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RESEARCH ARTICLE

Designing an Epitope-Based Vaccine against Bovine Viral Diarrhea using Immuno-informatics

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A B S T R A C T

Bovine Viral Diarrhea (BVD) is a major economic threat to the livestock industry due to its widespread infection among cattle around the world. Due to resistant strains and regulatory system inadequacies, traditional therapeutic vaccines failed to eradicate it. In this study, a Multi-Epitope Vaccine (MEV) with noticeable immunological properties is proposed that can mitigate these problems by decreasing cross-contamination, autoimmune responses, and inflammatory responses. Using Immunoinformatics approaches, putative CTL- and B-cell epitopes from four BVDV proteins (E1, E2, E^{rns}, and NS3) were predicted and scrutinized after immunogenic profiling. The robust binding of chimeric vaccine to bovine TLR3 was validated through protein-protein docking investigations. The molecular dynamic simulation proved it to be stable in a biological system. The proposed MEV proved vital in strengthening the host immunity upon infection as the immunological simulation was projected to induce immunoglobulins, interleukins, interferons, helper T cells, and cytotoxic T cells. Our results therefore imply that the proposed vaccine will aid in inducing a strong and durable immunity in animals against BVDV infection.

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INTRODUCTION

Bovine Viral Diarrhea (BVD) is a highly transmissible viral disease that affects cattle and ruminants. The virus transmits within herds through persistently infected animals (Schweizer *et al*., 2021), causing symptoms such as diarrhea, fever, respiratory infections, and developmental disorders. The immunosuppressive nature of the virus leads to high morbidity and mortality rates, affecting milk production, reproductive losses, veterinary costs, and trade restrictions (Chauhan *et al*., 2024). The virus encodes a polyprotein that produces four structural proteins (E1, E2, E^{rns}, Capsid) and seven non-structural proteins (N^{pro}, NS2-3, NS4A-B, NS5A-B, p7). The E2 glycoprotein induces immunogenicity by producing neutralizing antibodies, while capsid protein and NS3 are potential targets for inhibiting viral infection. However, these proteins differ in antigenicity and protective effectiveness, necessitating further research to determine their vaccination potential (Al-Kubati *et al*., 2021).

Cattle populations worldwide are susceptible to the virus, causing high morbidity and reproductive losses. Pakistan's dairy and meat industry is particularly affected. Despite this, the root causes of the disease have received little attention. A recent survey showed 23.7% sero-prevalence in cattle (Ain *et al*., 2023). Factors like animal pasturing, trade, and population density can cause virus prevalence shifts and introduce the virus into previously uninfected dairy territories.

Controlling BVD involves a multi-pronged approach including biosafety measures, culling of PI animals, vaccination, and regular herd monitoring (Moennig and Becher, 2018). Vaccination is crucial for preventing PI calves, reducing viral dissemination, providing herd-level protection, and addressing genetic diversity. Current vaccines, such as Modified-Live Virus (MLV) and Killed Virus (KV), have limited efficacy against multiple circulating genotypes (Sangewar *et al*., 2020) and are associated with immunosuppressive effects, reduced fertility and BNP risk (Antos *et al*., 2021).

Advancements in genome sequencing and bioinformatics have revolutionized recombinant vaccine development. Reverse vaccinology is a cutting-edge approach that minimizes trial-and-error and identifies previously unnoticed antigens (Khalid and Poh, 2023). This study aims to contribute to improved epitope-based vaccine design against Bovine Viral Diarrhea (BVD), providing a potential method for effective disease prevention and management in cattle populations. The multi-epitope-based Vaccine (MEV) targeting BVD is illustrated in Fig. 1.

