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RESEARCH ARTICLE

Multi Epitope Based Vaccine Design and Analysis against *Mycoplasma bovis* Using Immunoinformatic Approaches

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A B S T R A C T

Mycoplasma bovis belongs to Mollicutesclass, is responsible for the respiratory and reproductive diseases in dairy farms. Vaccination is essential for protection from *M. bovis* bacteria. Previously, several vaccines have been reported that are rendered useless with the passage of time due to versatile changes in bacteria. This study aims to identify the conserved regions for B and T cell epitopes of chromate transporter protein. Different bioinformatics tools including Ellipro, NetCTL and NETMHCIIpan were used to predict B cell, CTL and HTL epitopes, respectively. Altogether, we predicted 14 peptide epitopes (11 CTL and 3HTL epitopes) of chromate transporter protein that induce the immune system. These epitopes used to predict vaccine against *M. bovis*. Total length of predicted multi epitope vaccine consists upon 239 amino acids in its primary structure. To check interactions, molecular docking was performed by patchDock and analyzed through LigPlot. To evaluate multi epitope vaccine's immunogenic profile, an *Insilico* immune response was produced by C-ImmSim server. JCat was used for optimization of codon and the reverse translation resulting in a vaccine cDNA sequence that can be used for an efficient expression. Antigenicity and allergenicity was studied by Vaxijen 2.0 and Allertop server. It was observed that epitope-based vaccine helped to avoid the outbreaks of pandemics in dairy farms with more efficacy and fewer side effects. This research work will help researchers in testing the effectiveness of epitope-based vaccine design against *M. bovis*.

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INTRODUCTION

Mycoplasma bovis belongs to Mollicutes class and is characterized by lack of cell wall, small genome size (0.58–1.4 Mbp), and lower GC content (23 to 40 percent). *M. bovis* is known as a predominant pathogen that affects cattle around the world. It is a leading cause of cattle infections including mastitis, otitis media, arthritis, respiratory diseases, and reproductive diseases, but variation in the disease severity is quite common. It is also reported that *M. bovis*has also infected cattle lungs, eyes, ears, brain, and joints (Maunsell *et al.,* 2011; Parker *et al.,* 2018; Dudek *et al.,* 2020). Despite the fact, *Mycoplasma bovis* is main cause of mastitis and BRD in the cattle, *M. bovis* infection costs the US beef and dairy industry $32 million and $108 million per year, respectively. *M. bovis* was isolated in 1961 from infected cattle for the first time. It is frequently extracted from the lungs of the cattle suffering from pneumonia. *M. bovis* can also be isolated from cattle's upper respiratory tract. It spreads to many countries through animal movements. Infection has now been reported in nearly all over the world including majority of European countries (Nicholas and Ayling, 2003). In addition, *M. bovis* infections caused numerous economic losses in terms of laboratory diagnosis, treatment, and cattle production (Maunsell *et al.,* 2011; Li *et al.,* 2015).

Mycoplasma bovis is not prevalent but widespread in enzootically infected areas of bovine population. Basically, infection is introduced by both healthy calves and young cattle that shed mycoplasma, but once it has spread on multiple age sites, it is extremely hard to

eliminate. Generally, *M. bovis* transmitted from infected to uninfected cattle in different ways, such as close contact with aerosol and through contaminated insemination material which believed to be the main route of spread. As well as, infection can occur through maternal contact, nose to nose contact, from urine of infected animal, and through contaminated milk. Fomite-mediated transmission has been shown to occur due to *M. bovis* survival in severe conditions (Underwood *et al.,* 2015; Dudek *et al.,* 2020). Despite the fragile nature of many of the mycoplasmas in the environment, it can live for two months in sponges and milk at 4°C, and in water for more than two weeks. Cold or wet environment can increase chances of infection; at higher temperature, survival rate drops dramatically. However, in comparison to infected animals, the environment is not a major source of infection.(Nicholas and Ayling, 2003).

Basically *M. bovis* is smallest pathogen that present in animal's respiratory tract and adheres to mucosal surfaces where invades into tissues, here they survive, multiply and release toxins that can cause serious tissue damage. With the attack of this pathogen, there is increased respiration, frequent hacking cough, fever, runny nose, decreased appetite and swelling in joints etc. *M. bovis* has significant number of ways for evading and altering host responses. During *M. bovis* infection, anti-inflammatory and proinflammatory cytokines are stimulated (Maunsell *et al.,* 2019). Cytotoxic T lymphocytes (CTLs) act as drivers of immunity against *M. bovis* in cattle. CTLs not only lyse certain target cells, but it releases molecules, that have been found to kill pathogen directly such as granulysin.$WC1^{+}\gamma\delta T$ cells have an important role in *M. bovis* infection in cattle, while its exact role is unknown (Kennedy *et al.,* 2002; Endsley *et al.,* 2004). Diagnosis of *M. bovis* infection can be performed through microbial culture, but this conventional method is time taking. PCR and ELISA techniques can be used to detect infection of *Mycoplasma bovis* (Sachse *et al.,* 1993).

