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

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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 (AKVAAWTLKAAAC) 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.

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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).

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. 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 (Pahil *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 exoproteome 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

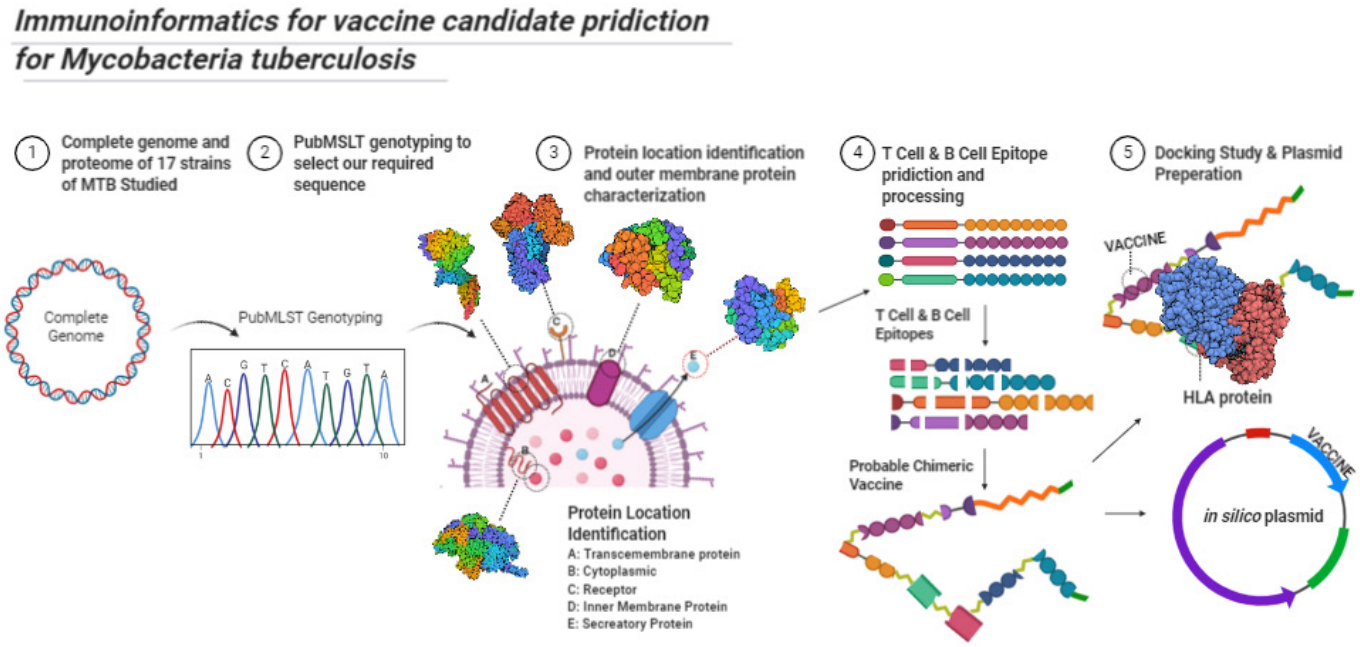

Figure 1. Protocol of study summarized.

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, OMPs (Laal and Zolla-Pazner, 2010), inner membrane, or cytoplasmic using PSORTb 3.0.2 (database for subcellular location of proteins of bacteria) (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, VaxiJen 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 physiochemical properties of proteins

TM of the non-allergic proteins were checked using the Transmembrane hidden Markov model (TMHMM) method (Krogh *et al*., 2001). ABTMpro 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://www.cbs.dtu.dk/services/NetMHCII-2.2/) under default parameter conditions.

Similarly, B-cell epitopes prediction was done by ABCPred (https://webs.iiitd.edu.in/raghava/abcpred/ABC_submission.html), the BCPred (https://webs.iiitd.edu.in/raghava/bcepred/bcepred_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 VaxiJen 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:

$$G = \frac{\varepsilon \text{ Ha}}{N}$$

G = grand average of hydropathy value; Ha = 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 MHCcluster 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 GGGS). 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 (AKVAAWTLKAAAC) 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 Expasy ProtParam server (Gasteiger *et al*., 2005). The 2° 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 Phre2 (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 Locust 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

properties are the characteristic features of the vaccine candidates described by Chauhan et al. (2019) here vaxign predicts that out of 982 proteins, only 165 OMPs possess the adhesion property. Majid and Andleeb (2019) suggested that the proteins having allergic properties cannot be considered for vaccine candidate prediction. Similarly, for vaccine designing, protein should be soluble in *E. coli* and host cell for protein production and biological reaction respectively (Magnan *et al*., 2010); therefore, the screened 165 OMPs were further refined on the above basis and only 35 proteins were found suitable for the analysis.

In the next step, signaling natures of these 165 OMPs were studied through SignalP, SecretomeP 2.0, and PredGPI. SignalP 4.1 based on the Sec-dependent pathway predicts 85 proteins under the "classical secreted proteins." Similarly, SecretomeP 2.0 predicted 135 proteins as the "non-classical secreted proteins" and PredGPI predicts the presence of 8 GPI-anchor proteins, i.e., the presence of the ω-site.