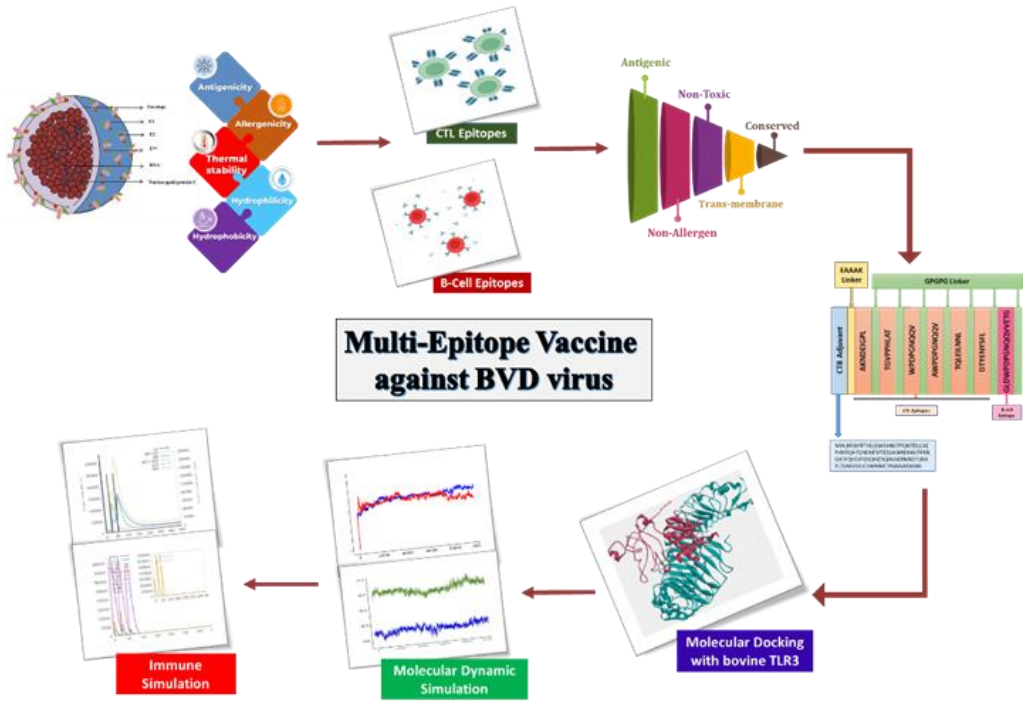


Fig. 1: Graphical illustration of the comprehensive study to develop Multi-Epitope based Vaccine (MEV) against BVD.

MATERIALS AND METHODS

Analysis of BVDV proteins: The primary protein sequences of E1, E2, E^{rns}, and NS3 proteins of BVDV 1b genotype (Accession No. AZB53078.1) were then analyzed for antigenicity and allergenicity through Vaxijen v2.0 (using 0.4 as a threshold value) and AllerTOP v.2.0 servers, respectively (Doytchinova and Flower, 2007; Dimitrov *et al.*, 2014). Afterwards, the physical and chemical parameters were assessed using ExPASy tool ProtParam (Gasteiger *et al.*, 2005).

Epitope prediction: Linear B-cell and cytotoxic T-cell (CTL) epitopes were predicted using BCPred and NetMHCpan4.1 in immunogenic viral proteins (Soto *et al.*, 2022), then examined for toxicity, allergenicity, antigenicity, conservation, and topology. The curated linear B-cell epitopes underwent further analyses based on flexibility, antigenicity, surface accessibility, hydrophilicity and the presence of beta-turns in them (Bui *et al.*, 2007).

Interaction analysis of CTL epitopes with BoLA alleles: The anticipated linear CTL epitopes were analyzed by molecular docking with Bovine Leukocyte Antigen (BoLA) alleles i.e., BoLA-2:018:01, BoLA-2:026:01, BoLA-3:017:01, and BoLA-3:027:01, representing haplotypes A11 and A12. PEP-FOLD 3.5v.2.0 server and SWISS-MODEL were used for predicting the structures of epitopes and BoLA alleles. PatchDock was used to measure the binding strength of the predicted CTL epitopes to the BoLA allele.

MEV design and its immunogenic profiling: The multi-epitope based vaccine (MEV) was designed by combining CTL- and B-cell epitopes through the GPGPG linker to preserve the immunogenic activity of each epitope. Furthermore, EAAAK linker was used to engineer mucosal adjuvant "Cholera Toxin Subunit B (CTB)" to the epitopes to uplift the immunogenic activity of the vaccine. The immunogenic profile, solubility and topology of the vaccine were assessed to validate the vaccine's structural stability and suitability for antigenic presentation.

Structural analysis of vaccine construct: The proposed vaccine construct's amino acid sequence was analyzed for secondary structure prediction using SOPMA and PSIPRED to access the frequency of beta-turns, the length of alpha-helices, and the existence of random coils. While the vaccine's structural modelling was conducted by comparative analysis of structures predicted by Robetta, RaptorX, I-TASSER and IntFOLD servers. The vaccine's structure was then predicted for conformational B-cell epitopes using the computational programme ElliPro.