Mycoplasma bovis infects cattle of all age groups and can survive in herd for a long time. Due to lack of cell wall, *M. bovis* exposed to membrane proteins that make primary contact with its host (Maunsell *et al.,* 2011). Cell wall absence makes *M. bovis* resistant to different antibiotics such as penicillin, amoxicillin, ampicillin, cephalosporins and tetracyclines. These antibiotics kill bacteria by inhibiting formation of cell wall in newly formed bacteria. As a result, Mycoplasmas without cell wall remains unaffected by these antibiotics. Some injectable antibiotics were used against *M. bovis*, but due to tissue damage in lungs and joints by *M. bovis*, it is difficult for injectable antibiotics to reach for long time in high concentrations to kill bacteria. Diagnosis and control of *M. bovis* related diseases might be difficult due to the inconsistency of disease expression and response to treatment and vaccines (Dudek *et al.,* 2020).

M. bovis has diverse set of variable surface proteins (Vsps) that include 13 important lipoproteins. VspA, VspB, and VspC are ideal vaccine targets out of the 13 important Vsps. (Lysnyansky *et al.,* 1996; Lysnyansky *et al.,* 1999). Latest advances in the field of genomics and proteomics enabled the development of vaccine that overcome the drawbacks of conventional vaccines. The epitope based vaccine approach could be a viable option for developing vaccine against those pathogens for which commercial vaccines have fail to do their work (Raza *et al.,* 2019). Live attenuated vaccines and epitope-based peptide vaccine can be used for treatment of *M. bovis* infections. Live virus particles with a low level of virulence included in the attenuated vaccines (Patronov and Doytchinova, 2013). This type of vaccine is delivered through intranasal direction and allow broader immune response that is more similar to natural immunity (He *et al.,* 2013). While epitope-based vaccines provide high specificity, good safety, stability and easy to produce and store, that's why it is preferred over conventional vaccines (Lei *et al.,* 2019). Immune cells such as T and B cells have ability to develop pathogen specific memory and, in this way, they are providing immunological protection. B and T-cells identify epitopes within their cognate antigens. Epitope prediction in antigens is of great interest for number of reasons, such as diagnostic tests, immunological monitoring, disease etiology recognition, and epitope-based vaccine prediction (Sanchez-Trincado *et al*., 2017). T-cell epitopes are the peptide fragments, while B-cell epitopes could be proteins, nucleic acids, lipids, or carbohydrates (Patronov and Doytchinova, 2013). Single epitope based peptide vaccine predict to target multiple strains by utilizing a multi epitope approach (Skwarczynski and Toth, 2016). To design vaccine for *M. bovis*, most virulent protein was predicted through whole genome analysis. The protein's B and T cell epitopes were predicted to design MEV.

To control *M. bovis* infections, there is need of effective treatment and vaccine. Until, epitope recognition was totally reliant on experimental approaches (Raza *et al.,* 2019). The traditional vaccine development methods not only time consuming, but they are also ineffective in controlling diseases because they are based on expression of antigens. It is also a possibility that expressed antigens might not be ideal candidates for vaccine. Due to these problems, it is impossible to control various types of epidemics (Oany *et al*., 2014). Now a days, immunoinformatic is a unique approach that identifies disease controlling strategies. Immunoinformatic based methods acquired lot of popularity as genomic and protein databases have grown (Rasheed *et al.,* 2021). Presently, there is no commercially available vaccine that based on epitope subunits while there is need of epitope-based vaccine against *M. bovis* to cure cattle herd. That is why, the primary goal of this research work was to predict T and B-cell epitopes that may be used to build epitope-based vaccine against *Mycoplasma bovis*.

MATERIALS AND METHODS

Retrieval of whole genome FASTA sequence: Whole-genome analysis was carried out to identify most antigenic protein of *M. bovis*. Protein sequences of most antigenic protein 'Chromate Transporter' of *Mycoplasma bovis* was retrieved by NCBI database whose accession no is TQF37121.

Selection of Most antigenic protein by whole genome analysis: VaxiJen server (http://www.ddg-pharmfac.net/vaxijen/) was used to identify the antigenicity and

SecretomeP server (http://www.cbs.dtu.dk/services/SecretomeP/) was used to predict protein secretion score. Server's default parameter was used to identify secretion score and antigenicity. A score of 0.6 was used to sort secretary proteins (threshold value for bacterial proteins).

Conserved regions identification: Multiple sequence alignment was performed using Clustal Omega program which aligned large number of sequences rapidly and precisely and available at https://www.ebi.ac.uk/Tools/msa/clustalo/. Multiple sequence alignment highlighted conserved sections as well as permitting the recognition of distantly correlated proteins. After that, conserved regions were used to find out linear B and T-cell epitopes.

