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Authors' Affiliation:
1. KBCMA College of
Veterinary and Animal
Sciences (Narowal Campus),
University of Veterinary and
Animal Sciences, Lahore –
2. Agricultural Bioinformatics
Key Laboratory of Hubei
Province, Hubei Engineering
Technology Research Center
of Agricultural Big Data,
College of Informatics,
Huazhong Agricultural
University, Wuhan – China
3. College of Life Sciences,
University of Science and
Technology of China, Hefei –
People's Republic of China
4. Department of health and
biological sciences, Abasyn
University Peshawar, Khyber
Pakhtunkhwa – Pakistan.
5. Department of Biomedical
and Diagnostic Sciences,
University of Tennessee
College of Veterinary
Medicine, Knoxville,
Tennessee – United States
of America
#These authors equally
contributed in the study.

*Corresponding Author:
Muhammad Ahsan Naeem
Email:
muhammadahsannaeem@ymail.com

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Reconnoitering *Mycobacterium tuberculosis* lipoproteins to design subunit vaccine by immunoinformatics approach

Muhammad Ahsan Naeem[#1]*, Muhammad Muzammal Adeel[#2], Ayesha Kanwal[3], Sajjad Ahmad[4], Waqas Ahmad[1], Qaiser Akram[1], Asif Saleh[1] , Waqas Ahmed[5]

Abstract

Background: Tuberculosis is an aerosol transmitted disease of human beings caused by *Mycobacterium tuberculosis* (*Mtb*). The only available vaccine for *Mtb* is Bacillus Calmette-Guérin (BCG). Currently no alternative or booster is available for BCG. The objective of this predictive approach was based on binding of MHC-I and MHC-II and B cell epitopes of *Mtb* for mouse host.

Methods: Immunoinformatics approach was used to design subunit vaccine (SV) by joining 8 MHC-I bindings, 6 MHC-II bindings, and 8 B-Cell epitopes with AAV, GPGPG, and KK amino acid linkers, respectively. The efficacy of the SV was enhanced through *Mtb* protein Rv3763 (LpqH, PDB ID= 4ZJM) as an adjuvant at the N-terminal of SV. The *in silico* analyses evaluated the SV to predict allergenicity, antigenicity, and physico-chemical properties.

Results: Predictions revealed that SV is non-allergic and highly antigenic. The physico-chemical analysis showed that the SV was stable and basic in nature. The three-dimensional structure of SV was stable with a high binding affinity against the mouse TLR2 receptor. *In silico* cloning suggested the effective transformation of SV into the eukaryotic expression vector.

Conclusion: This study permits preclinical validation of the designed SV in mouse host to confirm its immunogenic potential and efficacy, which will help in controlling tuberculosis.

Introduction

Bacille Calmette-Guérin (BCG) is the only available attenuated strain of *Mycobacterium bovis* against Tuberculosis (TB) [1]. As a vaccine, BCG can prevent neonates and children from the spread of TB [2]. However, the use of BCG vaccine has some severe limitations, as its overall efficacy is still in question [3, 4]. Several controversies about BCG include; failure to provide protection against pulmonary TB in adults, the absence of a booster dose, and difficulty in the differential diagnosis between diseased and vaccinated individuals by standard diagnostic test *e.g.* tuberculin skin test [5, 6]. Consequently, *Mtb* has undergone a dormant state in most cases after BCG vaccination and is associated with latent chronic infection development [7]. The latent *Mtb* becomes active when a person's immune status becomes weak, *e.g.*, individuals with genetic immune defects, aged people, and people taking immunosuppressant drugs [6]. To overcome these issues of the currently available vaccine, the development of an alternative vaccine is essential. It is necessary to design a vaccine that is safer, more immunogenic, and induces long-lasting protection. An answer to this could be a multi-epitope subunit vaccine (SV), which is a substitutive approach to immunization. It identifies the peptide epitopes on immunogens and generates the immunogenic response [6]. For this purpose, synthetic versions of peptide epitopes have been used for engineering the vaccine. Contrary to routine vaccines, SV are non-infective, synthetic, possess zero risks of pathogen activation, and are free from unwanted effects, which are common while using whole organism attenuated vaccine [6].