Molecular docking of MEV with Toll-Like Receptor (TLR): The binding conformation between MEV and TLR3 was investigated through docking analysis run on the ZDOCK server and the interfaced residues were visualized with the help of PDBsum and PDBe-PISA databases.

PRODIGY (PROtein BinDIng enerGY prediction) was used to assess the binding affinity of the docked complex, predicting the binding free energy, its dissociation constant (K_d), the nature and number of intermolecular interactions within a 5.5Å distance as a threshold, along with the percentages of polar and charged amino acid content on the non-interacting interface (Xue *et al.*, 2016).

Molecular dynamic simulation: Molecular dynamics (MD) modelling using Desmond software from the Schrodinger suite was used to evaluate the robustness of protein complexes (Ivanova *et al.*, 2018). The OPLS_2005 force field was used to run the simulation, which included a 100 ns trajectory of the best docked complex of bovine TLR3 and MEV against BVD.

Throughout the simulation, the physiological conditions were maintained by adding 0.15 M NaCl keeping the NPT ensemble at 300 K and 1 atm. RMSD (Root Mean Square Deviation), Root Mean Square Fluctuation (RMSF), quantitative analysis of hydrogen bonds and Radius of Gyration (RoG) was recorded during simulation study.

***In silico* immune simulation:** The C-ImmSim server was used to validate the immunological response generated by chimeric MEV construct by *in silico* simulating the thymus, bone marrow, and lymph nodes of the mammalian immune system. The ability of MEV to mimic several immune cell types included immunoglobulins, B-cells, cytotoxic T lymphocytes (CTL), helper T lymphocytes (HTL), natural killer (NK) cells, dendritic cells, cytokines, and interferons was assessed across 1050 simulation phases with injections administered at 28-day intervals (Castiglione *et al.*, 2012; Riaz *et al.*, 2023). A standard dose of 1000 units of MEV per injection was used to estimate the MEV-induced active cellular regulation. All other default settings for the simulation were left by default.

RESULTS

Immunogenic assessment and physicochemical properties of BVDV proteins: The protein sequences of retrieved BVDV proteins (E1, E2, E^{rns}, and NS3) were rigorously evaluated for allergenicity and antigenicity profiles. Significant antigenicity was shown by all four proteins with score of 0.4558 for E1, 0.5973 for E2, 0.4274 for E^{rns}, and 0.5833 for NS3, supporting their ability to activate an immunological response. The high scores indicate that these proteins are likely to have strong interaction with the immune system. It was also found that none of the proteins were allergenic highlighting that the exposure to these proteins is not likely to result in allergic responses. Thus, all the proteins suggested as a promising vaccine candidates against BVDV.

Some key physical and chemical parameters for these proteins were also investigated, noteworthy were the instability indices of 28.14, 29.62, 28.08, and 36.08 for E1, E2, E^{rns}, and NS3, respectively. All the four proteins appeared to have an unusually high degree of stability, with values well below the commonly used criterion of 40. Aliphatic indices for E1, E2, E^{rns}, and NS3 were 113.38, 81.62, 56.27, and 85.30, respectively, highlighting their potential as attractive options. This finding highlighted their ability of heat resilience that is critical for the potential vaccine. The proteins' negative GRAVY values indicated their hydrophilic character. The GRAVY values that were measured 0.287 for E1 protein, -0.235 for E2 protein, -0.758 for E^{rns} protein, and -0.242 for NS3 protein. Except for E1, which exhibits hydrophobic features, this study confirmed that all proteins are hydrophilic in nature. Interestingly, E1's capacity to form hydrogen bonds counteracts its hydrophobicity, indicating that it has favorable thermodynamic interactions with water and polar solvents. In conclusion, the data indicated that the evaluated proteins were not only hydrophilic but also thermostable. The comprehensive investigation revealed that the curated BVDV proteins have good antigenicity and physicochemical properties presented their potential for subunit vaccine targeting several epitopes.

Prediction and analysis of potential CTL epitopes: CTL epitopes play a crucial role in triggering long-lasting immune responses and are also essential for successful clearance of viruses and eradication of infected cells. In the present study, 9-mer CTL epitopes were predicted using the NetMHCpan 4.1 server. Epitopes were predicted in BVDV immunogenic proteins with prime focus on BoLA alleles of haplotypes A11 and A20. With a strong binder threshold set at 0.5%, epitopes were classified based on percentage values as strong, weak, or non-binding using default criteria. It resulted in a collection of CTL epitopes, with the proteins E1, E2, E^{rns}, and NS3 each contributing 11, 24, 1, and 40 epitopes, respectively. Using stringent criteria, including significant immunogenic profile (non-toxicity, antigenicity and non-allergenic), sequence conservancy, and location of epitope outside the membrane domain, only one epitope from E2-protein, one from E^{rns} protein, and four from NS3 protein were selected as the best possible candidates for further study.