Consensus sequence generation: Geneious Prime software was used to identify consensus sequence of target protein that have structurally and functionally conserved regions of importance. After uploading sequences manually to the software, these were aligned and assembled and a consensus sequence file was retrieved.

T Cell epitopes (CTL, HTL) prediction: For the prediction of T cell epitopes, NetCTL Server was used that is a specified tool used to predict T cell epitopes. Reliable Cytotoxic T lymphocyte (CTL) epitope prediction is vital for the rational vaccine design. Notably, it reduces time and effort required to identify the epitopes. NetCTL is a webtool that predicts CTL epitopes in any protein, available at http://www.cbs.dtu.dk/services/NetCTL/. NetMHCIIpan Server was used to find out binding of peptides to any known sequence of MHC II molecule that classified at default threshold. This web based server is freely available at http://www.cbs.dtu.dk/services/NetMHCIIpan/. Additionally, VaxiJen server was used to evaluate antigenicity of each peptide.

Designing of vaccine construct: To design vaccine construct, GPGPG spacer was chosen because G and P rich areas are related to β turns that would result in secondary and tertiary structures. GPGPG and AAY linkers inhibit production of the junctional epitopes, which is primary goal in designing of multiepitope vaccines; however, they promote immunization as well as epitope presentation (Nezafat *et al.,* 2016). For immune response enhancement, an adjuvant linked with first CTL epitope via EAAAK spacer, but the other epitopes linked via GPGPG and AAY spacers. The β-defensin adjuvant was preferred because it is a 45 amino acid long peptide which functions not only as an immunomodulator and antimicrobial agent, but also behave as chemoattractant molecule (Hoover *et al.,* 2003; Behmard *et al.,* 2020).

Tertiary structure prediction, Refinement and Validation: The purposed vaccine construct was further subjected for its 3D structure prediction process. For this purpose, trRosetta (https://yanglab.nankai.edu.cn/trRosetta/) was used, which is quick and precise algorithm of protein structure prediction. It predicts protein structure based on restrained energy minimizations with distance and orientation restraints derived from the network outputs. The quality of structure was assessed by using the pdbsum Ramachandran plot.

Prediction of B cell epitope: B-cells produce antibodies that bind to antigens; those specific parts recognized by antibodies are called B-cell epitopes. B-cell epitopes from the proteins of pathogen used linear B-cell epitopes to stimulate defensive immunity(Larsen *et al.,* 2006). Ellipro tool of IEDB-AR (http://tools.iedb.org/ellipro/) was used to find out the conformational B-cell epitopes for final MEV construct.

Physiochemical properties of vaccine: Using threshold value of 0.4, VaxiJen was used to assess the antigenicity of the vaccine construct. The vaccine's allergenicity was assessed by using AllerTOP server (https://www.ddg-pharmfac.net/AllerTOP/). AllerTOP is primary proper alignment free allergen prediction server. Furthermore, allergenicity of designed vaccine verified through Allergen FP server which provides different properties of amino acids. Other physicochemical properties of our predicted vaccine, such as its isoelectric point, molecular weight (M.W), aliphatic index, instability index, half-life, and the GRAVY score, were evaluated using the ExPASy ProtParam server (https://web.expasy.org/protparam/). TMHMM server v2.0 used to find out the transmembrane helices of target protein (Kar *et al.,* 2020).

Molecular docking of vaccine: To create stable immune response, designed vaccine must interact with 3RG1 target immune cell receptors. Molecular docking studies were conducted to investigate these interactions by using PatcDock. PatchDock server (https://bioinfo3d.cs.tau.ac.il/PatchDock/) is a freely available geometry-based docking algorithm. PatchDock's inputs are uploaded protein structures or either protein PDB codes. LIGPLOT was used to analyze the interactions of docked complexes. For a given complex file, the LIGPLOT software creates protein's schematic diagrams and also the ligand interactions.

Immune Simulation and Reverse translation, Codon optimization: C-ImmSim server was used to evaluate vaccine immune simulation that expresses immune response profile and immunogenicity. Java Codon Adaptation Tool (JCat) was applied to find out codon optimization and reverse translation, resulting in vaccine cDNA sequence which was used for efficient expression in *M. bovis* (strain PG1).

RESULTS

Selection of most Antigenic Protein of *M. bovis* through whole Genome Analysis: Protein sequences of virulent strains of Chromate Transporter of *Mycoplasma bovis* were retrieved from NCBI database, through whole genome analysis. Chromate transporter protein involves in chromate transmembrane transporter activity. Antigenic score (vaxijen2.0) is 0.7949, Secretory score is 0.316992, No. of amino acids are 222 and subcellular location is membrane.