Alongside, these 165 OMPs were also examined for their antigenicity properties using VaxiJen and ANTIGENpro web server. Results suggest that out of 165, only 36 OMPs found common in both possessing the antigenic property were selected 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 orthologs 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/PPEs 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 Membrane	0.651	0.476	-	-	1.3759	0.716231	NON-ALLERGEN
2	WP_104857303.1	CAP domain-containing protein	Unknown	0.530	0.781	0.609104	-	0.7376	0.915117	NON-ALLERGEN
3	WP_078800718.1	MULTISPECIES: PE family protein, partial	Cytoplasmic Membrane	0.695	0.495	-	-	1.0133	0.769487	NON-ALLERGEN
4	WP_031744040.1	PE family protein, partial	Cytoplasmic Membrane	0.637	-	0.519341	-	1.1921	0.732876	NON-ALLERGEN
5	WP_031666010.1	PE family protein	Cytoplasmic Membrane	0.651	0.567	0.856755	-	1.9838	0.734517	NON-ALLERGEN
6	WP_031663355.1	YncE family protein, partial	Extracellular	0.533	-	0.868289	-	1.2737	0.867664	NON-ALLERGEN
7	WP_031661316.1	MULTISPECIES: hypothetical protein	Unknown	0.661	0.494	-	Y	0.8716	0.928439	NON-ALLERGEN
8	WP_031647515.1	PE domain-containing protein	Cytoplasmic Membrane	0.610	0.595	0.641062	-	0.8733	0.92256	NON-ALLERGEN
9	WP_016330440.1	PE family protein	Cytoplasmic Membrane	0.717	0.472	0.870189	-	2.2221	0.893498	NON-ALLERGEN
10	WP_010886074.1	MULTISPECIES: PE family protein	Cytoplasmic Membrane	0.680	-	0.839125	-	1.9856	0.727407	NON-ALLERGEN
11	WP_009938654.1	MULTISPECIES: PE family protein	Unknown	0.686	0.475	0.926527	-	2.0814	0.75097	NON-ALLERGEN
12	WP_009938581.1	PE family protein	Extracellular	0.722	-	0.87177	-	2.1149	0.765368	NON-ALLERGEN
13	WP_003918025.1	Mce associated membrane protein	Unknown	0.551	-	0.75448	-	0.8091	0.937554	NON-ALLERGEN
14	WP_003910913.1	MULTISPECIES: PE family protein	Extracellular	0.701	0.566	0.831665	-	2.0993	0.817835	NON-ALLERGEN
15	WP_003910446.1	MULTISPECIES: PE family protein	Extracellular	0.709	-	0.885624	-	1.9053	0.704416	NON-ALLERGEN
16	WP_003909110.1	MULTISPECIES: hypothetical protein	Unknown	0.563	-	0.886396	-	0.7865	0.956754	NON-ALLERGEN
17	WP_003905853.1	resuscitation-promoting factor rpfE	Unknown	0.593	0.716	0.759912	-	0.7946	0.92811	NON-ALLERGEN
18	WP_003901898.1	DUF3060 domain-containing protein	Extracellular	0.575	0.638	0.82723	-	0.9613	0.894376	NON-ALLERGEN
19	WP_003901751.1	MULTISPECIES: type VII secretion system ESX-1 associated protein EspJ	Unknown	0.696	-	0.833429	-	0.7436	0.949298	NON-ALLERGEN
20	WP_003901367.1	hypothetical protein	Unknown	0.608	0.564	0.871834	-	0.8165	0.915544	NON-ALLERGEN
21	WP_003900461.1	hypothetical protein	Unknown	0.516	-	0.619065	-	0.8171	0.865637	NON-ALLERGEN

Continued

S.No.	Protein Accession	Protein Name	Localization1	Adhesin Probability2	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	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[1,2] and solubility property[3] during over-expression in plasmid vector in *E.coli* during vaccine production.

S.No.	Protein Accession	THMHH1	Trans-membrane helices ATBMPro2	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	1	INSOLUBLE
14	WP_003910913.1	Non Transmembrane protein	0	SOLUBLE
15	WP_003910446.1	Non Transmembrane protein	0	SOLUBLE
16	WP_003909110.1	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	1	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

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	A0A120J1X3	X7ZDK8	A0A120IWV9	G0TP77
2	WP_078800718.1	A0A1R3XWC7	A0A120IZZ7	X7Y8A8	A0A109SL38	G0TN80
3	WP_031744040.1	A0A1A9E8H7	A0A109SXW1	A0A1X0KMN3	A0A109SPU3	G0TMT4
4	WP_031663355.1	A0A1A9E4Q1	A0A109SVA7	-	A0A109SLK3	G0TG89
5	WP_031661316.1	-	-	A0A1X0KNU5	A0A109SPQ4	-
6	WP_031647515.1	A0A1A9E4Q4	A0A109SV94	X7XZK8	A0A109SLK3	G0TG89
7	WP_016330440.1	A0A1A9EC74	-	U5WT40	A0A109SM39	G0TGY4
8	WP_009938654.1	A0A1A9EC53	A0A109T0N7	U5WT45	A0A109SRH6	L0P2Z0
9	WP_009938581.1	A0A1A9EAI0	-	A0A163RQL8	A0A109SQH3	G0TFF2
10	WP_003910913.1	A0A1A9E9H2	A0A109SYL6	U5WZ44	-	G0TPW3
11	WP_003910446.1	A0A1A9E7G2	A0A120J183	X7ZE39	A0A109SNF0	G0TKT3
12	WP_003909110.1	A0A0H3MAA2	A0A109T229	A0A1V3WW93	A0A120IZ35	G0TL77
13	WP_003905853.1	A0A109S8N2	A0A109SYL0	A0A164DVN1	A0A120IXK7	G0TPD7
14	WP_003901367.1	A0A1A9E8T0	A0A120J1T3	X7Z041	A0A109SPE4	G0TNJ1
15	WP_003900461.1	A0A0H3M6X4	A0A109SYE1	A0A1X0KN27	A0A120IX73	G0TMK9
16	WP_003900236.1	A0A0H3M950	A0A109SUZ4	-	B2MVV3	G0TFS8
17	WP_003900226.1	A0A1A9E4C7	A0A109SV30	X7ZLG4	A0A109SM15	G0TFM5
18	WP_003416124.1	A0A0H3ME00	A0A120J2P4	A0A1X0KXC9	A0A109SRH5	G0TGV5
19	WP_003409568.1	P67225	A0A120J1C8	X7XXD2	A0A109SNM9	G0TLH5
20	WP_003409409.1	A0A0K2HXD6	A0A109SX79	X7XZ04	A0A120IWZ7	G0TLE4
21	WP_003405142.1	A0A0H3M4Y6	A0A109SV46	A0A1X0KR97	A0A109SLQ5	G0TGA3
22	WP_003404775.1	A0A0H3MBK5	A0A109SUW6	A0A1X0KR45	A0A109SLR5	G0TFS2

included 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 naive 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

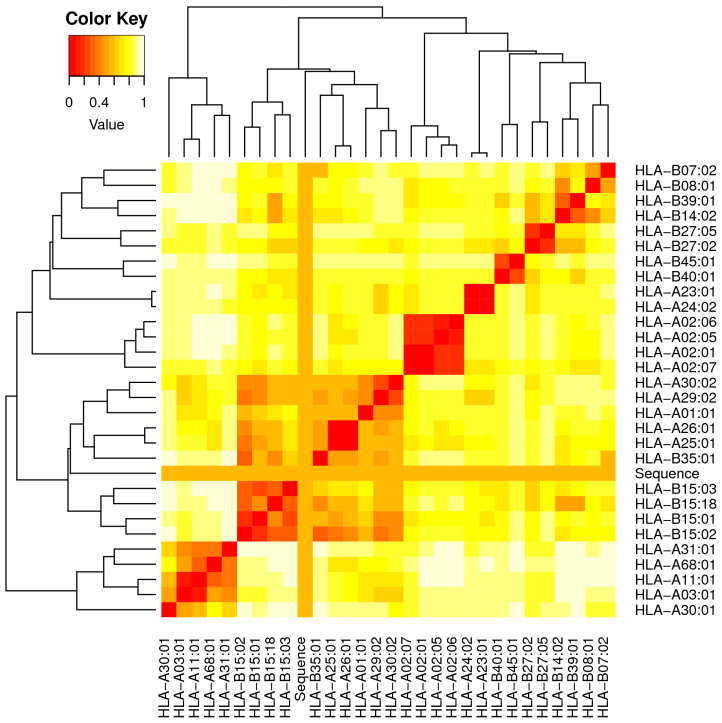

Figure 2. Heat map analysis of T-cell epitopes using MHC cluster.