Mtb lipoproteins are a diverse class of membrane-bound proteins with multiple functions [8]. These proteins have signal peptides which help in their secretion out of a bacterial cell and may cause post-translational modification in the host cell [8]. Immunoinformatics is a newly developed approach that predicts thermodynamically stable linker joined multi-epitopes novel vaccine(s) significantly less cost and time. We have used this approach in our study to predict MHC-I, MHC-II binding, and B cell epitopes to design a SV against tuberculosis. This vaccine was evaluated computationally for its physico-chemical characteristics, structure validation, antigenicity, allergenicity prediction, and *in silico* cloning in the eukaryotic expression vector. This study will help to provide a new immunogenic vaccine that may have the ability to induce the immune response against tuberculosis if evaluated in animal model, by complementing or boosting BCG challenges.

Methods

Collection of *Mycobacterium tuberculosis* protein sequences for vaccine design

Primary sequences of 89 lipoproteins of *Mycobacterium tuberculosis* H37Rv were retrieved from Mycobrowser (database of *Mycobacterium spp.*) in FASTA format. These protein sequences were employed for scheming multi-epitope SV construct potentiated against TB infection. Experimentally known crystal structure of *Mycobacterium tuberculosis* Rv3763 (LpqH, PDB ID= 4ZJM) TLR2 agonist protein and its primary sequence were retrieved from Protein Data Bank (PDB) [9], and Mycobrowser database respectively, to be used as an adjuvant.

Prediction of epitopes

MHC-I binding epitopes for all the selected lipoproteins of *Mtb* were predicted using Immune Epitope Database (IEDB) resources [10]. "IEDB recommended 2.19" prediction method. Six MHC alleles (H-2-Db, H-2-Dd, H-2-Kb, H-2-Kd, H-2-Kk, H-2-Ld) each of 9-mer in length were selected, and peptides were sorted based on their lowest percentile rank. Similarly, the IEDB tool (http://tools.immuneepitope.org/mhcii/) [11] was recruited for predicting MHC-II binding epitopes. Default parameters were used against the mouse (host) with locus H-2-I. All three alleles, namely, H2-IAb, H2-IAd and H2-IEd, were selected for this locus. 15-mer length MHC-II epitopes for mouse alleles were predicted for all the lipoproteins. B cell linear epitopes were predicted using ABCPRED server for the ultimate SV design. ABCPRED (http://crdd.osdd.net/raghava/abcpred/ABC_submission.html) [12] with threshold >0.9 and epitope length of 20 amino acids was used.

Multi-epitope SV construct

Epitopes of lowest percentile rank (high binding affinity), 8 of MHC-I and 6 of MHC-II, were joined together with AAY and GPGPG amino acid linkers, respectively. 8 B cell epitopes with score ≥ 0.95 were selected and joined with KK amino acid linker to form the final vaccine construct. Therefore, mouse TLR2 (Uniprot ID: Q9QUN7) agonist Rv3763/LpqH *Mtb* protein [9] was selected as an adjuvant to augment the antigen-specific immune reactions. Moreover, a 13 amino acid sequence (AKFVAAWTLKAAA) known as PADRE sequence was concatenated at the start and the end of the epitope sequence of the vaccine to enhance its efficacy [13, 14]. The final SV consisted of 593 amino acids.

Physiochemical parameter, allergenicity, and antigenicity predictions of SV construct

Physicochemical parameters of the vaccine were determined by using ProtParam [15] web server, a part of Expert Protein Analysis System (EXPASY). Various parameters were predicted using this tool, including; theoretical pI, estimated half-life, molecular weight (kDa), grand average of hydropathy (GRAVY), and aliphatic index. AlgPred web tool [12] was applied for the prediction of allergenicity of SV. AlgPred uses combined algorithms (SVMc+ IgEepitope + ARPs BLAST+ MAST) approach to give highly accurate results. Allergenicity measurement of a protein is essential because most of the allergens are protein in nature and can cause sensitization or severe allergic reactions. Thus, the predicted vaccine construct must be free from the allergenic nature. For antigenicity index prediction, ANTIGENpro tool was applied, as this tool is efficient and accurate. ANTIGENpro performs prediction by using primary protein sequences regardless of any pathogen and without performing their alignment. For categorizing a protein as antigenic or non-antigenic, ANTIGENpro

uses a support vector machine (SVM) algorithm based on its microarray data.

