of the TMHMM method validate the finding of vaxign, CELLO and PSORTb tool. Furthermore, when all the 36 OMPs were subjected to the AllergenPro web server to find out if their exist any allergic tendency, then 1 OMP (WP_003401880.1) was found to pose allergic behavior in the host cell, and therefore excluded from the further studies. The solubility of the above OMPs were examined through SOLPro suggesting that out of 35 OMPs, 22 OMPs were soluble whereas, rest 13 OMPs having insoluble nature during overproduction in vitro were excluded from the race of potential vaccine candidates (Supplementary Table 3).

To reduce the pathogenesis of TbM infection, a potential vaccine candidate should have a tendency to also identify the associated intra-pathogenic species. Therefore, the presence of the orthologous sequences of the MTbC in the above selected 22 OMPs were detected by the Ortho MCL server and the results are summarized in Supplementary Table 4. Results suggest that out of 22 OMPs, 6 proteins (WP_031663355.1, WP_031661316.1, WP_016330440.1, WP_009938581.1, WP_003910913.1 and WP_003900236.1) were not common in all the 5-members of MTbC, therefore, were excluded from the potential vaccine candidates list and the filtered 16 OMPs were selected for further studies. Palucci et al. (2016) observed that even a few GGA-GGN repeats of PE/PPE proteins can play an important role in Tb pathogenesis and provide immunity to host by activating the TLR2-dependent MTb entry into macrophages. Hence, suggests that the family group such as PPE, PE and PE_PGRS proteins influences the antigenic variation and immune system evasion. In another study Ocampo et al. (2014) depicted that antigen Rv1911c (LppC), are lipoproteins representing an important protein present on the cell envelope thereby enhancing MTb pathogen’s virulence. Therefore, considering their important feature these proteins were
Table 2: Proteome analysis of LJ319 (NZ_CP026742.1) strain of *M. tuberculosis* and outer membrane protein characterization using different servers. 1: Localization using CELLO, PSORBTb; 2: Adhesion property using Vaxign server; 3,4,5: Protein signaling and GPI-anchor by SignalP, SecretomeP and PredGPI; 6,7: Antigenicity prediction using Vaxijen, AntigenPro; 8: Allergenicity by AllergenPro Server.

| S.No. | Protein Accession | Protein Name                  | Localization1 | Adhesin Probability2 | SignalP3 | SecretomeP4 | PredGPI5 | VaxiJen6 | AntigenPro7 | AllergenPro8 |
|-------|------------------|-------------------------------|---------------|----------------------|----------|-------------|----------|----------|-------------|--------------|
| 1     | WP_104857305.1   | PE family protein            | Cytoplasmic   | 0.651                | 0.476    | -           | -        | 1.375    | 0.716       | NON-ALLERGEN |
| 2     | WP_104857303.1   | CAP domain-containing protein | Unknown       | 0.530                | 0.781    | 0.609       | -        | 0.737    | 0.915       | NON-ALLERGEN |
| 3     | WP_078800718.1   | MULTISPECIES: PE family protein, partial | Cytoplasmic Membrane | 0.695                | 0.495    | -           | -        | 1.013    | 0.769       | NON-ALLERGEN |
| 4     | WP_031744040.1   | PE family protein, partial   | Cytoplasmic Membrane | 0.637                | -        | 0.519       | 0.341   | 1.192    | 0.732       | NON-ALLERGEN |
| 5     | WP_031666010.1   | PE family protein            | Cytoplasmic Membrane | 0.651                | 0.567    | 0.857       | -        | 1.983    | 0.734       | NON-ALLERGEN |
| 6     | WP_031663355.1   | YncE family protein, partial | Extracellular  | 0.533                | -        | 0.868       | -        | 1.273    | 0.867       | NON-ALLERGEN |
| 7     | WP_031661316.1   | MULTISPECIES: hypothetical protein | Unknown     | 0.661                | 0.494    | -           | -        | Y        | 0.871       | 0.928439    |
| 8     | WP_031647515.1   | PE domain-containing protein | Cytoplasmic Membrane | 0.610                | 0.595    | 0.641       | -        | 0.873    | 0.922       | NON-ALLERGEN |
| 9     | WP_016330440.1   | PE family protein            | Cytoplasmic Membrane | 0.717                | 0.472    | 0.870       | -        | 2.222    | 0.893       | NON-ALLERGEN |
| 10    | WP_010886074.1   | MULTISPECIES: PE family protein | Cytoplasmic Membrane | 0.680                | -        | 0.839       | -        | 1.985    | 0.727       | NON-ALLERGEN |
| 11    | WP_009938654.1   | MULTISPECIES: PE family protein | Unknown     | 0.686                | 0.475    | 0.926       | -        | 2.081    | 0.750       | NON-ALLERGEN |
| 12    | WP_009938581.1   | PE family protein            | Extracellular  | 0.722                | -        | 0.871       | -        | 2.114    | 0.765       | NON-ALLERGEN |
| 13    | WP_003918025.1   | Mce associated membrane protein | Unknown     | 0.551                | -        | 0.754       | -        | 0.809    | 0.937       | NON-ALLERGEN |
| 14    | WP_003910913.1   | MULTISPECIES: PE family protein | Extracellular | 0.701                | 0.566    | 0.831       | -        | 2.093    | 0.817       | NON-ALLERGEN |
| 15    | WP_003910446.1   | MULTISPECIES: PE family protein | Extracellular | 0.709                | -        | 0.885       | -        | 1.905    | 0.704       | NON-ALLERGEN |
| 16    | WP_003909110.1   | MULTISPECIES: hypothetical protein | Unknown     | 0.563                | -        | 0.886       | -        | 0.786    | 0.957       | NON-ALLERGEN |
| 17    | WP_003905853.1   | resuscitation-promoting factor rpfE | Unknown     | 0.593                | 0.716    | 0.759       | -        | 0.794    | 0.928       | NON-ALLERGEN |
| 18    | WP_003901898.1   | DUF3060 domain-containing protein | Extracellular | 0.575                | 0.638    | 0.827       | -        | 0.961    | 0.894       | NON-ALLERGEN |
| 19    | WP_003901751.1   | MULTISPECIES: type VII secretion system ESX-1 associated protein EspJ | Unknown | 0.696                | -        | 0.833       | -        | 0.743    | 0.949       | NON-ALLERGEN |
| 20    | WP_003901367.1   | hypothetical protein          | Unknown       | 0.608                | 0.564    | 0.871       | -        | 0.815    | 0.915       | NON-ALLERGEN |
| 21    | WP_003900461.1   | hypothetical protein          | Unknown       | 0.516                | -        | 0.619       | -        | 0.817    | 0.865       | NON-ALLERGEN |