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 (^{107}CESGGNWSI^{115}, ^{398}WPIRAPSRL^{406}, ^{158}HYRFTLYHL^{166} and ^{104}RADRARNTY^{112}), 7 MHC II epitopes (^{184}YGNGGPGGA^{192}, ^{203}WIYGHGGHG^{211}, ^{205}YGHGGHGGA^{213}, ^{51}VEGHTHTIS^{59}, ^{57}IEGDDTDRR^{65}, ^{90}VSPPETTTD^{98}, and ^{54}YRTIDIRNH^{62}) and 15 B-cell epitopes (177AGAIGNGGDGGNGGTS192; 1 9 7 G S G G D G G N G G N A G L I G 2 1 2 ; 2 3 1 G T G G N G G L L L G F N G T N 2 4 6 ; 1 7 5 G G A G G N G G W L Y G N G G P 1 9 0 ; 1 3 2 G L L Y G N G G N G G A G D T A 1 4 7 ; 3 5 5 G G A G G A G G R G G W L V G N 3 7 0 ; 3 4 9 G H A G G A G G A G G A G G R G 3 6 4 ; 5 0 4 G G T G G D G G D G G H A G T G 5 1 9 ; 4 6 7 N G G I G G D G A G G G N A T S 4 8 2 ; 4 9 2 G G N G G A G G D A G H G G T G 5 0 7 ; 4 9 5 G G A G G N G A T G G T G V G N 5 1 0 ; 1 9 9 A G G G G G G T T P T G Y L G P 2 1 4 ; 1 6 9 G A G G G D V G G G G A G G T T 1 8 4 ; 263GNGNDGNTNFGSGNAG278 and 98GGVGNARADRA RNTYT113)} were used to design the chimeric vaccine. Two linkers HEYGAEALERAG 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 (AKVAAWTLKAAAC) 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 *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 hydropathic 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.

MHC I Epitopes													
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 HLA-A*32:01 0.2 HLA-A*02:03 1.2 HLA-B*15:01 0.91	H-2-Db 0.8179 HLA-B*07:02 0.5563 HLA-B*53:01 0.3353 HLA-B*44:03 0.2369	HLA-B*07:02 0.80	HLA-B*07:02 0.4701	198.9 64.17	1.1606	0.07651	Non-Toxin	1.3
		64	72	ALSAHVAAF	HLA-B*15:01 0.07 HLA-A*32:01 0.11 HLA-A*26:01 0.74	HLA-B*53:01 0.5923 HLA-B*44:03 0.5154 H-2-Db 0.3402 H-2-Kb 0.2168 HLA-A*02:01 0.1017 HLA-B*35:01 0.0648	HLA-B*15:01 0.10	HLA-B*15:01 0.0210	10.7 15.58	0.7437	0.11191	Non-Toxin	1.56
2	WP_078800718.1	79	87	ALSAGGGAY	HLA-B*15:01 0.40 HLA-B*53:01 0.38	HLA-B*44:03 0.7221 HLA-B*53:01 0.3820 HLA-B*35:01 0.0582 H-2-Kb 0.0569	HLA-B*15:01 0.40 HLA-A*01:07 0.80 HLA-A*01:10 0.40	HLA-B*15:01 0.1159 HLA-A*01:10 0.4631	25.7	1.2638	0.10203	Non	0.66
3	WP_031744040.1	46	54	EVSVAISAL	HLA-B*53:01 0.53 HLA-B*44:03 0.35 HLA-A*26:01 0.75	HLA-B*53:01 0.5356 HLA-B*44:03 0.3559 H-2-Db 0.2565 H-2-Kb 0.2371 HLA-B*35:01 0.0762 HLA-B*07:02 0.0542	HLA-A*26:01 0.30	HLA-A*26:01 0.0785	154.2	0.7269	0.03446	Non	1.69

MHC I Epitopes													
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*58:01 0.05	HLA-B*58:01 0.0412 HLA-B*15:01 0.2037	8.4	0.7621	0.05318	Non	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 HLA-B*53:01 0.3628 HLA-B*35:01 0.1000 H-2-Db 0.0703 H-2-Kb 0.0638	HLA-A*03:01 0.80 HLA-B*15:01 0.40 HLA-A*01:06 0.80 HLA-A*01:07 0.80 HLA-A*01:10 0.80 HLA-A*01:12 0.80	HLA-B*15:01 0.1468 HLA-A*01:10 0.4365	31.7	1.1074	0.03904	Non	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 0.80	HLA-B*39:01 0.4061	409.6	0.8331	0.0483	Non	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*44:03 0.5451 HLA-B*57:01 0.2541	HLA-B*58:01 0.80	HLA-B*58:01 0.2554	42.6	1.2630	0.14215	non	0.74

MHC I Epitopes													
S.No.	**Accession No.**	**Start**	**Stop**	**Epitopes**	**IEDB**	**MHC-NP**	**NetCTLpan**	**NetMHCpan**	**IC50**	**Vaxijen**	**Immunogen**	**Toxicity**	**GRAVY Score**
		79	87	ALTGAGGSY	HLA-B*44:03 0.21 HLA-B*53:01 0.48	HLA-B*44:03 0.8221 HLA-B*53:01 0.4128 HLA-B*35:01 0.1007 H-2-Kb 0.0879	HLA-B*15:01 0.80	HLA-B*15:01 0.2413	48.3	1.1445	0.04864	non	0.38
11	WP_003910446.1	66	74	ELSAHAVAF	HLA-B*53:01 0.72 HLA-A*26:01 0.80	HLA-B*53:01 0.7911 HLA-B*44:03 0.4024 H-2-Db 0.2867 HLA-B*35:01 0.0821	HLA-A*26:01 0.80	HLA-A*26:01 0.2280	590.8	0.8438	0.1117	n	0.97
13	WP_003905853.1	96	104	AYSVNWDAI	HLA-B*53:01 0.2174 HLA-A*24:02 0.80	H-2-Db 0.2396 HLA-B*53:01 0.2174	HLA-A*24:02 0.80	HLA-A*24:02 0.3759	210.2	1.3424	0.23163	n	0.26
		107	115	CESGGNWSI	HLA-B*53:01 0.53 HLA-B*40:01 0.88	HLA-B*53:01 0.4531	HLA-B*40:01 0.20	HLA-B*40:01 0.2436	99.5	1.5147	0.09759	n	-0.37
		113	121	WSINTGNGY	HLA-A*26:01 0.02 HLA-B*15:01 0.84 HLA-A*01:02 0.51 HLA-A*01:03 0.45	HLA-B*44:03 0.2895 HLA-B*53:01 0.2782	HLA-A*01:01 0.20 HLA-A*26:01 0.20 HLA-B*15:01 0.80 HLA-A*01:02 0.30 HLA-A*01:03 0.20 HLA-A*01:06 0.80 HLA-A*01:07 0.30	HLA-B*15:01 0.3899 HLA-A*01:02 0.3411 HLA-A*01:03 0.4806 HLA-A*01:04 0.4981 HLA-A*26:01 0.1288	72.6	0.9465	0.12073	n	-0.78
14	WP_003901367.1	106	114	DVNGFGISL	HLA-B*53:01 0.27 HLA-B*07:02 0.15 HLA-A*26:01 0.40	HLA-B*44:03 0.2504 H-2-Db 0.2488 HLA-B*53:01 0.2067 HLA-B*07:02 0.1145	HLA-A*26:01 0.80	HLA-A*26:01 0.3430		2.5625	0.19356	n	0.74