Prediction of SV secondary structure

For secondary structure prediction of SV protein, PSIPRED web-based server 4.0 (http://bioinf.cs.ucl.ac.uk/psipred/) was applied, which uses Psi-BLAST *i.e.,* Position-specific iterated BLAST method to find out the closest identical sequences of related protein templates or sequences.

Tertiary structure prediction of SV construct and molecular docking with TLR2 immune receptor

Tertiary structure prediction of the vaccine is crucial because it is more stable, and this stability is achieved by decreasing the energy of protein molecules. Three-dimensional SV vaccine structure was designed and configured by RaptorX server at default parameters. Energy minimization and structure visualization were carried out by (University of California, San Francisco) UCSF Chimera tool. To perform the interaction analysis, a molecular docking approach was used in which SV construct was docked against mouse TLR2 (PDB ID: 2Z7X) using PatchDock (http://bioinfo3d.cs.tau.ac.il/PatchDock/) [16] followed by the rescoring and refinement of docked solutions through FireDock (Fast Interaction Refinement in Molecular Docking) server. FireDock predicts the ten best way-outs based on binding score. Interacting amino acids of SV and TLR2 receptors were visualized through the LigPlot tool.

It is crucial to detect whether a designed vaccine has a binding affinity with its receptor or not [17]. If the receptor can recognize *Mtb,* then it leads to an immunogenic response in the host [18].

Reverse translation, codon optimization and *in silico* cloning of SV construct

To check the expression of the designed SV construct in the eukaryotic system, we first reverse-translate our protein using Sequence Manipulation Suite: reverse translation tool, and the resulting sequence was used for codon optimization. Java Codon Adaptation Tool (JCAT) [19] was used to optimize the codon of SV construct in eukaryotic organisms (*Mus musculus*). Three additional parameters, including prokaryote ribosome binding site, restriction enzymes cleavage sites, and avoid the rho-independent transcription termination, were selected for appropriate codon optimization, followed by *Xho*I restriction site digestion and cloning into adeno-associated virus vector (pAAV) by using SnapGene tool.

Results

Epitopes collection and design of SV

After predicting MHC-I binding, MHC-II binding, and B cell epitopes, the immuno-informatics approach was used to construct a SV. MHC epitopes were selected according to the instructions of IEDB *i.e* low percentile epitopes have a high binding affinity with MHC molecules. Thus, for percentile rank, a threshold of less than or equal to 0.3 was self-created for shortlisting of epitopes of different proteins. The shortlisted MHC-I and

MHC-II binding epitopes for lipoproteins are presented in table 1 and 2, respectively. B cell epitopes were selected based on their highest score. To shortlist these epitopes, a self-created threshold of more than 0.9 score was used in table 3.

Protein Name	Peptide Seq	Start	End	Allele	Percentile Rank	ANN IC50 (nM)
LprB	LGPHFSFSW	91	99	H-2-Dd	0.1	268.42
LpqK	YAHTNYLLL	188	196	H-2-Db	0.1	4.36
LpqM	YQLVNAFFS	140	148	H-2-Db	0.1	106.03
LprM	LELQWGRTI	340	348	H-2-Kk	0.1	5.74`
pstS3	DGPTLAKIF	148	156	H-2-Dd	0.1	1085.98
ugpG	RGPQSWSEF	172	180	H-2-Dd	0.1	217.85
proX	ASCANADPL	25	33	H-2-Db	0.1	19.28

Table 1: Predicted MHC-I binding epitopes.
(≤ 0.3 percentile rank as threshold, against mice alleles H-2- (Db, Dd, Kb, Kd, Kk, Ld)