Continued
| S.No. | Protein Accession | Protein Name | Localization | Adhesin Probability | SignalP3 | SecretomeP4 | PredGPI5 | VaxiJen6 | AntigenPro7 | AllergenPro8 |
|-------|------------------|--------------|--------------|---------------------|----------|-------------|---------|---------|-------------|-------------|
| 22    | WP_003900236.1   | MULTISPECIES: phosphate-binding protein PstS | Unknown      | 0.555               | 0.578    | 0.632093    | Y       | 0.7332   | 0.860693    | NON-ALLERGEN |
| 23    | WP_003900226.1   | MULTISPECIES: PPE family protein PPE13 | Cytoplasmic Membrane | 0.573               | -        | 0.714397    | -       | 0.7535   | 0.650685    | NON-ALLERGEN |
| 24    | WP_003898733.1   | MULTISPECIES: hypothetical protein | Cytoplasmic Membrane | 0.552               | -        | 0.751933    | -       | 0.7079   | 0.917996    | NON-ALLERGEN |
| 25    | WP_003898652.1   | MULTISPECIES: phosphate-binding protein PstS | Extracellular | 0.689               | 0.567    | 0.882589    | -       | 0.7777   | 0.938864    | NON-ALLERGEN |
| 26    | WP_003420544.1   | MULTISPECIES: hypothetical protein | Unknown      | 0.565               | 0.578    | 0.821116    | -       | 1.1519   | 0.954752    | NON-ALLERGEN |
| 27    | WP_003416124.1   | MULTISPECIES: hypothetical protein | Cytoplasmic Membrane | 0.584               | -        | 0.971893    | -       | 0.7155   | 0.890375    | NON-ALLERGEN |
| 28    | WP_003409568.1   | MULTISPECIES: hypothetical protein | Unknown      | 0.661               | -        | 0.669988    | -       | 0.8804   | 0.908044    | NON-ALLERGEN |
| 29    | WP_003409409.1   | MULTISPECIES: hypothetical protein | Cytoplasmic Membrane | 0.550               | 0.588    | 0.605829    | Y       | 0.7521   | 0.891223    | NON-ALLERGEN |
| 30    | WP_003407152.1   | MULTISPECIES: membrane protein | Unknown      | 0.569               | -        | 0.939834    | -       | 0.7517   | 0.938426    | NON-ALLERGEN |
| 31    | WP_003405142.1   | MULTISPECIES: FmdB family transcriptional regulator | Unknown | 0.692               | -        | 0.854831    | -       | 1.2480   | 0.765297    | NON-ALLERGEN |
| 32    | WP_003404775.1   | MULTISPECIES: phosphate-binding protein PstS | Unknown      | 0.622               | 0.546    | 0.876684    | -       | 0.8066   | 0.936169    | NON-ALLERGEN |
| 33    | WP_003402239.1   | MULTISPECIES: PPE family protein PPE10 | Cytoplasmic Membrane | 0.558               | -        | 0.742658    | -       | 0.7709   | 0.863596    | NON-ALLERGEN |
| 34    | WP_003401880.1   | MULTISPECIES: hypothetical protein | Extracellular | 0.602               | -        | 0.918108    | -       | 2.1849   | 0.893926    | ALLERGEN     |
| 35    | WP_003400534.1   | MULTISPECIES: single-stranded DNA-binding protein | Cytoplasmic | 0.574               | -        | 0.541185    | -       | 0.7244   | 0.841163    | NON-ALLERGEN |
| 36    | WP_003399940.1   | MULTISPECIES: type VII secretion system ESX-1 WXG100 family target CFP-10 | Extracellular | 0.512               | -        | 0.855303    | -       | 0.7826   | 0.891476    | NON-ALLERGEN |
Table 3. Screening of potential vaccine candidates for transmembrane regions\textsuperscript{1,2} and solubility property\textsuperscript{3} during over-expression in plasmid vector in *E. coli* during vaccine production.

| S.No. | Protein Accession       | THMHH1 | Trans-membrane helices | SolPro3       |
|-------|-------------------------|--------|------------------------|---------------|
| 1     | WP_104857305.1          | Non Transmembrane protein | 0            | SOLUBLE       |
| 2     | WP_104857303.1          | Non Transmembrane protein | 0            | INSOLUBLE     |
| 3     | WP_078800718.1          | Non Transmembrane protein | 0            | SOLUBLE       |
| 4     | WP_031744040.1          | Non Transmembrane protein | 0            | SOLUBLE       |
| 5     | WP_031666010.1          | Non Transmembrane protein | 0            | INSOLUBLE     |
| 6     | WP_031663355.1          | Non Transmembrane protein | 0            | SOLUBLE       |
| 7     | WP_031661316.1          | Non Transmembrane protein | 0            | SOLUBLE       |
| 8     | WP_031647515.1          | Non Transmembrane protein | 0            | SOLUBLE       |
| 9     | WP_016330440.1          | Non Transmembrane protein | 0            | SOLUBLE       |
| 10    | WP_010886074.1          | Non Transmembrane protein | 0            | INSOLUBLE     |
| 11    | WP_009938654.1          | Non Transmembrane protein | 0            | SOLUBLE       |
| 12    | WP_009938581.1          | Non Transmembrane protein | 0            | SOLUBLE       |
| 13    | WP_003918025.1          | Non Transmembrane protein | 0            | INSOLUBLE     |
| 14    | WP_003910913.1          | Non Transmembrane protein | 0            | SOLUBLE       |
| 15    | WP_003910446.1          | Non Transmembrane protein | 0            | SOLUBLE       |
| 16    | WP_003901367.1          | Non Transmembrane protein | 0            | SOLUBLE       |
| 17    | WP_003905853.1          | Non Transmembrane protein | 0            | SOLUBLE       |
| 18    | WP_003901898.1          | Non Transmembrane protein | 0            | INSOLUBLE     |
| 19    | WP_003901751.1          | Non Transmembrane protein | 0            | INSOLUBLE     |
| 20    | WP_003901367.1          | Non Transmembrane protein | 0            | SOLUBLE       |
| 21    | WP_003900461.1          | Non Transmembrane protein | 0            | SOLUBLE       |
| 22    | WP_003900236.1          | Non Transmembrane protein | 0            | SOLUBLE       |
| 23    | WP_003900226.1          | Non Transmembrane protein | 0            | SOLUBLE       |
| 24    | WP_003898733.1          | Non Transmembrane protein | 0            | INSOLUBLE     |
| 25    | WP_003898652.1          | Non Transmembrane protein | 0            | INSOLUBLE     |
| 26    | WP_003420544.1          | Non Transmembrane protein | 0            | INSOLUBLE     |
| 27    | WP_003416124.1          | Non Transmembrane protein | 1            | SOLUBLE       |
| 28    | WP_003409568.1          | Non Transmembrane protein | 0            | SOLUBLE       |
| 29    | WP_003409409.1          | Non Transmembrane protein | 1            | SOLUBLE       |
| 30    | WP_003407152.1          | Alpha Helical Transmembrane protein | 1      | INSOLUBLE     |
| 31    | WP_003405142.1          | Non Transmembrane protein | 0            | SOLUBLE       |
| 32    | WP_003404775.1          | Non Transmembrane protein | 0            | SOLUBLE       |
| 33    | WP_003402239.1          | Non Transmembrane protein | 0            | INSOLUBLE     |
| 34    | WP_003401880.1          | Non Transmembrane protein | 0            | INSOLUBLE     |
| 35    | WP_003400534.1          | Non Transmembrane protein | 0            | INSOLUBLE     |
| 36    | WP_003399940.1          | Non Transmembrane protein | 0            | INSOLUBLE     |
including among above selected 16 OMPs (Abraham et al., 2018; Kavvas et al., 2018; Phelan et al., 2016).

T-cell (MHC-I, MHC-II) and B-cell epitopes prediction

All the 16 OMPs when subjected to IEDB server for epitopes prediction, then based on higher affinity [Inhibitory Concentration (IC) < 50 nM] and good percentile rank (≤0.2), 221 MHC-I, 69 MHC-II, and 81 B-cell epitopes were filtered. To further refine the IEDB prediction for MHC-I and II binding epitopes, MHC-NP, netCTL, netMHC, and Propred tools were used. As a result, 159 MHC-I and 41 MHC-II epitopes, found common in the results of these tools were selected for characterization studies. Similarly, B-cell epitopes were filtered by IEDB, BepiPred linear epitope prediction servers, BCPREDS and ABCPred tools suggesting 31 common B-cell epitopes were suitable for the study.

Epitope characterization

Immunogenicity, antigenicity and toxicity prediction of epitopes

The above selected MHC-I (159), MHC-II (41), and B cell epitopes (31) were subjected to the IEDB immunogenicity prediction tool to check immunological behavior of the epitopes. Using immunogenicity score (>0.038 cutoff value), 98 and 34 MHC-I and MHC II epitopes respectively out of 159 MHC-I and 41 MHC-II epitopes, were picked for further studies that showed higher potency to stimulate naïve T cells and also to induce cell-mediated immunity, results are in (Supplementary Table 5). Furthermore, the antigenicity of selected MHC I and II epitopes was evaluated by the VaxiJen web server. A total of 51 (29 MHC-I + 22 MHC-II) epitopes containing antigenicity values more than 0.7 were considered as the potent epitopes (Supplementary Table 5). Similarly, out of 31 B-cell epitopes, only 19 were found immunogenic and antigenic epitopes (Supplementary Table 5).