Identification of conserved regions: The multiple sequence alignment recognizes regions of protein sequences that are similar and provide most conserved regions among *M. bovis* strains. Guide trees and HMM profile-profile algorithms were used by the Clustal Omega alignment tool to align sequences of CR protein of *Mycoplasma bovis.* The results of Clustal Omega are shown in Fig. S1.

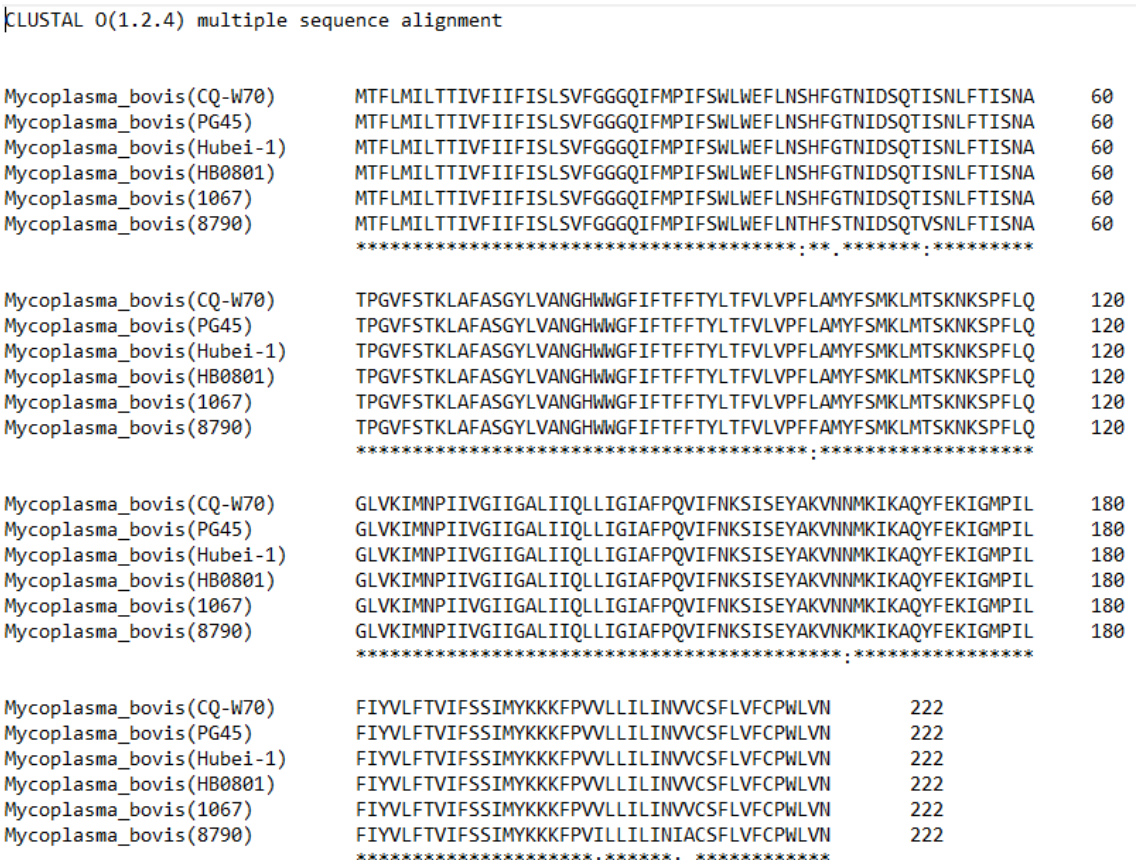

Fig. S1: The multiple sequence alignment highlighted regions of protein sequences that are similar and provide most conserved regions among *M. bovis* strains. Guide trees and HMM profile-profile algorithms were used by the Clustal Omega alignment tool to align sequences of CR protein of *Mycoplasma bovis*.

Generation of consensus sequence: Consensus sequence contains those residues which are frequently present. It may be a nucleotide or an amino acid, it appears at each location in a sequence alignment. Consensus sequence of the CR protein are generated by using the Geneious Prime Tool. Consensus sequence is shown below:

MTFLMILTTIVFIIFISLSVFGGGQIFMPIFSWLWEFL
NSHFGTNIDSQTISNLFTISNATPGVFSTKLAFASGY
LVANGHWWGFIFTFFTYLTFVLVPFLAMYFSMKLM
TSKNKSPFLQGLVKIMNPIIVGIIGALIIQLLIGIAFPQ
VIFNKSISEYAKVNNMKIKAQYFEKIGMPILFIYVLF
TVIFSSIMYKKKFPVVLLILINVVCSFLVFCPWLVN

Prediction of T cell epitope: CTL epitopes were predicted by using NetCTL server. A perfect vaccine should be able to mimic the natural immunity triggered through an infection while also generating long lasting adaptive immunity, in which CTL and HTL epitopes both playing vital role. CTL epitopes develop long lasting cellular immunity capable of eliminating circulating virus as well as virus infected cells. While HTL epitopes play vital role in the development of both humoral and the cellular immune response. So, an ideal vaccine candidate should contain significant CTL and HTL epitopes that are receptor specific (Kar *et al.,* 2020). HTL epitope was predicted using NetMHCIIpan 4.0 Server. All the predicted CTL epitopes are shown in Table 1 while HTL epitopes in Table 2.