Protein Name	Peptide Seq	Start	End	Allele	Percentile Rank
LpqU	RRWLRAVAVIGATAM	4	18	H2-IAd	0.01
LprI	ALAHRRSAALEAAQS	66	80	H2-IAd	0.01
LpqO	RRGARMAALLAAAAL	4	18	H2-IAd	0.01
LppN	VYLRAPVAVDAAVVS	98	112	H2-IAd	0.01
LpqF	RRAGRRMLALSAAAA	9	23	H2-IAd	0.01
GlnH	MTRRALLARAAAPLA	1	15	H2-IAd	0.01

Table 2: Predicted MHC-II binding epitopes
(≤ or = 0.3 percentile rank as threshold, against mice alleles H-2-I (Ab, Ad, Ed)

Lipoprotein	Epitope	Score	Start position
LppJ	LDHSYGEMILDGECRNTTDH	0.95	152
LpqW	GIDWIGPGFNPHLLSDLSPV	0.95	58
LprD	NAYLAELAKDDARKQNRTTA	0.95	107
LpqM	LPMQSPWTALRTACLTGVAH	0.96	408
Rv2585c	RTRRALALCVPRDAIARDAG	0.95	330
Rv2864c	SPFGMALVAATVAAGKTPVP	0.95	481
LppL	PTIEPAQPAVSPPTSQDPAG	0.95	40
GlnH	MADQPYGVGINLDNTGLVRF	0.95	271

Table 3: Predicted B cell epitopes of lipoproteins.

These epitopes were linked together by using a linker in such a way that MHC-I epitopes are linked by AAY, MHC-II by GPGPG, and B cell epitopes by KK linkers, respectively. Further, the Rv3763 (LpqH, PDB ID= 4ZJM) protein of *Mtb* [9] was used as an adjuvant at N-terminal to improve the immunogenicity of the vaccine. A sequence of 13 amino acids, the PADRE sequence [14], was incorporated after adjuvant and at the end of the last epitope to further enhance vaccine efficacy (Fig. 1).
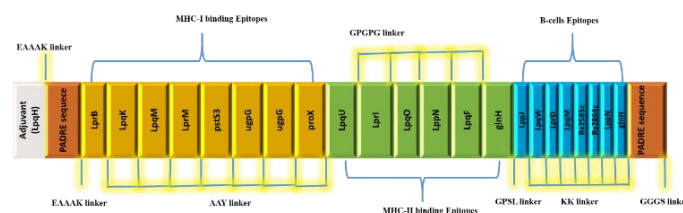

Figure 1: Schematic diagram of final SV construct. The multi epitope vaccine sequence consisted of 593 amino acid residues. Among these, the first 159 residues are represented as an adjuvant followed by MHC-I binding epitopes (160–280 amino acids), MHC-II binding epitopes (281–400 amino acids) and B cell epitopes (401-593 amino acids).

This Diagram clearly showed that the EAAAK linker joined the adjuvant and MHC-I binding epitopes, while MHC-I and MHC-II binding epitopes were connected through AAY and GPGPG linkers. The MHC-II binding

epitope and first B cell epitope were joined by a GPSL linker.

Allergenicity, Antigenicity and Physicochemical Predictions of vaccine

The results of allergenicity prediction by AlgPred revealed that SV is non-allergen with a -0.69 score, which is less than the threshold value of -0.4. However, antigenicity prediction by ANTIGEN pro revealed that SV is a probable antigen with a score of 0.7730, which is more than the threshold value of 0.4. Physico-chemical properties were obtained by using ProtParam, which showed that SV protein has a total of 593 amino acids with a molecular weight of 60.1 kDa. The theoretical PI is 9.89, which depicts that our SV is basic in nature, while the computed instability index of SV is 37.31, demonstrating that SV is stable in nature. The grand average of GRAVY value is -0.018 which means that SV is hydrophilic *i.e.* easily dissolvable in water.

Secondary Structure Prediction

The secondary structure of SV protein was predicted by PSIPRED server (Fig. 2). The results were obtained in the form of a strand (yellow), helix (pink), and coil (grey line), which are presented with different color schemes. Percentage of strand, helix and coil in SV is 17.87%, 40.64%, and 41.49%, respectively.