In the next step, cross-reactivity induced by epitopes in the host tissue was figured out through the ToxinPred server and all the 70 epitopes were found to be non-toxic.

Physiochemical analysis of epitopes

The physiochemical properties of epitopes were explored by GRAVY analysis through the ProtParam tool. According to the considered criteria, 40 epitopes (13 from MHC I, 12 from MHC II, and 15 from B cell) having VaxiJen score value >1.0 were subjected to the GRAVY analysis. Among 40 epitopes, only 26 epitopes (MHC I: 4, MHC II: 7, and B cell: 15) having negative score values were predicted as hydrophilic. Above screened hydrophilic epitopes are possibly present in the outer surface, and therefore have a greater tendency to initiate the immunogenicity

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### Table 4: Screening of orthologs associated intra-pathogenic species in the Mycobacterium tuberculosis complex (MTbC) using OrthoMCL.

| S.No. | Protein Accession | M. bovis | M. africanum | M. kansasii | M. microti | M. canettii |
|-------|------------------|----------|--------------|-------------|------------|------------|
| 1     | WP_104857305.1   | A0A0H3M749 | A0A1201X13   | X7ZDK8     | A0A1201WV9 | G0TP77     |
| 2     | WP_078800718.1   | A0A1R3XWC7 | A0A1201ZZ7   | X7Y8A8     | A0A109SL38 | G0TN80     |
| 3     | WP_021744040.1   | A0A1A9E8H7 | A0A109SXX1   | A0A1X0KMN3 | A0A109SPU3 | G0TM4      |
| 4     | WP_021663355.1   | A0A1A9E4Q1 | A0A109SVA7   | -           | A0A109SLK3 | G0TG89     |
| 5     | WP_021661316.1   | A0A1A9E4Q4 | A0A109SV94   | X7ZK8      | A0A109SLK3 | G0TG89     |
| 6     | WP_021664515.1   | A0A1A9EC74 | -            | U5WT40     | A0A109SM93 | G0TG84     |
| 7     | WP_021630440.1   | A0A1A9EC53 | A0A109T0N7   | U5WT45     | A0A109SRH6 | L0PZ0      |
| 8     | WP_009938654.1   | A0A1A9EA0  | -            | A0A163RQ5L8 | A0A109SCH3 | G0TPF2     |
| 9     | WP_009938581.1   | A0A1A9E9H2 | A0A109SYL6   | U5WZ44     | -          | G0TPW3     |
| 10    | WP_003910913.1   | A0A1A9E7G2 | A0A1201J183  | X7Z3E9     | A0A109SNF0 | G0RK73     |
| 11    | WP_009091110.1   | A0A0H3MAA2 | A0A109T229   | A0A1V3WW93 | A0A1201Z35 | G0TL77     |
| 12    | WP_00905853.1    | A0A109S8N2 | A0A109SYLO   | A0A164DV1  | A0A1201X77 | G0TPD7     |
| 13    | WP_00901367.1    | A0A1A9E8T0 | A0A1201T3    | X7Z041     | A0A109SPE4 | G0TN11     |
| 14    | WP_00900461.1    | A0A1A9E6X4 | A0A109SYE1   | A0A1X0KN27 | A0A1201X73 | G0TMK9     |
| 15    | WP_00900236.1    | A0A1A9E5M0 | A0A109SUZ4   | -          | B2MV3     | G0TFS8     |
| 16    | WP_0090226.1     | A0A0A9E4C7 | A0A109SV30   | X7ZLG4     | A0A109SM5 | G0TFM5     |
| 17    | WP_003146124.1   | A0A0H3ME00 | A0A1202P4    | A0A1X0KXC9 | A0A109SRH5 | G0TGV5     |
| 18    | WP_003409568.1   | P67225    | A0A1201C8    | X7XDD2     | A0A109SNM9 | G0TLM5     |
| 19    | WP_003409490.1   | A0A0KHXD6 | A0A109SX79   | X7ZX04     | A0A1201WZ7 | G0TLE4     |
| 20    | WP_003405142.1   | A0A0H3M4Y6 | A0A109SV46   | A0A1X0KR97 | A0A109SLQ5 | G0TGA3     |
| 21    | WP_003404775.1   | A0A0H3MBK5 | A0A109SUV6   | A0A1X0KR45 | A0A109SLR5 | G0TFS2     |

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[Note: The table includes orthologs associated with intra-pathogenic species in the Mycobacterium tuberculosis complex (MTbC) using OrthoMCL.]
in the host cell. Hence, chimeric vaccine constructs were designed using all the 26 epitopes.

**MHC restriction and cluster analysis of selected epitopes**

After physiochemical analysis, the selected epitopes were further validated for the MHC interaction using the MHC cluster and the results are shown as a heat map (Fig. 2) and dynamic tree. The epitopes are clustered according to the interaction with HLA. The red color suggests strong interaction, while the yellow color indicates weak interaction. Selected 4 MHC I and 7 MHC II epitopes showed strong interaction with HLA genes.

**Construction of chimeric vaccine**

All the shortlisted 26 epitopes {4 MHC I epitopes (\texttt{CESGGNWSI}, \texttt{WPIRAPSRL}, \texttt{HYRFTLYHL}, and \texttt{RADRARNTY}), 7 MHC II epitopes (\texttt{YGNGGPGGA}, \texttt{WIYGHGGHG}, \texttt{VEGHTHTIS}, \texttt{IEGDDTDRR}, \texttt{VEGHTHTIS}, \texttt{YGNGGPGGA}, and \texttt{VEGHTHTIS}), and 15 B-cell epitopes (\texttt{AGAIGNGGDGGNGGTS}, \texttt{GSGGDGGNGGNAGLIG}, \texttt{GTGGNGGLLLGFNGTN}, \texttt{GGAGGNGGWLYGNGGP}, \texttt{GLLYGNGGNGGAGDTA}, \texttt{GGAGGAGGRGGWLVGN}, \texttt{GHAGGAGGAGGAGGRG}, \texttt{GGTGGDGGDGGHAGTG}, \texttt{NGGIGGDGAGGGNATS}, \texttt{GGNGGAGGDAGHGGTG}, \texttt{GGAGGNGATGGTGVGN}, \texttt{AGGGGGGTTPTGYLGP}, \texttt{GAGGGDVGGGGAGGTT}, \texttt{GNGNDGNTNFGSGNAG}, and \texttt{GGVGNARADRA RNTYT113)} were used to design the chimeric vaccine. Two linkers HEYGAERALER and GGGS were used to join the epitopes. 50S ribosomal protein L7/L12 (rplL) (Accession no. WP_003403353.1), beta-defensin (Accession no. WP_031737436.1), HBHA (Accession no. WP_094028633.1), and PADRE (AKVAWTLKAAAC) were successfully used as adjuvant (Lee et al., 2014) and linker (Alexander et al., 2000), respectively, for the construction of vaccine candidates by a linker “EAAAK” at both termini (N and C). Satyam et al. (2020) also used same adjuvant during the vaccine construction against Mycobacteroids. VC1, VC2, and VC3 prove their efficacy through their antigenic, allergenic, and toxicity analysis.

**Characterization of vaccine constructs**

**Antigenicity, allergenicity, and solubility prediction**

Antigenicity, allergenicity, and solubility of VC1, VC2, and VC3 were predicted by the ANTIGENpro, VaxiJen 2.0, AlgPred, and SOLpro server. The antigenicity score value >0.569 in ANTIGENpro and >1.5596 in VaxiJen 2.0 indicates a satisfactory antigenic property of all the three vaccines constructs. AlgPred server predicted the non-allergenic behavior of VC1, VC2, and VC3. Similarly, SOLpro showed good solubility (>0.9820) of these vaccine constructs during their heterologous expression in the \textit{E. coli}.