Construction of MEV: To build multiepitope peptide-based vaccine construct, all 14 selected epitopic peptides (3 HTL and 11 CTL) fused together via AAY and GPGPG spacers. β-defensin (GIINTLQKYYCRVRG GRCAVLSCLPKEEQIGKCSTRGRKCCRRKK) added via EAAAK spacer and first CTL epitope also attach there as an adjuvant to amino terminus of polypeptide to increase the immunogenicity of multi-epitope-based vaccine. EAAAK linker increases stability. Epitopes were sequentially linked together based on the compatibility of their interactions(Hoover *et al.,* 2003; Behmard *et al.,* 2020). The MEV construct contained a total of 239 amino acids in its primary structure shown in Fig. 1.

Structure prediction and evaluation of MEV: MEV polypeptide sequence submitted to trRosetta, which generated 3D model of vaccine construct shown in Fig. 2. For refinement of 3D structure of MEV pdbsum Ramachandran plot that showed most favored regions 89.5 percent, additional allowed regions 8.6%, generously allowed regions 0.5%, and disallowed regions 1.4%.

Prediction of B cell epitope: The ElliPro server find out continuous and discontinuous B cell epitopes by using default parameters. Discontinuous B cell epitopes are shown in Table 3 and continuous B cell epitopes in Table 4.

Physiochemical properties of Vaccine: For assessing effectiveness and safety of vaccine candidate, various physicochemical properties must be assessed. Immuno-genicity was observed in multiepitope vaccine construct and predicted by VaxiJen v2.0, which gave the vaccine a score of 1.1788 for antigenicity. The allergenicity of vaccine

GIINTLQKYYCRVRGGRCAVLSCLPKEEQIGKCSTRGRKCCR
RKKEAAAKFPVVLLILIAAYIYVLFTVIFAAYWWGFIFTFFAAYIF
MPIFSWLAAYTTIVFIIFIAAYHWWGFIFTFAAYFLVFCPWLVA
AYMPILFIYVLAAYFIIFISLSVAAYCSFLVFCPWAAYNMKIKAQ
YFGPGPGAFPQVIFNKSISEYAGPGPGISEYAKVNNMKIKAQ
GPGPGSISEYAKVNNMKIKA

Fig. 1: The multi epitope vaccine construct. To construct MEV, all 14 selected epitopic peptides (3 HTL and 11 CTL) fused together via AAY and GPGPG spacers. β-defensin (GIINTLQKYYCRVRGGRCAVLSCL PKEEQIGKCSTRGRKCCRRKK) was added via EAAAK spacer. The MEV construct contained a total of 239 amino acids**.**

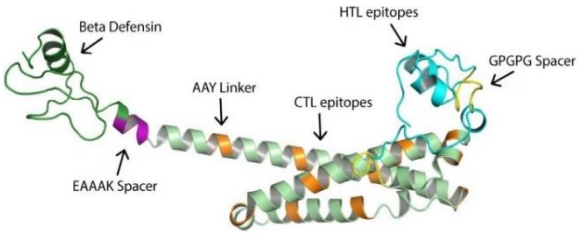

Fig. 2: The structure of the predicted protein. MEV polypeptide sequence submitted to trRosetta, which generated 3D model of vaccine construct. The quality of the protein highlighted most favored regions 89.5 percent, additional allowed regions 8.6%, generously allowed regions 0.5%, and disallowed regions 1.4%.