Prediction and validation of the tertiary structure in protein

This pattern of the protein was predicted through RaptorX and the resultant SV has been shown in Fig. 3a. 3D model of SV was predicted for 3 domains at different amino acid, including; domain 1 (1-268) 2.40 x 10^{-10}, domain 2 (366, 581) 8.60 x 10^{-5} and domain 3 (269, 365) 1.20 x 10^{-4}. The calculated overall un-normalized GDT (uGDT) of SV structure was 347. 100% residues (593) in the SV protein were modeled and 11% (64) residues were in the disordered region.

The quality or checking of any error in the 3D model of protein was performed using ProSA-web [26]. ProSA-web calculated Z-score -1.52 for SV (Fig. 3b). The Ramachandran plot validated the tertiary structure of SV. The plot showed (Fig. 3c) that 423 out of 593 (87.9%) residues were in the favored regions [A, B, L], 40 residues (8.3%) were in additional allowed regions [a,b,l,p], 7 (1.5%) residues were in generously allowed regions [~a, ~b, ~l, ~p] and only 11 (2.3%) residues were in disallowed regions. The number of glycine residues (shown in a triangle) was 69, and the number of proline was 41. Thus, this tertiary structure is of good quality as most of its residues are present in the most favored regions.

Docking of SV with TLR2 receptor

Molecular docking was performed through PatchDock server. SV protein was docked with mouse TLR2 receptor containing adjuvant. PatchDock server produced docked solutions based on refined geometry and electrostatic affinity of the protein surface. Resulting docked complexes were subjected to Fast Interaction Refinement in Molecular Docking (FireDock) server for refinement and re-scoring based on respective binding

energy. The top-ranked complex was selected, having global energy -7.46 and attractive Van Der Waals interactions score -11.71 (Fig. 4a).
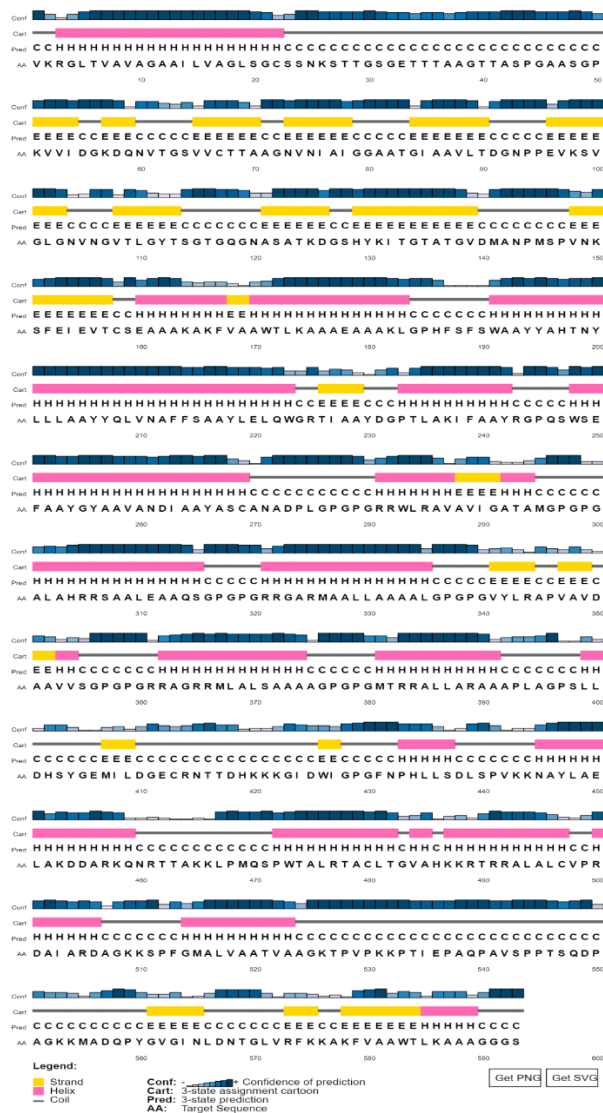