**Physicochemical analysis of designed vaccine constructs**

ProtParam server suggests the molecular weight of all vaccine constructs ranges between 59 and 72 kDa. All three vaccine constructs are steady in the corresponding pH (Table 6). A negative value (−0.544) of GRAVY (a hydrophilic index) analysis suggests...
Table 5. Identification of potent MHC I, MHC II and B cell epitopes and their characterization such as antigenicity, immunogenicity toxicity and hydrophilicity was performed using various servers.

| S.No. | Accession No. | Start | Stop | Epitopes            | IEDB      | MHC-NP     | NetCTLpan | NetMHCpan | IC50 | Vaxijen | Immunogen | Toxicity | GRAVY Score |
|-------|---------------|-------|------|---------------------|-----------|------------|-----------|-----------|------|---------|-----------|----------|-------------|
| 1     | WP_104857305.1 | 108   | 116  | AINAPTLAL           | HLA-B*07:02 0.26 | H-2-Db 0.8179 | HLA-B*07:02 0.80 | HLA-A*32:01 0.2 | 198.9 | 1.1606  | 0.07651   | Non-Toxin | 1.3         |
|       |               |       |      |                     | HLA-A*02:03 1.2 | HLA-B*07:02 0.5356 | HLA-B*07:02 0.4701 | HLA-B*15:01 0.8179 | 0.2 |         |           |          |             |
|       |               |       |      |                     | HLA-B*44:03 0.2369 | HLA-B*53:01 0.3353 | HLA-A*01:01:10 0.4631 | HLA-B*15:01 0.1159 | 0.11 |         |           |          |             |
|       |               |       |      |                     | HLA-B*35:01 0.0648 | HLA-A*02:01 0.1017 | HLA-A*01:01:10 0.4631 | HLA-B*35:01 0.0582 | 25.7 | 1.2638  | 0.10203   | Non       | 0.66        |
| 2     | WP_078800718.1 | 79    | 87   | ALSAGGAY            | HLA-B*15:01 0.07 | HLA-B*15:01 0.7221 | HLA-B*15:01 0.10 | HLA-A*01:07 0.80 | 10.7 | 0.7437  | 0.11191   | Non-Toxin | 1.56        |
|       |               |       |      |                     | HLA-B*35:01 0.38 | HLA-B*53:01 0.3556 | HLA-B*15:01 0.0210 | HLA-A*01:10 0.40 | 15.58 |         |           |          |             |
|       |               |       |      |                     | HLA-B*44:03 0.3559 | HLA-B*53:01 0.3082 | HLA-A*26:01 0.30 | HLA-A*01:10 0.40 | 0.7437 | 0.11191 |           |          |             |
| 3     | WP_031744040.1 | 46    | 54   | EVSVAISAL           | HLA-B*53:01 0.53 | HLA-B*53:01 0.5559 | HLA-B*15:01 0.10 | HLA-A*26:01 0.0785 | 154.2 | 0.7269  | 0.03446   | Non       | 1.69        |
|       |               |       |      |                     | HLA-B*35:01 0.3559 | HLA-B*44:03 0.3559 | HLA-A*26:01 0.30 | HLA-A*01:10 0.40 | 0.7437 | 0.11191 |           |          |             |
|       |               |       |      |                     | HLA-B*07:02 0.0542 | HLA-B*07:02 0.0542 | HLA-A*26:01 0.30 | HLA-A*01:10 0.40 | 0.7437 | 0.11191 |           |          |             |
| S.No. | Accession No. | Start | Stop | Epitopes | IEDB  | MHC-NP | NetCTLpan | NetMHCpan | IC50 | Vaxijen | Immunogen | Toxicity | GRAVY Score |
|-------|---------------|-------|------|----------|-------|--------|-----------|-----------|------|---------|------------|----------|-------------|
| 47    |               | 55    |      | VSVAISALF | HLA-B*44:03 0.48 | HLA-B*58:01 0.05 | HLA-B*15:01 0.20 | HLA-B*44:03 0.4831 | H-2-Db 0.4565 | HLA-B*53:01 0.4089 | HLA-B*57:01 0.2049 | H-2-Kb 0.1861 | HLA-B*35:01 0.0636 | HLA-B*53:01 0.0412 | HLA-B*15:01 0.2037 | 2.39 |
| 127   |               | 135   |      | ALNAGAGSY | HLA-B*44:03 0.29 | HLA-B*53:01 0.62 | HLA-B*35:01 0.15 | HLA-B*44:03 0.7629 | H-2-Db 0.3628 | HLA-B*35:01 0.1000 | H-2-Kb 0.0703 | H-2-Kb 0.0638 | HLA-B*44:03 0.5490 | HLA-B*53:01 0.0533 | HLA-B*35:01 0.0463 | 0.31 |
| 8     | WP_009938654.1 | 43    | 51   | GADEVSAAL | HLA-B*44:03 0.27 | HLA-B*07:02 0.61 | HLA-B*53:01 0.5490 | HLA-B*44:03 0.2987 | H-2-Db 0.2455 | HLA-A*02:01 0.1771 | HLA-B*07:02 0.0761 | H-2-Kb 0.0592 | HLA-B*35:01 0.0533 | HLA-B*39:01 0.4096 | 0.58 |
| 210   |               | 218   |      | GAGGAAGLW | HLA-B*53:01 0.08 | HLA-B*58:01 0.24 | HLA-B*53:01 0.9008 | HLA-B*58:01 0.80 | HLA-B*53:01 0.9008 | HLA-B*58:01 0.80 | HLA-B*57:01 0.2541 | HLA-B*44:03 0.5451 | HLA-B*57:01 0.2541 | HLA-B*58:01 0.2554 | 0.74 |
| S.No. | Accession No. | Start | Stop | Epitopes | IEDB     | MHC-NP            | NetCTLpan | NetMHCpan | IC50 | Vaxijen | Immunogen | Toxicity | GRAVY Score |
|-------|---------------|-------|------|----------|----------|--------------------|-----------|-----------|------|---------|------------|----------|-------------|
| 79    | WP_003910461  | 66    | 74   | ALTGAGSY | HLA-B*44:03:02 | HLA-B*44:03 | HLA-B*15:01 | 48.3 | 1.1445 | 0.04864   |         | 0.38       |
| 67    | WP_003905853.1| 96    | 104  | ELSAHAVAF | HLA-B*53:01:07 | HLA-B*53:01 | HLA-A*26:01 | 590.8 | 0.8438 | 0.1117 | n        | 0.97      |
| 117   | WP_003901367.1| 106   | 114  | CESGGNWSI | HLA-B*53:01:05 | HLA-B*53:01 | HLA-B*40:01 | 72.6 | 0.9465 | 0.12073 | n        | -0.78     |
| 13    |             | 113   | 121  | WSINTGNGY | HLA-A*26:01:02 | HLA-B*15:01 | HLA-A*15:01 | 72.6 | 0.9465 | 0.12073 | n        | -0.78     |
| S.No. | Accession No. | Start | Stop | Epitopes     | IEDB   | MHC-NP       | NetCTLpan | NetMHCpan | IC50  | Vaxijen | Immunogen | Toxicity | GRAVY Score |
|-------|---------------|-------|------|--------------|--------|--------------|-----------|-----------|-------|---------|-----------|----------|-------------|
| 15    | WP_003900461.1| 105   | 113  | ALSGALGGV    | HLA-A*02:01 0.28 | HLA-A*02:01 0.2358 | HLA-A*02:01 0.40 | HLA-A*02:01 0.4591 | 34.6  | 0.8589  | 0.05646 | n        | 1.49      |
| 17    |               | 25    |      | RPGPVPLAL    | HLA-B*07:02 0.26 | HLA-B*07:02 0.5260 | HLA-B*07:02 0.10 | HLA-B*07:02 0.0182 | 8.8   | 0.9348  | 0.0431   | n        | 0.43      |
| 98    | WP_003900461.1| 104   | 112  | RAATAHPAL    | HLA-A*02:01 0.40 | HLA-B*07:02 0.80 | HLA-B*07:02 0.2968 | HLA-B*39:01 0.2162 | 100.2 | 1.0803  | 0.19336 | Non-Toxic | 1.31      |
| 409   | WP_003900461.1| 417   |      | WRTRATTAR    | HLA-B*27:05 0.80 | HLA-B*27:05 0.1382 | HLA-B*27:05 0.80 | HLA-B*27:05 0.4276 | 129.2 | 0.8849  | 0.19494 | Non-Toxic | -1.43     |
| S.No. | Accession No. | Start | Stop | Epitopes | IEDB | MHC-NP | NetCTLpan | NetMHCpan | IC50 | Vaxijen | Immunogen | Toxicity | GRAVY Score |
|-------|--------------|-------|------|----------|------|--------|-----------|-----------|------|---------|------------|----------|-------------|
| 398   | WP_003416124.1 | 24    | 32   | VPRAFLAV | HLA-B*44:03 0.28 | HLA-B*44:03 0.2598 | HLA-A*02:01 0.80 | HLA-A*02:01 0.1168 | 10.0 | 1.0189 | 0.24437 Non-Toxic | 3.19 |
| 92    | GAKAAAAY    | 100   | 100  | WPRAFLAV | HLA-B*44:03 0.65 | HLA-B*44:03 0.6895 | HLA-B*15:01 0.30 | HLA-B*15:01 0.1807 | 36.8 | 0.9617 | 0.10144 Non-Toxic 0.84 |
| 18    | WP_003409568.1 | 25    | 33   | WPRAFLAV | HLA-B*53:01 0.51 | HLA-B*53:01 0.6969 | HLA-B*53:01 0.40 | HLA-B*53:01 0.2441 | 40.0 | 1.2067 | 0.21277 Non-Toxic 2.62 |
| 19    | WP_003409568.1 | 158   | 166  | WPRAFLAV | HLA-B*44:03 0.35 | HLA-B*44:03 0.2455 | HLA-A*24:02 0.15 | HLA-A*24:02 0.1941 | 89.9 | 1.2841 | 0.1777 Non-Toxic -0.42 |
### MHC I Epitopes