MHC I Epitopes													
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-B*44:03 0.1876	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*53:01 0.50	HLA-B*07:02 0.5260 HLA-B*53:01 0.2450 HLA-A*02:01 0.0838	HLA-B*07:02 0.10	HLA-B*07:02 0.0182	8.8	0.9348	0.0431	n	0.43
		98	106	MASGIGGAL	HLA-B*53:01 0.44 HLA-B*39:01 0.62	HLA-B*53:01 0.7044 H-2-Db 0.6406 HLA-B*44:03 0.3601 H-2-Kb 0.1921 HLA-B*07:02 0.1609 HLA-A*02:01 0.0809	HLA-B*07:02 0.80 HLA-B*39:01 0.40	HLA-B*07:02 0.2968 HLA-B*39:01 0.2162	100.2	1.0803	0.19336	Non-Toxic	1.31
17	WP_003900226.1	104	112	RAATAHPAL	HLA-A*02:01 0.40 HLA-B*57:01 0.17 HLA-B*07:02 0.08	H-2-Db 0.7492 HLA-B*53:01 0.5460 H-2-Kb 0.3652 HLA-A*02:01 0.2140 HLA-B*57:01 0.1107 HLA-B*44:03 0.1063 HLA-B*07:02 0.1033	HLA-B*07:02 0.30	HLA-B*07:02 0.0918	25.3	0.9399	0.13381	Non-Toxic	0.11
		409	417	WRTRATTAR	HLA-B*27:05 0.80	HLA-B*53:01 0.1382	HLA-B*27:05 0.80	HLA-B*27:05 0.4276	129.2	0.8849	0.19494	Non-Toxic	-1.43

MHC I Epitopes													
S.No.	**Accession No.**	**Start**	**Stop**	**Epitopes**	**IEDB**	**MHC-NP**	**NetCTLpan**	**NetMHCpan**	**IC50**	**Vaxijen**	**Immunogen**	**Toxicity**	**GRAVY Score**
		398	406	WPIRAPSRL	HLA-B*07:02 0.38 HLA-B*53:01 0.12	HLA-B*07:02 0.3878 H-2-Kb 0.3336 H-2-Db 0.1643 HLA-B*53:01 0.1296	HLA-B*07:02 0.20	HLA-B*07:02 0.1569 HLA-B*39:01 0.4982	44.5	1.2351	0.01356	Non-Toxic	-0.42
		92	100	GAKAAAAVY	HLA-B*44:03 0.65 HLA-B*53:01 0.44 HLA-B*57:01 0.49	HLA-B*44:03 0.6895 HLA-B*53:01 0.5444 HLA-B*57:01 0.4559 HLA-B*35:01 0.3642 H-2-Db 0.1513	HLA-B*15:01 0.30	HLA-B*15:01 0.1807	36.8	0.9617	0.10144	Non-Toxic	0.84
18	WP_003416124.1	24	32	VLIAAFLAV	HLA-B*44:03 0.28 HLA-A*02:01 0.87	H-2-Db 0.3278 HLA-B*44:03 0.2598 H-2-Kb 0.1140	HLA-A*02:01 0.80	HLA-A*02:01 0.1168	10.0	1.0189	0.24437	Non-Toxic	3.19
		26	34	IAAFLAVWW	HLA-B*44:03 0.8421 HLA-B*53:01 0.7595	HLA-B*44:03 0.8421 HLA-B*53:01 0.7595 HLA-B*57:01 0.6010	HLA-B*58:01 0.30	HLA-B*58:01 0.0578	10.4	1.7392	0.32079	Non-Toxic	2.1
		25	33	LIAAFLAVW	HLA-B*53:01 0.51 HLA-B*58:01 0.20	HLA-B*44:03 0.6969 HLA-B*53:01 0.5801 H-2-Db 0.2905 HLA-B*57:01 0.2702	HLA-B*58:01 0.40	HLA-B*58:01 0.2441	40.0	1.2067	0.21277	Non-Toxic	2.62
19	WP_003409568.1	158	166	HYRFTLYHL	HLA-B*44:03 0.35	HLA-B*53:01 0.2455 H-2-Kb 0.2328 H-2-Db 0.1658 HLA-B*44:03 0.1359	HLA-A*24:02 0.15	HLA-A*24:02 0.1941	89.9	1.2841	0.17774	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	RADRARNTY	HLA-B*53:01 0.96 HLA-A*01:02 0.30	HLA-B*53:01 0.5906 HLA-B*57:01 0.4844 HLA-B*44:03 0.3501 HLA-B*35:01 0.3262	HLA-A*01:01 0.30 HLA-A*01:02 0.20	HLA-A*01:02 0.4373 HLA-A*01:07 0.4580	1013.7 945.8	1.2267	0.16332	Non-Toxic	-2.1
22	WP_003404775.1	313	321	ATYEIVCSK	HLA-A*03:01 0.25 HLA-B*44:03 0.64	HLA-B*44:03 0.5864 H-2-Kb 0.1714 H-2-Db 0.1228	HLA-A*03:01 0.05	HLA-A*03:01	32.0	0.8318	0.12585	Non-Toxic	0.31
		121	129	SPAWNLPVV	HLA-B*07:02 0.20	HLA-B*07:02 0.2927 H-2-Db 0.1639	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*07:02 0.42	H-2-Db 0.2901 HLA-B*07:02 0.2142 H-2-Kb 0.2084 HLA-B*53:01 0.0880	HLA-B*07:02 0.80	HLA-B*07:02 0.4793	205.1	0.7412	0.15241	Non-Toxic	0.76