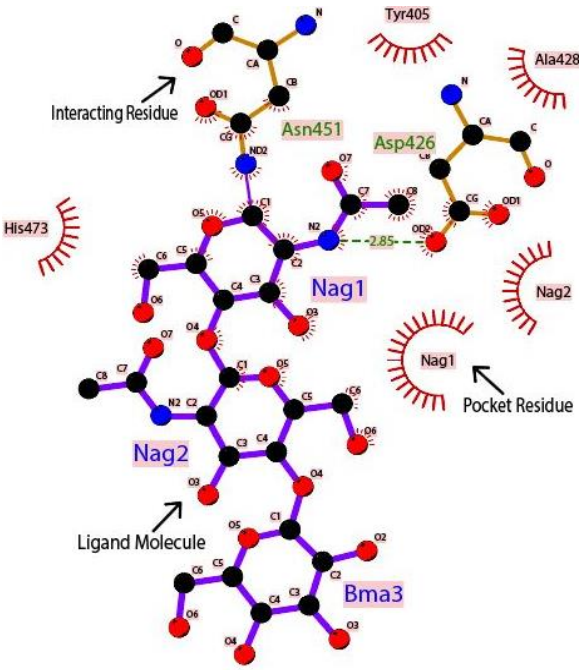

Fig. 3: The docking interaction of MEV protein structure. Ligplot was used to check intermolecular interactions of the predicted protein against 3rg1. Fig. show that Ala428 (A), Nag2 (R), Tyr405 (A), Nag1 (R) and His473(A) is a neighboring amino acid residue for the pocket region. The lines see in purple Asn451(A), Asp426(A), Nag1(Q), Nag2(Q) and Bma3(Q) are ligands. The lines see in brown are interacting residues. The interacting residues linked with ligand through hydrogen bonding.

candidate was tested to confirm that it does not cause allergic reactions when injected into the body. As predicted by the web server AllerTOP, discovered vaccine candidate to be non-allergenic. ProtParam tool within ExPASy server was used in this study to predict various physiochemical parameters associated with the vaccine. Aliphatic index of vaccine suggests that vaccine will be of thermostable nature. GRAVY score shows that vaccine candidate can interact by aqueous environment. The vaccine candidate's instability index indicates that protein is stable.

Table 1: The predicted CTL epitopes from the consensus sequence of the protein. NsetCTL server was used to forecast the CTL epitopes. The epitopes were predicted by NetMHCIIpan 4.0 Server. The predicted epitopes consist of 11 CTL epitopes

No	Peptide sequence	position	Prediction score	Antigenicity score
1	FPVVLLILI	199	0.8616	1.9818
2	IYVLFTVIF	182	1.8551	1.5768
3	WWGFIFTFF	82	1.6271	2.5425
4	IFMPIFSWL	26	1.6013	2.6930
5	TTIVFIIFI	8	1.1238	2.0368
6	HWWGFIFTF	81	1.7729	1.9172
7	FLVFCPWLV	213	1.4269	3.4701
8	MPILFIYVL	177	1.2835	2.8346
9	FIIFISLSV	12	1.4103	1.5285
10	CSFLVFCPW	211	1.7892	1.9393
11	NMKIKAQYF	164	1.5107	1.8280

Table 2: The predicted HTL epitopes from the consensus sequence of the protein. NetCTL server was used to forecast the HTL epitopes. The epitopes were predicted by NetMHCIIpan 4.0 Server. The predicted epitopes consist of 3 HTL epitopes

No	Peptide sequence	Position	Allele	Score	Antigenicity score
1	AFPQVIFNKSISEYA	146	DRB3_0303	1.93	0.8328
2	ISEYAKVNNMKIKAQ	156	DRB3_0303	2.10	1.1394
3	SISEYAKVNNMKIKA	155	DRB3_0303	2.87	1.0883

Molecular Docking of vaccine with 3RG1: For analyzing multiepitope vaccine polypeptide's binding pattern with immune cell protein the molecular docking was done by using PatchDock server. 3RG1 ligand of Bostaurus used because the host animal is cattle. 3RG1 is Crystal structure of RP105/MD-1 complex, in Bostaurus. RP105 is TLR family member and is considered to function as an accessory molecule to signaling/recognition cell surface receptors (Kimoto *et al*., 2003). Ligplot was used to check intermolecular interactions shown in Fig. 3. Molecular docking of Multi epitope-based vaccine was performed with 3rg1 and interactions were analyzed by using Ligplot. Fig. shows that Ala428 (A), Nag2 (R), Tyr405 (A), Nag1 (R) and His473(A) is a neighboring amino acid residue for the pocket region. The lines are in purple Asn451(A), Asp426(A), Nag1(Q), Nag2(Q) and Bma3(Q) are ligands. The lines see in brown are interacting residues. The interacting residues linked with ligand through hydrogen bonding.

Immune simulation: To evaluate multi epitope vaccine's immunogenic profile, an in silico immune response was produced by C-ImmSim server. In comparison to primary response, the secondary and tertiary responses were considerably higher. In addition, B cell isotypes suggested possible isotype switching potentials and memory formation. Pre activation of TCs during vaccination, also increased the response of TH (helper) and TC (cytotoxic) cell populations (Fig. 4).

Reverse translation and codon optimization: JCat tool was used to optimize vaccine's codon usage for maximum protein expression in *M. bovis*. In general, in the host system protein expression is defined as a codon adaptation index (CAI) whose value is >0.8 and the GC content of round about 30 to 70%. Our vaccine had CAI value of 1.0 and the GC content of reverse translated vaccine was 48.25%.