| S.No. | Accession No. | Start | Stop | Epitopes         | IEDB       | MHC-NP     | NetCTLpan | NetMHCpan | IC50 | Vaxijen | Immunogen | Toxicity | GRAVY Score |
|-------|---------------|-------|------|------------------|------------|------------|-----------|-----------|------|---------|-----------|----------|-------------|
| 20    | WP_003409409.1 | 104   | 112  | RADRANTRY        |            | HLA-B*53:01 | 0.96      | HLA-B*53:01 | 0.5906 | HLA-A*01:01 | 0.30   | HLA-A*01:01 | 0.373 | 1013.7 | 945.8 | Non-Toxic | -2.1       |
|       |               |       |      |                  |            |            |           |           |      |         |           |          |             |
| 22    | WP_003404775.1 | 313   | 321  | ATYEIVCSKK       |            | HLA-B*44:03 | 0.25      | HLA-B*44:03 | 0.5864 | HLA-A*01:02 | 0.05   | HLA-A*01:02 | 0.20  | 32.0 | 0.8318 | 0.12585 | Non-Toxic | 0.31       |
|       |               |       |      |                  |            | H-2-Kb     | 0.1714    | H-2-Db    | 0.1228 |            |         |            |       |      |        |          |            |
| 121   |               | 129   |      | SPAWNLPVV        |            | HLA-B*07:02 | 0.20      | HLA-B*07:02 | 0.2927 | HLA-B*07:02 | 0.40   | HLA-B*07:02 | 0.1510 | 42.7 | 1.4014 | 0.23361 | Non-Toxic | 0.62       |
| 131   |               | 139   |      | GPIAVTYNL        |            | HLA-B*53:01 | 0.88      | HLA-B*53:01 | 0.0880 |            |         |            |       |      |        |          |            |