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 0.20 DRB1_0301 0.15 DRB1*0405 0.68	HLA-DRB1_0402 0.33 HLA-DQA10301-DQB10301 0.22 HLA-DQA10501-DQB10301 0.22	DRB1_0301 22.11 DRB1_0309 11.58 DRB1_1501 15.31 DRB1_1506 15.31	DRB1*0101 7.528 DRB1*0301 5.585 DRB1*0401 7.046 DRB1*0405 7.268 DRB1*1501 6.492 DPA1*0103/DPB1*0201 5.586 DPA1*0103/DPB1*0301 6.512 DPA1*0103/DPB1*0401 4.927 DPA1*0103/DPB1*0402 5.375	27.4	0.9209	0.15562	Non-Toxic	0.98

MHC I Epitopes													
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 DQA1*0301/ DQB1*0302 0.42 DRB1_0301 0.22	DRB1_0101 0.72	DRB1_0101 48.00 DRB1_0102 48.00 DRB1_0301 42.11 DRB1_1311 16.87 DRB5_0101 34.69	DRB1*0101 7.62 DRB1*1501 5.239 DQA1*0301/ DQB1*0302 7.021 DQA1*0401/ DQB1*0402 7.364 DPA1*0103/ DPB1*0201 8.633 DPA1*0103/ DPB1*0301 9.1 DPA1*0103/ DPB1*0402 8.59	7.1	0.8767	0.21885	Non-Toxic	1.79
		184	192	YGNGGPGGA	DQA1*0501/ DQB1*0301 0.80 DRB1_0305 0.02	HLA-DQA10301-DQB10301 0.20	DRB1_0301 11.58 DRB1_0305 23.08	DRB1*0101 6.651 DRB1*0401 6.114 DQA1*0501/ DQB1*0301 7.021 DPA1*0103/ DPB1*0201 4.235	70.1	2.2643	0.10296	Non-Toxic	-0.73
6	WP_031647515.1	193	201	VGGIGGAGG	DRB1*0701 0.24	HLA-DQA10301-DQB10301 0.32	DRB1_0410 6.38 DRB1_1506 10.20	DRB1*0701 7.054 DRB1*0901 7.061	39.4	2.0089	0.26264	Non-Toxic	0.9
		179	187	LARAGTAGG	DRB1_1301 21.59 DQA1*0501/ DQB1*0301 0.64	DRB1_0301 20.00 DRB1_0423 18.18 DRB1_1301 21.59	HLA-DQA10102-DQB10602 0.49 HLA-DQA10301-DQB10301 0.31	DQA1*0102/ DQB1*0602 6.628 DQA1*0501/ DQB1*0301 6.494 DRB1*0101 7.334	71.7 18.3	0.9301	0.17853	Non-Toxic	0.31

MHC I Epitopes													
S.No.	**Accession No.**	**Start**	**Stop**	**Epitopes**	**IEDB**	**MHC-NP**	**NetCTLpan**	**NetMHCpan**	**IC50**	**Vaxijen**	**Immunogen**	**Toxicity**	**GRAVY Score**
8	WP_009938654.1	179	187	LIGDGAVGT	DQA1*0501/ DQB1*0301 0.26 DRB1_0421 0.26	HLA-DQA10501-DQB10301 0.37	DRB1_0301 34.74 DRB1_0305 17.58 DRB1_0421 14.22	DPA1*0103/ DPB1*0301 7.089 DQA1*0301/ DQB1*0302 7.164 DQA1*0501/ DQB1*0301 7.296 DRB1*0101 7.242 DRB1*0401 7.181 DRB1*1302 7.322		1.1340	0.15779	Non-Toxic	0.99
		194	202	IGGNAIVAG	DRB1*1501 0.08	DRB1_0403 0.51	DRB1_0421 10.89 DRB1_1102 4.76	DRB1*0101 8.265 DRB1*0405 8.039 DRB1*1501 6.408	489.5	1.0480	0.22557	Non-Toxic	1.34
11	WP_003910446.1	333	341	LVGNGGAGG	DQA1*0501/ DQB1*0301 0.84 DRB1_0421 0.42	HLA-DQA10301-DQB10301 0.44	DRB1_0102 20.67 DRB1_0421 12.22	DPA1*0103/ DPB1*0301 6.342 DQA1*0501/ DQB1*0301 6.894 DRB1*0101 7.177 DRB1*0405 7.129	54.7	2.3016	0.12221	Non-Toxic	0.48
		203	211	WIYGHGGHG	DQA10301-DQB10301 0.70 DQA1*0401/ DQB1*0402 6.436	DRB1_0402 0.32 HLA-DQA10301-DQB10301 0.27	DRB1_1502 16.33	DQA1*0501/ DQB1*0301 7.149 DQA1*0401/ DQB1*0402 6.436 DRB1*0101 7.488 DRB1*0701 7.574	42.3	1.8502	0.1438	Non-Toxic	-0.63

MHC I Epitopes													
S.No.	**Accession No.**	**Start**	**Stop**	**Epitopes**	**IEDB**	**MHC-NP**	**NetCTLpan**	**NetMHCpan**	**IC50**	**Vaxijen**	**Immunogen**	**Toxicity**	**GRAVY Score**
		123	131	IGNGANGVA	DRB1_0301 0.50	DRB1_1302 0.79	DRB1_1506 17.35 DRB1_0301 10.53	DQA1*0102/ DQB1*0602 6.968 DQA1*0501/ DQB1*0301 6.658	7.3	1.1329	0.11673	Non-Toxic	0.46
		205	213	YGHGGHGGA	DQA1*0102/ DQB1*0602 0.14	HLA-DQA10301-DQB10301 0.24	DRB1_0305 13.19	DQA1*0501/ DQB1*0301 6.719 DQA1*0102/ DQB1*0602 6.614 DRB1*0405 6.04	32.4	2.8692	0.15645	Non-Toxic	-0.88
		66	74	LSAHAVAFH	DPA1*0301/ DPB1*0402 0.65 DRB1_1304 0.14	HLA-DQA10101-DQB10501 0.73 HLA-DQA10102-DQB10502	DRB1_1107 10.77 DRB1_1114 17.62 DRB1_1304 41.11	DPA1*0103/ DPB1*0201 6.549 DPA1*0103/ DPB1*0301 7.205 DPA1*0301/ DPB1*0402 6.356 DRB1*0401 7.435	83.2	0.9708	0.22363	Non-Toxic	1
12	WP_003909110.1	196	204	VGAGGGGGG	DRB1_0301 0.42 DQA1*0501/ DQB1*0301 0.28	HLA-DQA10301-DQB10301 0.29	DRB1_0301 8.42	DQA1*0102/ DQB1*0602 6.791 DQA1*0501/ DQB1*0301 6.428	55.4	4.7600	0.1601	Non-Toxic	0.36
14	WP_003901367.1	7	15	VRIAVGATS	DRB1_0301 0.46	HLA-DPA10103-DPB10301 74.9 0.51 HLA-DPA10201-DPB11401 0.35	DRB1_0101 35.00 DRB1_0102 51.67 DRB1_0301 43.16 DRB1_0402 30.21	DRB1*0101 6.694 DRB1*0405 6.485		0.8114	0.21037	Non-Toxic	1.12