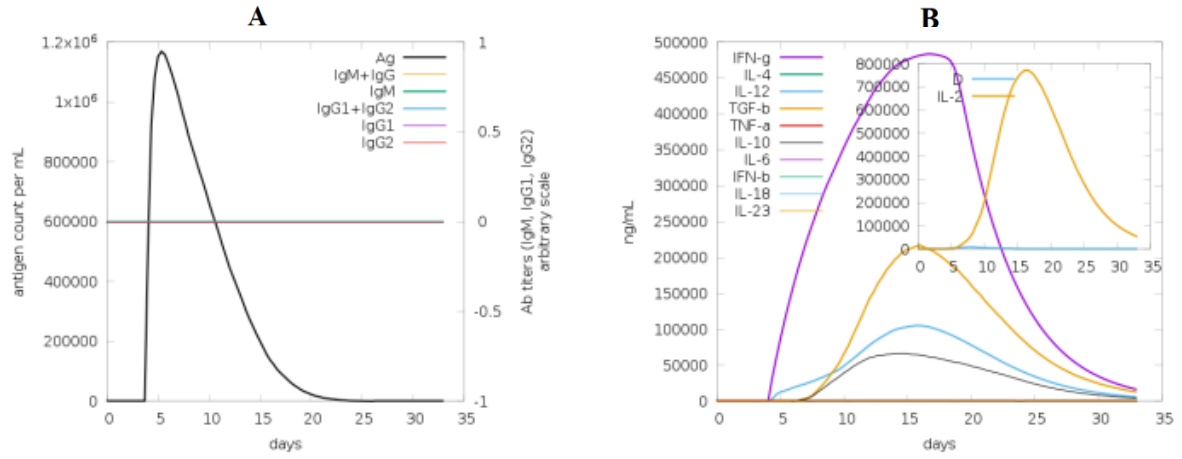

Fig. 4: Immune simulation results for the predicted MEV. C-IMMSIM immune server was used to assess the immunological profile of the predicted MEV. The responses (secondary and tertiary) produced by the simulation were significantly higher in comparison with primary response.

Table 3: Conformational or discontinuous B cell epitopes in a vaccine with many epitopes as predicted by ElliPro. The epitopes were predicted using default parameters

Discontinuous epitopes	Score
A:G1, A:I2, A:I3, A:N4, A:T5, A:L6, A:Q7, A:K8, A:Y9, A:Y10, A:C11, A:V13, A:R14, A:G15, A:G16, A:R17, A:C18, A:A19, A:V20, A:L21, A:S22, A:C23, A:L24, A:P25, A:K26, A:E27, A:E28, A:Q29, A:I30, A:G31, A:K32, A:C33, A:S34, A:T35, A:R36, A:G37, A:R38, A:K39, A:C40, A:C41, A:R42, A:K44	0.842
A:C165, A:V189, A:I190, A:F191, A:N192, A:K193, A:S194, A:I195, A:S196, A:E197, A:Y198, A:A199, A:G200, A:P201, A:G202, A:P203, A:G204, A:I205, A:S206, A:Y208, A:A209, A:K210, A:V211, A:N212, A:N213, A:M214, A:K215, A:I216, A:K217, A:A218, A:Q219, A:G220, A:P221, A:G222, A:P223, A:G224, A:S225, A:I226, A:S227, A:Y229, A:A230, A:K231, A:V232, A:N233, A:N234, A:M235, A:K236	0.693
A:T118, A:F119, A:A120, A:A121, A:Y122, A:F123, A:L124, A:V125, A:F126, A:C127, A:P128, A:W129, A:L130, A:V131, A:A132, A:A133, A:Y134, A:M135	0.655
A:F92, A:S93, A:L95, A:A96, A:A97, A:T99, A:T100, A:I101	0.544

Table 4: Linear/continuous B cell epitopes in the Vaccine construct, predicted by ElliPro server using default parameters. The presence of these epitopes in the vaccine design is essential for eliciting a successful immune response.

Linear epitopes	Position	Score
GIINTLQKYYCRVRGGRCAVLSCLPKEEQIGKCSTRGRKCCRRKKE	46	0.823
VIFNKSISEYAGPGPGISEYAKVNNMKIKAQGPGPGSISEYAKVNNMK	48	0.693
TFAAYFLVFCPWLVAAYM	18	0.655

DISCUSSION

M. bovis transmitted from infected animal to uninfected animal via close contact, maternal contact and through contaminated milk (Dudek *et al.,* 2020). To control the infections of *M. bovis*, there is a need to develop therapeutics like drugs or vaccines against the disease. Vaccination is widely acknowledged as the most efficient method of disease prevention. Basically, vaccines provoke an immune response by introducing foreign antigens to the immune system. Attenuated vaccines commonly used to treat *M. bovis* related diseases, but their usage is linked to biosafety concerns such as autoimmune or severe allergic reactions, as well as synthesis and manufacturing difficulties. In addition, live attenuated vaccines elicit minimal immune response and mostly they consist upon large proteins. Multiple epitope-based vaccine is a good alternative that uses short multiple peptide fragments. These peptides elicit immune response and mostly are antiallergens.