### MHC II Epitopes

| S.No. | Accession No. | Start | Stop | Epitopes | IEDB       | NetMHC II | Propred | EpiTop | IC50 | Vaxijen | Immunogen | Toxicity | GRAVY Score |
|-------|---------------|-------|------|----------|------------|-----------|---------|--------|------|---------|-----------|----------|-------------|
| 1     | WP_104857305.1 | 149   | 157  | MLYGAGGVG | DQA10301 - DQB10301 |            | DRB1*0301 | 0.20   | DRB1*0301 | 0.22   | DRB1*0301 | 0.68   | 27.4 | 0.9209 | 0.15562 | Non-Toxic | 0.98       |
| S.No. | Accession No. | Start | Stop | Epitopes | IEDB     | MHC-NP     | NetCTLpan | NetMHCpan | IC50 | Vaxijen | Immunogen | Toxicity | GRAVY Score |
|-------|---------------|-------|------|----------|----------|------------|-----------|-----------|------|---------|------------|----------|--------------|
| 3     | WP_031744040.1| 2     | 10   | FVIAAPEVM| DRB1_0102 0.08 | DRB1_0101 0.72 | DRB1_0101 48.00 | DRB1_0101 0.72 | 7.1  | 0.8767  | 0.21885 | Non-Toxic | 1.79        |
|       |               |       |      |          | DQA1*0301/ DQBI*0302 0.42 | DRB1_0101 0.72 | DQA1*0301/ DQBI*0302 0.42 | DQA1*0301/ DQBI*0302 0.42 |       |         |          |           |             |
|       |               |       |      |          | DRB1_0301 0.22 | DRB1_0102 48.00 | DRB1_0102 48.00 | DRB1_0102 48.00 |       |         |          |           |             |
|       |               |       |      |          | DRB1_0301 42.11 | DRB1_0301 42.11 | DRB1_0301 42.11 | DRB1_0301 42.11 |       |         |          |           |             |
|       |               |       |      |          | DRB1_1311 16.87 | DRB1_1311 16.87 | DRB1_1311 16.87 | DRB1_1311 16.87 |       |         |          |           |             |
|       |               |       |      |          | DRB1_0101 34.69 | DRB1_0101 34.69 | DRB1_0101 34.69 | DRB1_0101 34.69 |       |         |          |           |             |
|       |               |       |      |          | DQA1*0301/ DQBI*0302 0.80 | HLA-DQA10301- DQB10301 0.20 | HLA-DQA10301- DQB10301 0.20 | HLA-DQA10301- DQB10301 0.20 | 70.1 | 2.2643  | 0.10296 | Non-Toxic | -0.73      |
| 184   | 192           | 193   | 201  | YGGNGPGGA | DQA1*0301/ DQBI*0301 0.80 | DQA1*0301/ DQBI*0301 0.80 | DQA1*0301/ DQBI*0301 0.80 | DQA1*0301/ DQBI*0301 0.80 |       |         |          |           |             |
|       |               |       |      |          | DRB1_0305 0.02 | DRB1_0301 11.58 | DRB1_0305 23.08 | DRB1_0305 23.08 |       |         |          |           |             |
| 6     | WP_031647515.1| 193   | 201  | VGGIGGAGG | DRB1*0701 0.24 | HLA-DQA10301- DQB10301 0.32 | HLA-DQA10301- DQB10301 0.32 | HLA-DQA10301- DQB10301 0.32 | 39.4 | 2.0089  | 0.26264 | Non-Toxic | 0.9        |
|       |               |       |      |          | DRB1_0410 6.38 | DRB1_0410 6.38 | DRB1_0410 6.38 | DRB1_0410 6.38 |       |         |          |           |             |
|       |               |       |      |          | DRB1_1506 10.20 | DRB1_1506 10.20 | DRB1_1506 10.20 | DRB1_1506 10.20 |       |         |          |           |             |
|       |               |       |      |          | DRB1_1301 21.59 | DRB1_1301 21.59 | DRB1_1301 21.59 | DRB1_1301 21.59 |       |         |          |           |             |
| 179   | 187           | 193   | 201  | LARAGTAGG | DRB1_1301 21.59 | DQA1*0501/ DQBI*0301 0.64 | DQA1*0501/ DQBI*0301 0.64 | DQA1*0501/ DQBI*0301 0.64 | 71.7 | 0.9301  | 0.17853 | Non-Toxic | 0.31       |
|       |               |       |      |          | DRB1_0301 20.00 | HLA-DQA10301- DQB10301 0.31 | HLA-DQA10301- DQB10301 0.31 | HLA-DQA10301- DQB10301 0.31 |       |         |          |           |             |
|       |               |       |      |          | DRB1_0423 18.18 | HLA-DQA10301- DQB10301 0.31 | HLA-DQA10301- DQB10301 0.31 | HLA-DQA10301- DQB10301 0.31 |       |         |          |           |             |
|       |               |       |      |          | DRB1_1301 21.59 | HLA-DQA10301- DQB10301 0.31 | HLA-DQA10301- DQB10301 0.31 | HLA-DQA10301- DQB10301 0.31 |       |         |          |           |             |
|       |               |       |      |          | DQA1*0501/ DQBI*0301 0.64 | HLA-DQA10301- DQB10301 0.31 | HLA-DQA10301- DQB10301 0.31 | HLA-DQA10301- DQB10301 0.31 |       |         |          |           |             |
|       |               |       |      |          | DRB1*0901 7.061 | DQA1*0102/ DQBI*0602 6.628 | DQA1*0102/ DQBI*0602 6.628 | DQA1*0102/ DQBI*0602 6.628 |       |         |          |           |             |
|       |               |       |      |          | DRB1*0901 7.061 | DQA1*0102/ DQBI*0602 6.628 | DQA1*0102/ DQBI*0602 6.628 | DQA1*0102/ DQBI*0602 6.628 |       |         |          |           |             |
|       |               |       |      |          | DRB1*0101 7.334 | DRB1*0101 7.334 | DRB1*0101 7.334 | DRB1*0101 7.334 |       |         |          |           |             |
| S.No. | Accession No. | Start | Stop | Epitopes      | IEDB                  | MHC-NP                           | NetCTLpan          | NetMHCpan | IC50  | Vaxijen | Immunogen | Toxicity | GRAVY Score |
|-------|---------------|-------|------|---------------|-----------------------|----------------------------------|--------------------|------------|-------|--------|-----------|----------|-------------|
| 8     | WP_009938654.1| 179   | 187  | LIGDGA VGT    | DQA1*0501/  | HLA-DQA10501- | DRB1_0301 | 34.74 |       | 1.1340 | 0.15779   | Non-Toxic | 0.99        |
|       |               |       |      |               | DQB1*0301  | DQB1*0301 | 0.26 | DRB1_0421 | 0.26 |
|       |               |       |      |               | DRB1_0305 | 17.58     | DRB1_0241 | 14.22 |
|       |               | 194   | 202  | IGGNAIVAG     | DRB1*1501  | DRB1_0403 | 0.08 | DRB1_0421 | 10.89 |
|       |               |       |      |               | 0.51       | DRB1_1102 | 4.76 |
| 11    | WP_003910446.1| 333   | 341  | LVGNGGAGG     | DQA1*0501/  | HLA-DQA10301- | DRB1_0102 | 20.67 |       | 54.7   | 2.3016   | Non-Toxic | 0.48        |
|       |               |       |      |               | DQB1*0301  | DQB1*0301 | 0.84 | DRB1_0421 | 0.42 |
|       |               |       |      |               | DRB1_0102 | 20.67     | DRB1_0421 | 12.22 |
|       |               | 203   | 211  | WIYGHGGHG     | DQA1*0301- | DRB1_0402 | 0.32 | DRB1_1502 | 16.33 |
|       |               |       |      |               | DQB1*0301  | 0.70      | DRB1_0402 | 6.436|
|       |               |       |      |               | DQA1*0401/  | HLA-DQA10301- | DQA1*0501/ | 42.3  |       | 1.8502 | 0.1438   | Non-Toxic | -0.63       |
|       |               |       |      |               | DQB1*0402  | DQB1*0301 | 0.70 | DQB1*0301 | 0.27 |
|       |               |       |      |               | 0.32       | DRB1_0402 | 6.436|
|       |               |       |      |               | 0.70      | DRB1_0402 | 6.436|
|       |               |       |      |               | 7.149     | DRB1_0101 | 7.488 |
|       |               |       |      |               | 7.129     | DRB1*0101 | 7.488 |
|       |               |       |      |               | 7.574     | DRB1*0101 | 7.574 |

References:

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## MHC I Epitopes

| S.No. | Accession No. | Start | Stop | Epitopes | IEDB | MHC-NP | NetCTLpan | NetMHCpan | IC50 | Vaxijen | Immuneogen | Toxicity | GRAVY Score |
|-------|---------------|-------|------|----------|------|--------|-----------|------------|------|---------|------------|----------|-------------|
| 123   | WP_003909110.1| 196   | 204  | WGAGGGGG | DQA1*0102/ | DRB1_0301 | 0.50 | DRB1_1302 | 0.79 | DQA1*0102/ | DQB1*0602 | 6.968    | 0.46        |
| 205   | WP_003901367.1| 7     | 15   | VRIVAVGATS | DRB1_0301 | 0.46 | HLA-DPA10103/ | DRB1_1301 | 74.9 | HLA-DPA10201/ | DPB1*0405 | 6.648 | 0.88        |

*Note: S.No. refers to sequence number, Accession No. to the accession number, Start and Stop to the start and stop positions of the epitope, Epitopes to the epitope sequence, IEDB to the source of the data, MHC-NP to the MHC class I molecules, NetCTLpan and NetMHCpan to the prediction scores, IC50 to the half-maximal inhibitory concentration, Vaxijen to the vaccine score, Immuneogen to the immunogenicity, Toxicity to the toxicity score, and GRAVY Score to the grand average of hydrophobicity score.*
| S.No. | Accession No. | Start | Stop | Epitopes     | IEDB         | MHC-NP       | NetCTLpan | NetMHCpan | IC50  | Vaxijen | Immunogen | Toxicity | GRAVY Score |
|-------|---------------|-------|------|--------------|--------------|--------------|-----------|-----------|-------|--------|-----------|----------|-------------|
| 51    | 59            | VEGHTHTIS |      |              | DRB1*1302 0.32 | DRB1_0402 0.34 | HLA-DQA10201-DQB10303 0.46 | DRB1_0301 12.63 | DRB1_0402 9.38 | DRB1_1102 17.86 | DRB1*0103/DBB1*0301 7.963 |   |       | 0.22232 | Non-Toxic | -0.42 |
| 15    | WP_003900461.1 | 57    | 65   | IEGDDTDRR   | DPA1*0201/DPB1*0101 0.30 | H-2-Iak 0.26 | DRB1_0301 34.74 | DRB1_0305 8.79 | DRB1_0421 31.11 | DPA1*0103/DBB1*0301 7.202 | DPA1*0201/DBB1*0101 6.134 | 3362.2 | 2.3721 | 0.14042 | Non-Toxic | -2.18 |
| 174   | 182           | VGGGGAGGT |     |              | DRB1_0301 0.42 | DRB1*0701 0.49 | HLA-DQA10301-DQB10301 0.49 | DRB1_1107 1.10 | DRB1_1501 5.10 | DRB1_0301 8.42 | DQA1*0102/DQB1*0601 6.816 | DQA1*0501/DQB1*0301 6.829 | DRB1*0101 7.077 | 29.2 | 4.2556 | 0.16333 | Non-Toxic | 0.32 |
| 17    | WP_003900226.1 | 28    | 36   | VAWDGLAAE   | DQA1*0102/DBB1*0602 0.24 | DRB1_0309 0.40 | DRB1_0301 12.63 | DQA1*0101/DQB1*0501 6.874 | DQA1*0102/DQB1*0602 7.184 | DRB1*0101 6.905 | 661.9 | 0.7727 | 0.17266 | Non-Toxic | 0.57 |
| 408   | 416           | WRTRATTAR |     |              | DRB1*0101 0.84 | DRB1*0405 0.36 | HLA-DQA10201-DQB10402 0.81 | DRB1_0301 23.16 | DRB1_0309 12.63 | DPA1*0103/DBB1*0301 7.874 | DPA1*0301/DBB1*0402 6.284 | 9.1 | 0.8849 | 0.19494 | Non-Toxic | -1.43 |