Figure 2: Yellow color blocks representing stand, pink; Helix and Gray lines representing coils of the secondary structure composed for SV.
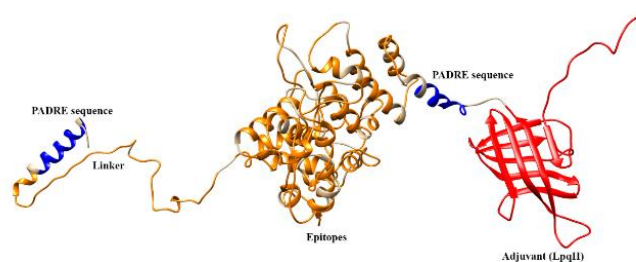

Figure 3a: The multi-epitope vaccine and its 3D model that has been procured refinement and homology modeling.
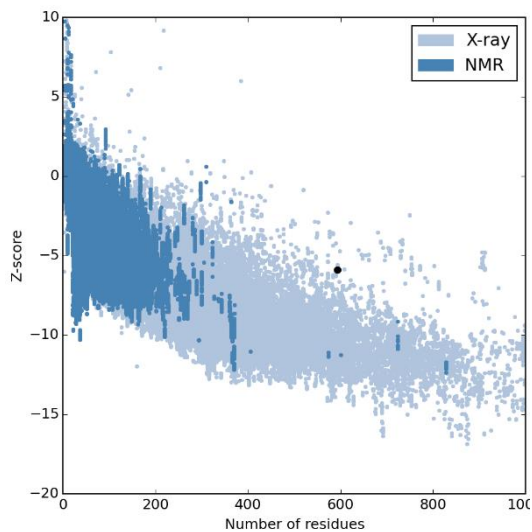
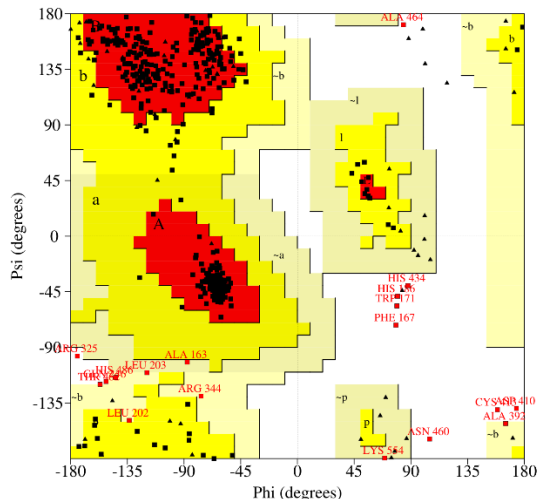**Figure 3b:** 3D structure showing Z-score and PROSA validation (-1.52).

Figure 3c: Plot analysis through Ramachandran model and various percentages of structures have also been mentioned in the lower set of the image.

U-shaped binding pocket amino acid residues (Glu310, Arg340, Arg395, Glu363, Gln390, Leu 525, Gln526 and Asn552) of TLR2 receptor were involved in hydrogen bonding with SV residues (Gly45, Ala46, Ser48, Asn93, Thr116, Gly117, Asn120, and Ala121) after molecular docking. The docked result is shown in Fig. 4b.
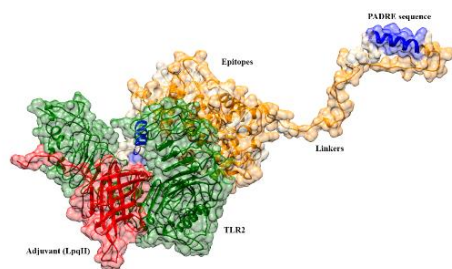

Figure 4a: Mouse TLR2 is docked with Mycobacterium tuberculosis Rv3763 (PDB ID: 4ZJM) adjuvant of SV construct.
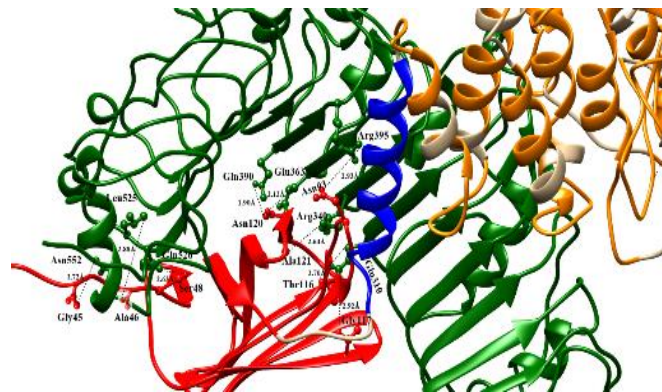

Figure 4b: Closed view of SV and mouse TLR2 receptor docked complex. SV as a ligand have adjuvant (red), epitopes (yellow), linkers (white) and PADRE sequence (Cyan) whereas TLR2 receptor has been shown in green color.