Introduction of immunoinformatic approaches that are both cost-effective and time-efficient, assisted researchers in predicting multi epitope-based vaccine. For the prediction of multi epitope-based vaccine, most conserved, consensus and antigenic epitopes are required for desired immune response. The antigenic, immunogenic, and antiallergenic properties were also focused which can play significant role in viral clearance mechanism. The best quality structure of consensus sequence required to find out appropriate B and T cell epitopes for this purpose I-TASSER and pdbsum Ramachandran plot was used.

Including various immunodominant sites of pathogen in vaccine construct aids to boost the antigenic effect. When epitope-based vaccines administered to cattle, short immunogenic peptides induce strong and focused immune responses. According to different studies, multiple peptides elicit more stronger Band T cell immune response unlike unconjugated epitopic peptides (Li *et al.,* 2014). Immune system is responsible for body's protection against any pathogen. Mostly immune system comes into action when pathogen invades into body. After that immune system prepares mechanisms to kill pathogen. While Pathogens, on the other hand, proliferate quickly. To avoid these circumstances, there is an option of epitope-based vaccine which assist immune system. When epitope-based vaccines administered to cattle, the immune system of cattle recognize it as pathogen. B cells recognize antigen through B cell receptor. After activation of B cells, its secret antibodies that help to neutralize the effect of pathogen and these antibodies memorize for further use. T cells, on the other hand, recognize antigens with MHC molecules (Raza *et al.,* 2019). Thus, successive sequences of 3 HTL and 11 CTL peptide

epitopes were combined using specified linkers. EAAAK and GPGPG were added as a spacer between epitopes. GPGPG boosted immunogenicity and improved proteasome processing and EAAAK spacer increase catalytic activity and stability (Livingston *et al*., 2002). trRosetta was used to predict structure of vaccine construct. Immunoinformatic and modeling studies were used to observe potential binding with host protein. The multi epitope vaccine demonstrated in vivo efficacy and protective immunity, and it was approved for phase-I clinical trials. To further confirm authenticity of our proposed vaccine, more thorough analysis and experimentation will be required.

When used alone, epitope-based peptide vaccines elicit comparatively poor immune response. Immunoreactivity of the vaccine may be enhanced by using appropriate adjuvants. In this research work, epitopic peptides were fused to β-defensin as an immunogenic adjuvant to create the vaccine construct. β-defensin work as antimicrobial agent. As well as by introducing β-defensin in vaccine construct, it will help to relatively enhance immune response.

B cells are essential elements of immune system because they produce antibodies against the invading pathogens. Antibodies seems to be more popular and useful therapeutic agent in the treatment of infectious diseases (Sormanni *et al*., 2015). However, conventional methods for developing therapeutic antibodies are time consuming and labor intensive. The present study enables researchers to develop therapeutic antibodies computationally using the predicted epitopes. Rather than relying on screening from large libraries, researchers will be able to rationally design functional therapeutic antibodies. Four conformational and three linear B cell epitopes predicted by Ellipro. Constructed vaccine's ability to bind to the immune receptor 3RG1 was tested via molecular docking and immune simulation. Triggering 3RG1 can aid to elicit signaling networks that activate pathogen specific immune pathways.

In immune simulation, constructed vaccine elicited higher macrophage activity and the immune responses. The designed vaccine is immunogenic, non-toxic, antigenic, as well as non-allergic. Various physicochemical properties indicate that designed vaccine is stable, polar in nature, soluble and thermostable. However, designed Vaccine efficiently elicit immune response and provide immunity against *M. bovis* related infections. Because of mRNA codon inconsistency, foreign gene expression can vary within host cell genome; thus, codon optimization is required for the higher expression. Designed vaccine has codon adaptation index 1:00 and the GC content of reverse translated vaccine was 48.25% indicating higher expression within *M. bovis* system.

Conclusions: This study proposes the prediction of MEVs against *M. bovis* using CTL and HTL epitopes. Altogether, we predicted 14 peptide epitopes including 11 CTL and 3HTL epitopes for our target chromate transporter protein of the *M. bovis* bacteria. Total length of predicted multi epitope vaccine was 239 amino acids in its primary structure. MEV found to be antigenic, antiallergic as well as immunogenic. Molecular Docking studies were used to confirm the stable interactions of vaccine with immune receptors. The immune simulation studies also confirmed the ability of vaccine to trigger an immune response. This research work will help researchers in testing effectiveness of this epitope-based vaccine against *M. bovis*.

Authors contribution: IA wrote the manuscript and performed analysis. TS, TP and SR performed the analysis. FJ, SK and MI reviewed the manuscript. Muhammad Asif Rasheed conceived the idea and reviewed the manuscript.

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