MHC I Epitopes													
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	DPA1*0103/ DPB1*0301 7.963 DPA1*0103/ DPB1*0402 6.314 DRB1*0405 7.019 DRB1*1302 6.732	37.3	1.6901	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/ DPB1*0301 7.202 DPA1*0201/ DPB1*0101 6.134	3362.2	2.3721	0.14042	Non-Toxic	-2.18
		174	182	VGGGGAGGT	DRB1_0301 0.42 DRB1*0701 .15	HLA-DQA10301-DQB10301 0.49 HLA-DQA10501-DQB10301 0.41	DRB1_1107 1.10 DRB1_1501 5.10 DRB1_0301 8.42	DQA1*0102/ DQB1*0602 6.816 DQA1*0501/ DQB1*0301 6.829 DRB1*0101 7.077 DRB1*0701 7.151 DRB1*1501 6.095	29.2	4.2556	0.16333	Non-Toxic	0.32
17	WP_003900226.1	28	36	VAWDGLAAE	DQA1*0102/ DQB1*0602 0.24 DRB1_0309 0.40	DRB1_0403 0.40	DRB1_0301 23.16 DRB1_0309 12.63	DQA1*0101/ DQB1*0501 6.874 DQA1*0102/ DQB1*0602 7.184 DRB1*1302 6.554	661.9	0.7727	0.17266	Non-Toxic	0.57
		408	416	WRTRATTAR	DRB1*0101 0.84 DRB1*0405 0.36 DRB1*1101 0.92 DRB1*1302 0.25	HLA-DQA10201-DQB10402 0.81 HLA-DQA10303-DQB10402 0.70	DRB1_0806 10.47 DRB1_0813 54.02	DPA1*0103/ DPB1*0301 7.874 DPA1*0301/ DPB1*0402 6.284	9.1	0.8849	0.19494	Non-Toxic	-1.43

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	VSPPETTTD	DRB1*0701 0.47	HLA-DQA10201-DQB10202 0.48	DRB1_0405 10.64	DRB1*0701 5.397	342.8	1.1017	0.17472	Non-Toxic	-0.99
		29	37	LAVWWIYET	DRB1_1301 23.64 DPA1*0201/ DPB1*0101 7.9	HLA-DQA10501-DQB10402 0.34 HLA-DQA10601-DQB10402 0.25 H-2-IEd 0.25	DRB1_1102 5.95 DRB1_1301 13.64	DPA1*0103/ DPB1*0201 6.468 DPA1*0201/ DPB1*0101 6.9 DRB1*0101 7.377	32.1	0.8173	0.63265	Non-Toxic	0.78
20	WP_003409409.1	54	62	YRTIDIRNH	DRB1*0101 0.84 DRB1_0401 0.70	HLA-DQA10201-DQB10402 0.47 HLA-DQA10303-DQB10402 0.48	DRB1_0305 28.35 DRB1_0401 20.70 DRB1_0703 28.45	DPA1*0103/ DPB1*0301 7.188 DPA1*0201/ DPB1*0101 7.701 DRB1*0101 6.581 DRB1*0901 6.762	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	AGAIGNGGDG GNGGTS	-	-	-	-	-	**2.8149**	0.4038	Non-Toxic	-0.44
		197	212	GSGGDGGNGG NAGLIG	-	-	-	-	-	**2.9137**	0.3245	Non-Toxic	-0.3
		231	246	GTGGNGGLLL GFNGTN	-	-	-	-	-	**1.4744**	0.26459	Non-Toxic	-0.03
2	WP_078800718.1	123	138	PGTGANGGP GGWLIGN	-	-	-	-	-	**0.8741**	0.53708	Non-Toxic	-0.28
3	WP_031744040.1	175	190	GGAGGNGGW LYGNGGP	-	-	-	-	-	**1.2080**	0.41611	Non-Toxic	-0.55
		132	147	GLLYGNGGNG GAGDTA	-	-	-	-	-	**2.1567**	0.25648	Non-Toxic	-0.26