Reverse translation and codon optimization
SV sequence was reverse translated to the nucleotide sequence. The total length of the reverse translated sequence is 1794 bp. Then this sequence was codon-optimized for expression in the mice host. Codon adaptation index (CAI) value of optimized sequence was 0.71, GC content of improved sequence was 73.24 and GC content of *Mus musculus* was 41.76%. Graphical representation of optimized or adapted sequence is shown in Fig. 5.

***In silico* optimization and cloning of vaccinal protein**
The SV nucleotide sequence was codon adapted/optimized according to the mammalian (mouse) vector by introducing BsmI and BamHI restriction sites in N- and C-terminal of the vaccine sequence. Finally, the improved sequence with restriction sites was adjusted in the pAAV-CMV-eGFP vector by means of SnapGene tool to guarantee protein expression in the vaccine (Fig. 6).
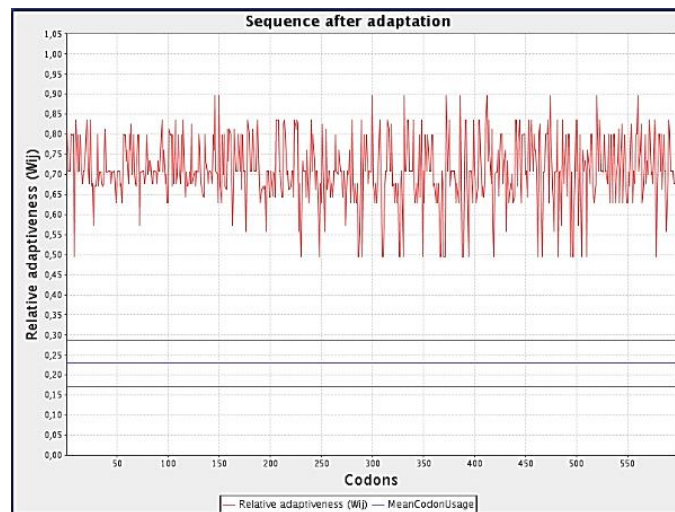

Figure 5: Graphical presentation of codon optimized sequence. Red peaks indicate that almost every codon has been optimized. Y-axis represents the relative addictiveness of each codon.
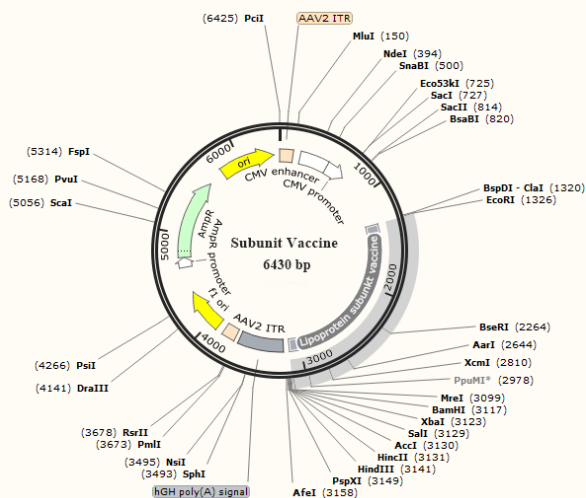
Figure 6: *In silico* cloning of final SV nucleotide sequence into eukaryotic expression vector (pAAV-CMV). Gray part labeled as "lipoprotein SV" has represented the vaccine insert.