MHC I Epitopes
### MHC I Epitopes

| S.No. | Accession No. | Start | Stop | Epitopes | IEDB   | MHC-NP              | NetCTLpan  | NetMHCpan | IC50 | Vaxijen | Immunogen | Toxicity          | GRAVY Score |
|-------|---------------|-------|------|----------|--------|---------------------|------------|------------|------|---------|-----------|------------------|-------------|
| 18    | WP_003416124.1 | 90    | 98   | VSSPETTD | DRB1*0701 | 0.47 | HLA-DQA10201-DQB10202 | DRB1_0405 10.64 | DRB1*0701 | 5.397 | 342.8 | 1.1017 | 0.17472 | Non-Toxic | -0.99 |
| 29    | LAVWWIYET     | 37    | 52   | VSPPETTD | DRB1_1301 | 23.64 | HLA-DQA10501-DQB10402 | DRB1_1102 5.95 | DRB1_1301 | 13.64 | 342.8 | 1.1017 | 0.17472 | Non-Toxic | -0.99 |
| 20    | WP_003409409.1 | 54    | 62   | YRTIDIRNH | DRB1*0101 | 0.84 | HLA-DQA10201-DQB10402 | DRB1_0305 28.35 | DRB1_0401 | 28.45 | 25.9 | 1.0538 | 0.3333 | Non-Toxic | -1.36 |

### B cell Epitopes

| S.No. | Accession No. | Start | Stop | Epitopes | IEDB   | ABCPred | BCPred | IC50 | Vaxijen | Immunogen | Toxicity          | GRAVY Score |
|-------|---------------|-------|------|----------|--------|---------|--------|------|---------|-----------|------------------|-------------|
| 1     | WP_104857305.1 | 177   | 192  | AGAIGNGDDG | GNGGTS | -       | -      | 2.8149 | 0.4038 | Non-Toxic | -0.44 |
| 197   | GSGGDGGNNGG | NAGLIG | -    | -       | -      | -      | -      | 2.9137 | 0.3245 | Non-Toxic | -0.3  |
| 231   | GTGGNGGLLL | GFNIGHTN | -    | -       | -      | -      | -      | 1.4744 | 0.26459 | Non-Toxic | -0.03 |
| 2     | WP_078800718.1 | 123   | 138  | PGTGANGG | GGWLIGN | -       | -      | 0.8741 | 0.53708 | Non-Toxic | -0.28 |
| 3     | WP_031744040.1 | 175   | 190  | GGAGGNGGW | LGNNGGP | -       | -      | 1.2080 | 0.41611 | Non-Toxic | -0.55 |
| 132   | GLLYGNGNG | GAGDTA | -    | -       | -      | -      | -      | 2.1567 | 0.25648 | Non-Toxic | -0.26 |
| S.No. | Accession No. | Start  | Stop   | Epitopes                  | IEDB | MHC-NP | NetCTLpan | NetMHCpan | IC50   | Vaxigen | Immunogen | Toxicity | GRAVY Score |
|-------|---------------|--------|--------|---------------------------|------|--------|-----------|-----------|--------|---------|-----------|----------|-------------|
| 6     | WP_031647515.1 | 118    | 133    | RKLIGDGAHGAPGTTQ          | -    | -      | -         | -         | 0.8585 | 0.43228 | Non-Toxic | -0.69    |
| 8     | WP_009938654.1 | 355    | 370    | GGAGGAGGREGWLVGN          | -    | -      | -         | -         | 2.0259 | 0.5602  | Non-Toxic | -0.06    |
|       |               | 349    | 364    | GHAGGAGGAGGAGGRG          | -    | -      | -         | -         | 3.6489 | 0.41684 | Non-Toxic | -0.28    |
| 11    | WP_003910446.1 | 495    | 510    | GGAGGGNGATGGTG            | -    | -      | -         | -         | 3.3778 | 0.37274 | Non-Toxic | -0.26    |
| 12    | WP_003909110.1 | 199    | 214    | AGGGGGGTTPGYLGP           | -    | -      | -         | -         | 2.3354 | 0.28666 | Non-Toxic | -0.26    |
| 13    | WP_003905853.1 | 123    | 138    | GGQLQTAFTWGTNARNGGS      | -    | -      | -         | -         | 0.8303 | 0.48705 | Non-Toxic | -0.4     |
| 15    | WP_003900461.1 | 169    | 184    | GAGGGDVGGGGAGGTT          | -    | -      | -         | -         | 3.9887 | 0.39318 | Non-Toxic | -0.07    |
| 17    | WP_003900226.1 | 263    | 278    | GNGNDGNTFNGSGNAG         | -    | -      | -         | -         | 2.3171 | 0.11949 | Non-Toxic | -1.27    |
| 19    | WP_003409568.1 | 110    | 125    | VHHVWTGIAHSGSTA          | -    | -      | -         | -         | 0.8218 | 0.26058 | Non-Toxic | 0.69     |
| 20    | WP_003409409.1 | 98     | 113    | GGVGNARADRRNTRYT          | -    | -      | -         | -         | 1.5330 | 0.35281 | Non-Toxic | -1.14    |
**Table 6.** Vaccine construct and their physiochemical characterization.