MHC I Epitopes													
S.No.	**Accession No.**	**Start**	**Stop**	**Epitopes**	**IEDB**	**MHC-NP**	**NetCTLpan**	**NetMHCpan**	**IC50**	**Vaxijen**	**Immunogen**	**Toxicity**	**GRAVY Score**
6	WP_031647515.1	118	133	RKLIGDGAH GAPGTGQ	-	-	-	-	-	**0.8585**	0.43228	Non-Toxic	-0.69
8	WP_009938654.1	355	370	GGAGGAGGRG GWLVGN	-	-	-	-	-	**2.0259**	0.5602	Non-Toxic	-0.06
		349	364	GHAGGAGGAG GAGGRG	-	-	-	-	-	**3.6489**	0.41684	Non-Toxic	-0.28
		504	519	GGTGGDGGD GGHAGTG	-	-	-	-	-	**3.7693**	0.37451	Non-Toxic	-0.86
		467	482	NGGIGGDGA GGGNATS	-	-	-	-	-	**3.1482**	0.45223	Non-Toxic	-0.44
		492	507	GGNGGAGGDA GHGGTG	-	-	-	-	-	**3.5999**	0.37648	Non-Toxic	-0.71
11	WP_003910446.1	495	510	GGAGGNGAT GGTGVGN	-	-	-	-	-	**3.3778**	0.37274	Non-Toxic	-0.26
12	WP_003909110.1	199	214	AGGGGGGTT PTGYLGP	-	-	-	-	-	**2.3354**	0.28666	Non-Toxic	-0.26
13	WP_003905853.1	123	138	GGLQFTAGTW RANGGS	-	-	-	-	-	**0.8303**	0.48705	Non-Toxic	-0.4
15	WP_003900461.1	169	184	GAGGGDVGGG GAGGTT	-	-	-	-	-	**3.9887**	0.39318	Non-Toxic	-0.07
17	WP_003900226.1	263	278	GNGNDGNTNF GSGNAG	-	-	-	-	-	**2.3171**	0.11949	Non-Toxic	-1.27
19	WP_003409568.1	110	125	VHWIVTGIAP GSGSTA	-	-	-	-	-	**0.8218**	0.26058	Non-Toxic	0.69
20	WP_003409409.1	98	113	GGVGNARADR ARNTYT	-	-	-	-	-	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	Antigen Pro	Immunogen	SolPro	AlgPred	Negative/ positive residues
VC1	WP_003403353.1	EAAAKMAKLSTDELL-DAFKEMTLLELSD-FVKKFEETFEVTA-AAPVAVAAAGAAPA-GAAVEAAEEQSEF-DVILEAAGDKKI-GVIKVVREIVSGL-GLKEAKDLVDGAPK-PLLEKVAKEAADEAK-AKLEAAGATVT-VKEAAAKAKFVAAW-TLKAAAHEYGAEAL-ERAGYGNGGPGGAG-GGSCESGGNWSIGGG-SAGAIGNGGDGGNG-GTSGGGSWIYGHGG-HGGGGSGNGNDGNT-NFGSGNAGGGGSRVR-RGATRTGSGGAAQG-GGSYGHGGHGGAG-GGSGSGGDGGNGGNA-GLIGGGGSGTGGNG-GLLLGFNGTNGGGST-DHVREADDANID-DLLGGGSGGAGGNG-GWLYGNGGPGGGS-GLLYGNGGNGGAG-DTAGGGSVEGHTH-TISGGGSGGAGGAG-GRGGWLVGNGGG-SIEGDDTDRRGGGSDH-VREADDANIDGGGS-RADRARNTYGGGS-GHAGGAGGAGGAG-GRGGGGSGGTGGDGG-DGGHAGTGGGGSVSP-PETTTDGGGSWPIRAPS-RLGGGSNGGIGGD-GAGGGNATSGGGSHY-RFTLYHLGGGSGGNG-GAGGDAGHGGTGG-GGSGGAGGNGATGGT-GVGNGGGSYRTIDIRNH-GGGSAGGGGGGTTPT-GYLGPGGGSGGVGNAR-ADRARNTYTGGGS-GAGGGDVGGGGAGGT-THEYGAEALERAGAK-FVAAWTLKAAAHEY-GAEALERAG	697	63286.82	5.25	23.5	50.6	-0.425	2.1003	0.840546	11.82166	SOLUBLE	NON ALLERGEN	67 / 46

Construct Name	Adjuvant Accession No.	Vaccine Construct	A.A Length	Mol Weight	pI	instability index	Aliphatic index	GRAVY	Vaxijen	Antigen Pro	Immunogen	SolPro	AlgPred	Negative/ positive residues
VC2	WP_031737436.1	EAAAKMAENSNID-DIKAPLLAALGAAD-LALATVNELITNLRE-RAEETRRSRVEES-RARLTKLQEDLPEQL-TELREKFTAEELRKAA-EGYLEAATSELVER-GEAALERLRSQQS-FEEVSARAEGYVD-QAVELTQEALGT-VASQVEGRAAKLVGIE-LEAAAKAKFVAAW-TLKAAAHEYGAEAL-ERAGYGNGGPGGAG-GGSCESGGNWSIGGG-SAGAIGNGGDGGNG-GTSGGGSWIYGHGG-HGGGGSGNGNDGNT-NFGSGNAGGGGSRVR-RGATRTGSGGAAQG-GGSYGHGGHGGAG-GGSGSGGDGGNGG-NAGLIGGGGSGTGG-NGGLLLGFNGTNG-GGSTDHVREADDANID-DLLGGGSGGAGGNG-GWLYGNGGPGGGS-GLLYGNGGNGGAG-DTAGGGSVEGHTH-TISGGGSGGAGGAG-GRGGWLVGNGGG-SIEGDDTDRRGGGSDH-VREADDANIDGGGS-RADRARNTYGGGS-GHAGGAGGAGGAG-GRGGGGSGGTGGDGG-DGGHAGTGGGGSVSP-PETTTDGGGSWPIRAPS-RLGGGSNGGIGGD-GAGGGNATSGGGSHY-RFTLYHLGGGSGGNG-GAGGDAGHGGTGG-GGSGGAGGNGATGGT-GVGNGGGSYRTIDIRN-HGGGSAGGGGGGTTPT-GYLGPGGGSGGVGNAR-ADRARNTYTGGGS-GAGGGDVGGGGAGGT-THEYGAEALERAGAK-FVAAWTLKAAAHEY-GAEALERAG	717	66356.75	5.16	30.66	50.96	-0.536	2.092	0.851725	13.52716	SOLUBLE	NON ALLERGEN	73 / 49

Construct Name	Adjuvant Accession No.	Vaccine Construct	A.A Length	Mol Weight	pI	instability index	Aliphatic index	GRAVY	Vaxijen	Antigen Pro	Immunogen	SolPro	AlgPred	Negative/ positive residues
VC3	WP_094028633.1	EAAAKMAENPNID-DLPAPLLAALGAAD-LALATVNDLIANLRE-RAEETRAETRTRVEER-RARLTKFQEDLPEQ-FIELRDKFTTEEL-RKAAEGYLEAATNRY-NELVERGEAALQRLR-SQTAFEDASARAE-GYVDQAVELTQEAL-GTVASQTRAVGER-AAKLVGIELEAAAKA-KFVAAWTLKAAAHEY-GAEALERAGYGNGG-PGGAGGGSCESGGN-WSIGGGSAGAIGNGG-DGGNGGTSGGGSWI-YGHGGHGGGGSGNG-NDGNTNFGSGNAGG-GGSRVRRGATRTGS-GGAAQGGGSYGHGG-HGGAGGGSGSGGDGG-NGGNAGLIGGGGSGT-GGNGGLLLGFNGTNG-GGSTDHVREADDANID-DLLGGGSGGAGGNG-GWLYGNGGPGGGS-GLLYGNGGNGGAG-DTAGGGSVEGHTH-TISGGGSGGAGGAG-GRGGWLVGNGGG-SIEGDDTDRRGGGSDH-VREADDANIDGGGS-RADRARNTYGGGS-GHAGGAGGAGGAG-GRGGGGSGGTGGDG-GDGGHAGTGGGGSVSP-PETTTDGGGSWPIRAPS-RLGGGSNGGIGGD-GAGGGNATSGGGSHY-RFTLYHLGGGSGGNG-GAGGDAGHGGTGG-GGSGGAGGNGATGGT-GVGNGGGSYRTIDIRN-HGGGSAGGGGGGTTPT-GYLGPGGGSGGVGNAR-ADRARNTYTGGGS-GAGGGDVGGGGAGGT-THEYGAEALERAGAK-FVAAWTLKAAAHEY-GAEALERAG	726	67474.98	5.24	28.57	49.94	-0.549	2.0723	0.831047	14.89276	SOLUBLE	NON ALLERGEN	73 / 51