Discussion

Vaccination is an efficient way to control infectious diseases and improve public health [20]. Now-a-days, much genomic, transcriptomic, and proteomic information is available for *Mtb* and other pathogenic microbes. Computational approaches were applied using this information to identify potential antigenic targets [21]. In our study, we focused to design a SV from 89 lipoproteins of *Mtb*. Instead of using the whole organism as a vaccine agent, only the immunogenic part of these proteins was used to construct the vaccine. Lipoproteins of *Mycobacterium tuberculosis* belong to the functionally broad class of membrane-bound proteins [8]. These proteins are involved in providing virulence and immunogenicity to Mtb as the interruption in the biosynthesis of lipoproteins leads to the weakening of *Mtb* activity [22].

Mtb epitopes that showed resemblance with the host proteome were identified. The protein sequences of lipoproteins were imperiled to BLASTp search against the human host proteome. These proteins of *Mtb* did not show any sequence homology with human proteins. Thus, these proteins could be excellent immunogenic targets for synthesizing vaccines against *Mtb*. In this study, epitopes for B and T cells of 89 lipoproteins were predicted. Epitopes of T cells are presented on the surface of antigen-presenting cells by combining with MHC molecules [23]. MHC molecules are antigen-presenting moieties, mainly of two types, *i.e.* class I and class II [24]. Class I molecules, mainly represented to cytotoxic T lymphocytes or CD8+ T cells, can be presented on any kind of nucleate cell that represents the endogenous processed protein or the engulfed processed protein that were digested and processed in cytosolic pathways [25]. Class II molecules are mainly combined and present exogenous proteins, such as pathogens' surface proteins. These are processed in the cellular endocytic pathways and presented to helper CD4+ T lymphocytes [20].

The immunoinformatics approach to analyze SV demonstrated that our final SV protein has a tremendous binding affinity with MHC-I, MHC-II, and B-cell epitopes based on structural and physicochemical characteristics. Model quality of the 3-dimensional vaccine protein structure predicted by homology modeling suggested the refined amino acid distribution. Ramachandran plot shows that most of the vaccine residues are present in the favorable region, and very few are located in the disallowed regions. This showed that the overall model quality of protein structure is acceptable.

The conformational stability of the vaccine was done by docking with the TLR2 receptor to determine the immunological response of the vaccine construct. Energy consumption was reduced to decrease the potential energy of the protein-receptor complex system. This process also keeps the dismissed arrangements of protein structure by replacing a few subcellular structures in the protein to yield appropriate stereochemistry of a stable protein framework.

The optimum level of translation expression of a vaccine in the eukaryotic system is necessary for vaccine functionality. The process was carried out by codon optimization of the nucleotide sequence of the vaccine to refine transcriptional and translational efficiency [26]. The parameters used for analyzing the codon optimization are; total GC content of DNA sequence and CAI. The important requirement for various physiological and biochemical studies is the solubility of the overexpressed recombinant protein in the eukaryotic system. Augmenting the strength of proteins is a domineering target in several mechanical and bio-medical uses. Therefore, unique immunoinformatics tools were incorporated in the current study for a safe, potentiated and immunogenic SV design which may have the controlling ability against tuberculosis.

Tuberculosis disease has become an intense problem at the international level. It causes many deaths in the developing states of Asia and Africa, and there is still no permanent control and remedial measure for the disease. The immunoinformatics technique was used for the development of a multi-epitope SV. This work begins with the recovery protein sequences of 89 *Mtb* lipoproteins. After that the immunogenic B- and T-cell epitopes were predicted, epitopes were joined by linkers for the effective separation of epitopes in *in-vivo* system, adjuvant was added in the start to enhance the immunogenicity and then design the SV to get the cell-mediated and humoral immunity. Allergenicity and antigenicity parameters were predicted to confirm that the vaccine is non-reactive in nature. The binding affinity with the host cell receptor was evaluated by molecular docking. Finally, the effective expression of SV protein was made sure by *in silico* cloning. Furthermore, the recommendation obtained from this study declares that the proposed vaccine requires experimental validation to analyze the efficacy of the vaccine in the sense of generating adequate immunological memory.

Competing interests

None.

Author Contributions

MMA and MAN conceived and designed this study; MAN, MMA and AK performed the experiments; SA, QA, WA, AS and WA analyze the data/results; MAN, MMA and SA wrote the manuscript. All authors read and approved the final manuscript.

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