| Construct Name | Adjuvant Accession No. | Vaccine Construct | A.A Length | Mol Weight | pI | instability index | Aliphatic index | GRAVY | Vaxijen Proteins | Immunogen | SolPro | AlgPred | Negative/positive residues |
|----------------|------------------------|-------------------|------------|------------|----|-------------------|-----------------|--------|-----------------|-----------|-------|--------|----------------------------|
| VC1            | WP_003403353.1         | EAAKMAKLSTDELL-   | 697        | 63286.82   | 5.25| 23.5              | 50.6            | -0.425 | 2.1003         | 0.840546  | 11.82166 | SOLUBLE | NON ALLERGEN 67 / 46     |
| Construct Name | Vaccine Construct Name | A.A Accession No. | Mol Weight | pI | Instability Index | Aliphatic Index | GRAVY | ProImmunogen | SolPro | AlgPred | Antigen | Soluble/Non-Allergen |
|----------------|------------------------|------------------|------------|----|------------------|----------------|-------|---------------|--------|---------|----------|---------------------|
| VC2            | WP_01737336.1          | 717              | 66356.75   | 5.16 | 30.66            | -0.536         | 2.092 | 0.851725      | 2.092  | 2.092   | 13.52716  | SOLUBLE ALLERGEN     |
| Construct Name | Adjuvant Accession No. | Vaccine Construct | A.A Length | Mol Weight | pI | instability index | Alphatic index | GRAVY | Vaxijen | Antigen Pro | Immunogen | SolPro | AlgPred | Negative/positive residues |
|----------------|------------------------|-------------------|------------|------------|----|------------------|----------------|--------|----------|-------------|------------|--------|---------|---------------------------|
| VC3            | WP_09402863.3.1        | EAAAAKMAENPID-DLPPELLALGAAD-LALATYNLIALRE-RAEETRAETRTRVEER-RARLTKFQDLPEQFIELRDKFTTEEL-RKAAEGYLEAATNRY-NEVEREAAAQLRL-RQJATFEDASARAE-GYIYDQAVELTQEAEL-GTVAQTTRAVGER-AAKLVGEIEAAAKA-KFVAATLKAAAHEY-GAEALERAGYNGG-PGGAGGSGCESGNN-WSIIQNGSAGAIGNGG-DGGNGGTSGGGGSWL-YGHGJHGGGSSNG-NDGNTNFSGNAGG-GGSRVIRGRATRTG-GGAAPQGGSYIHHGG-HGGAGGSGSGDDGG-NGNAGLIGGGSGTT-GGNGLLLGFNGTNG-GGSTDHVREADDANID-DLLPGGSGAGGNG-GWLYGNGGPGGGS-GLLYGNGGNGAG-DTAGGSVEGHIT-HISGGGSGAGAG-GRGGWLNNGGGG-SIEGDODRRGGGSDHDH-VREADDAINDGGGS-RADRARNTYGGGS-GHAGGGAGGAGG-GRGGGSGGTTGDD-GDGGAHTGGGGSVSP-PETTDGGGSWPIAPS-RLGGSGGSGDG-GAGGGNATSGGGSHY-RFTLYHLGGSGGNG-GAGDAGHGGTG-GGSGGAAGNGATGT-GVGNNGGSSYRTDIRN-HGGSSAGGGGGGTTP-GYLGPGGGSGGVGNNRA-ADRARNTYGGGS-GAGGDDVGGGAGGT-THEYGAEALERAG-FVAATLKAAAHEY-GAEALERAG
the hydrophilic character of the designed constructs which indicates strong interactions with water molecules. Further, the aliphatic index ranges from 49.85 to 59.07 for all vaccines construct suggest protein stability in a defined temperature range. Instability score of all vaccine constructs is <40 showed indicates the good stability of protein to commence an immunogenic reaction. The non-toxic and non-allergenic vaccine may be the good immunotherapy against the pathogenic MTb (Solanki et al., 2019). Based on physiochemical behavior, the shortlisted vaccine constructs (VC1, VC2, and VC3) were subjected for interaction studies.

**Figure 3.** Secondary structure prediction of vaccine constructs using PSIPRED. (a) Vaccine construct 1 (VC1) secondary structure shows helix, β-sheets and turns; (b) Vaccine construct 2 (VC2) secondary structures shows helix and β-sheets; (c) Vaccine construct 3 (VC3) secondary structures shows only helix.

**Figure 4.** (a) Tertiary structure prediction of vaccine constructs VC1 using Phre2. (b) Ramachandran plot analysis of VC1 vaccine construct using RAMPAGE with 90.3% amino acids in most favored region and 8.9% in allowed region.

The interaction of vaccine constructs with HLA allele’s protein

To observe the interaction of vaccine constructs with different HLA alleles of human, vaccine constructs (VC1, VC2, and VC3) were docked with 10 different HLA allele’s retrieved from literature and the results are summarized in Table 7. VC1 have the least global binding energy value with different HLA alleles, i.e., 6EQA (HLA-A*02:01); −35.39, 4F7M (HLA-A*24:02); −21.04, 1XR8 (HLA-B*15:01); −23.17, 1A1N (HLA-B*35:01); −8.29, 4O2E (HLA-B*39:01); −7.69, 1N2R (HLA B*44:02); −11.42,
Table 7. Vaccine constructs [VC1, VC2 and VC3] were docked with 10 different HLA allele’s proteins that correspond to *M. tuberculosis* susceptibility or pathogenicity.

| S.No. | HLA Allele | PDB ID | Vaccine Constructs |
|-------|------------|--------|--------------------|
| 1     | HLA-A*02:01| 6EQA   | VC1: -35.39, VC2: -9.28, VC3: -12.08 |
| 2     | HLA-A*24:02| 4F7M   |                    |
| 3     | HLA-B*15:01| 1XR8   |                    |
| 4     | HLA-B*35:01| 1A1N   |                    |
| 5     | HLA-B*39:01| 4O2E   |                    |
| 6     | HLA-B*44:02| 1N2R   |                    |
| 7     | HLA-B*58:01| 5IM7   |                    |
| 8     | HLA-DR2 (DRA*01:01-DRB1*15:01) | 1BX2 | VC1: -32.5, VC2: 2.13, VC3: -40.98 |
| 9     | HLA-DRA1*0101/DRB5*0101 | 1H15 | VC1: -28.08, VC2: 1.89, VC3: -9.82 |
| 10    | HLA-DQ2.3 (DQA1*03:01/DQB1*02:01) | 4D8P | VC1: -1.64, VC2: 1.46, VC3: 9.04 |

5IM7 (HLA-B*58:01); -16.4, 1BX2 (HLA-DR2 (DRA*01:01-DRB1*15:01)); -32.5, 1H15 (HLA-DRA1*0101/DRB5*0101); -28.08, and 4D8P (HLA-DQ2.3 (DQA1*03:01/DQB1*02:01)); -1.64.

Docking analysis elucidates the efficacy of the designed vaccine in term of binding affinity with HLA alleles. Based on docking analysis, the VC1 was screened as a potential vaccine construct having a tendency to stimulate the immune response as and when required in the host cell. Different adjuvants were also used in the designing process to improve the immune response. Through docking studies, the interaction of VC1 with TLR4/MD2 complex was validated. Satyam et al. (2020) also used TLR4/MD2 complex to predict the efficacy off the vaccine construct. TLR4/MD2 complex has a role in activating Dendritic Cells against a role in Tb.

Best docking was with HLA-A*02:01, HLA-A*24:02, HLA-B*15:01, and HLA-DR2 (DRA*01:01-DRB1*15:01) having binding energies -35.39, -21.04, -23.17, and -32.5 respectively; having interactions of alanine (ALA15) and serine of (SER42 and SER132) amino acids (data not shown); docking results are shown in Supplementary Figure 5a–d (Red depicts vaccine construct VC1 3D structure and blue-green depicts HLA protein 3D structure).

**In-silico cloning of VC1 construct for its heterologous expression in E. coli**

JCAT was used for cloning and expression prediction of constructed vaccine within the pET28a vector. For in silico cloning experiment the required cDNA sequences were obtained through reverse translation. Codon optimization results suggest 77.50% of constructs was made up of Guanine and Cytosine (GC) content. For the heterologous expression of VC1 in *E. coli*, its sequences was in-silico cloned into pET28a vector using EcoRI and NdeI restriction enzyme for the addition at 5′ and 3′ ends respectively (Fig. 6). The Codon Adaptation Index (CAI) value (1.0 for VC1) indicates the efficient heterologous expression of VC1 in *E. coli* cell.

**CONCLUSION**

The work performed is the stepwise proteomic screening for the identification of a multi-epitope chimeric vaccine targeting the MTb. Filters like subcellular localization, antigenicity, allergenicity, transmembrane α-helices, and solubility were utilized and three vaccine constructs (VC1, VC2, and VC3) were designed. Their secondary and tertiary structures were established through online tools. Based on in silico interaction studies with 10 HLA alleles, the VC-1 construct was found most potential. An in silico cloning studies using pET-28a (+) vector suggests the satisfactory expression and translation efficiency of the VC-1. The proposed anti-tubercular vaccine construct VC1 seems capable to initiate the immune response in the host cell and interact efficiently with HLA alleles. During the designing of VC1, besides, adjuvant (L7/L12 ribosomal protein) linker and PADRE epitopes were also added to enhance the anti-tubercular immune responses. Therefore, vaccine construct VC1, possess all the possible factors which are required to bring about the immunogenicity and feasibility against MTb. Further in vitro and in vivo expression studies in wet lab are needed to validate long-term immunological efficacy of predicted vaccine candidate. Further studies are also needed to detect the vaccine interaction with cell mediated and humoral immunity of the host.

**CONFLICT OF INTERESTS**

The authors declare no conflict of interests.

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