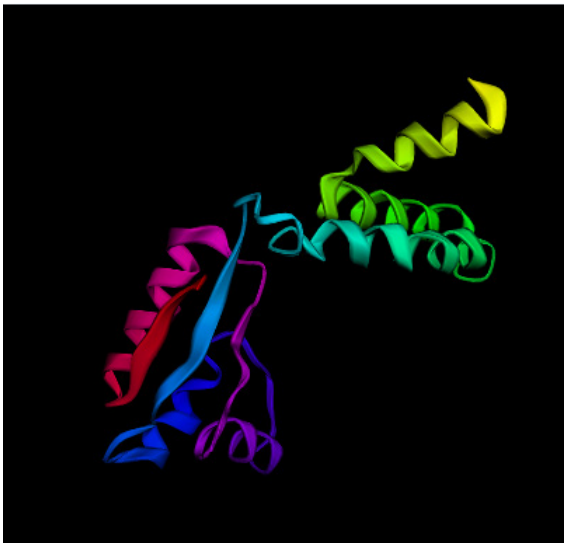

Figure 3. Secondary structure prediction of vaccine constructs using PSIPRED. (a) Vaccine construct 1 (VC1) secondary structure shows helix, β-sheets and turns; (b) Vaccine construct 2 (VC2) secondary structures shows helix and β-sheets; (c) Vaccine construct 3 (VC3) secondary structures shows only helix.

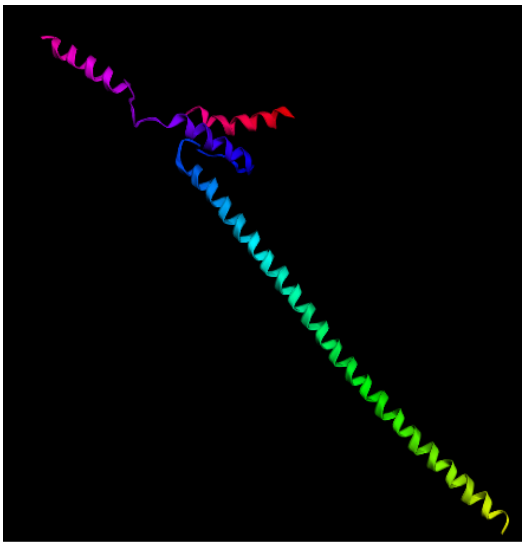

Figure 4. (a) Tertiary structure prediction of vaccine constructs VC1 using Phre2. (b) Ramachandran plot analysis of VC1 vaccine construct using RAMPAGE with 90.3% amino acids in most favored region and 8.9% in allowed region.

the 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.

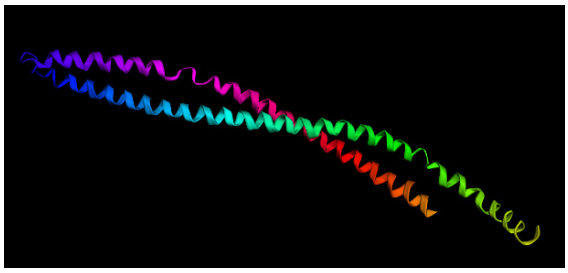

Figure 5. *In-silico* Docking of VC1 vaccine construct (red) with Human HLA alleles (blue and green). (a) HLA-A*02:01 docking with −35.39 binding energy; (b) HLA-A*24:02 with −21.04 binding energy; (c) HLA-B*15:01 with −23.17 binding energy; (d) HLA-DR2 (DRA*01:01-DRB1*15:01) with −32.5 binding energy.

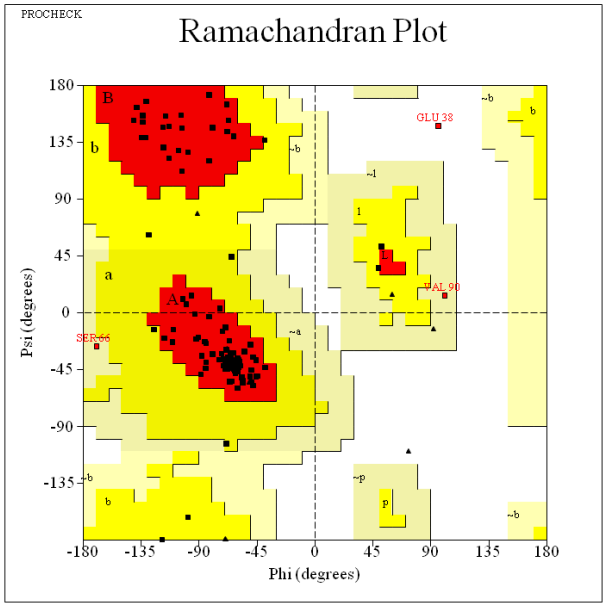

Figure 6. *In-silico* cloning of VC1 vaccine construct into pET28a vector for its heterologous expression in *E. coli* using EcoRI and NdeI restriction enzyme.

Structure prediction of selected vaccine constructs

Secondary and tertiary structures of the final vaccine constructs (VC1, VC2, and VC3) were predicted by PSIPRED and Phre2 (Fig. 3a–c, respectively). The predicted secondary structure of VC1, VC2, and VC3 consists of alpha-helix, beta-sheet and beta-turn. The model of VC1, VC2, and VC3 constructs were validated by the Ramachandran plot (Fig. 4).

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 Alelle	PDB ID	Vaccine Constructs		
			VC1	VC2	VC3
1	HLA-A*02:01	6EQA	-35.39	-9.28	-12.08
2	HLA-A*24:02	4F7M	-21.04	2	4.15
3	HLA-B*15:01	1XR8	-23.17	-10.07	-8.42
4	HLA-B*35:01	1A1N	-8.29	1.13	11.75
5	HLA-B*39:01	4O2E	-7.69	10.54	4.06
6	HLA B*44:02	1N2R	-11.42	13.34	10.94
7	HLA-B*58:01	5IM7	-16.4	-0.14	6.1
8	HLA-DR2 (DRA*01:01-DRB1*15:01)	1BX2	-32.5	2.13	-40.98
9	HLA-DRA1*0101/DRB5*0101	1H15	-28.08	1.89	-9.82
10	HLA-DQ2.3 (DQA1*03:01/DQB1*02:01)	4D8P	-1.64	1.46	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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None.

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