Structural modeling of CTL epitopes and BoLA alleles: For the highly antigenic and stable CTL epitopes AKNDEIGPL, TGVPPHLAT, WPDPGNQQV, AWPDPGNQQV, TQLEILNNL, and DTYENYSFL, the soPEP energy values were calculated to be -5.7022, -3.60819, -4.01853, -4.23799, -12.6186, and -7.52914, respectively.

BoLA-2:018:01 was modelled based on the sequence identity of 6at5.1.A (Template), which was outstanding i.e., 75.93%, as well as GMQE and QMEAN values of 0.69 and 1.09, respectively. The Ramachandran plot demonstrated an outstanding distribution of 98.53% amino acids within the target region providing further evidence of the accuracy of this modelling. Modelling of BoLA-2:026:01 with the same template yielded 75.07% sequence identity, 0.70 GMQE, and 0.87 QMEAN. Moreover, 98.90% of BoLA-2:026:01 residues were in the Ramachandran preferred area. BoLA-3:017:01 was modelled using the 6at5.1.A as a reference having 73.95% sequence similarity and has GMQE and QMEAN values of 0.68 and 0.76, respectively. Ramachandran plot analysis verified the accuracy of model, showing that 99.90% of the amino acids reside inside the optimal region. The sequence identity of modelled BoLA-3:027:01 was 72.93% with template, GMQE and QMEAN values were 0.68 and 0.53, respectively, and 98.90% of the amino acids were in the target region.

Models with higher structural integrity are given lower ratings by MolProbity on a scale from 0 to 1. For BoLA alleles under consideration, MolProbity scores vary from 0.66 to 0.94. A low MolProbity value (close to zero) denotes a well-built structure with minimal anomalies. The minimal number of steric conflicts, bond angle and length variations give structure of BoLA-3:017:01 and BoLA-3:027:01 alleles a MolProbity score of 0.66, indicating a high structural quality. The BoLA-2:018:01 and BoLA-2:026:01 alleles both have scores of 0.72, indicating structurally sound models, although the BoLA-2:026:01 allele may have slight deviations.

Molecular interaction of CTL epitopes and BoLA class-I alleles: Selected BoLA alleles and CTL epitopes were docked blindly using PatchDock server, and the resulting docking configurations were improved using the FireDock server. Three hydrogen bonds between Ala^{1} and Asp^{46} (2.39Å), Glu^{5} and Ser^{20} (2.45Å), and Gly^{7} and Ser^{18} (2.58Å) were responsible for strong binding affinity of AKNDIEGPL epitope with BoLA-2:026:01 (Fig. 2A). There were also complex molecular interactions between TGVPPHLAT epitope and BoLA-3:017:01, as evidenced by the presence of three hydrogen bonds between Gly^{2} and His^{24} (2.45Å), His^{24} and Asp^{50} (2.74Å), and Thr^{1} and Glu^{285} (2.46Å) (Fig. 2B). Binding of the WPDPGNQQV epitope to BoLA-2:018:01 resulted in the formation of a single hydrogen bond between Arg^{171} and Gln^{8} at 2.80Å (Fig. 2C). The AWPDPGNQQV epitope, on the other hand, formed two hydrogen bonds with the identical BoLA-allele, one between Val^{10} and Tyr^{43} (2.48Å) and the other between Asp^{4} and Ile^{229} (2.51Å) (Fig. 2D). Five hydrogen bonds were also identified as the molecular interaction signature between TQLEILNNL and BoLA-2:026:01. These involved the interactions between Ser^{20} and Glu^{4} (2.59Å), Glu^{4} and Arg^{22} (2.50Å), Ser^{18} and Thr^{1} (2.65Å), Thr^{1} and Glu^{228} (3.08Å), and Thr^{1} and Glu^{228} (2.59Å) (Fig. 2E). It was found that the DTYENYSFL epitope, which interacts with BoLA-3:027:01, forms five hydrogen bonds, including those between Gln^{172} and Glu^{4} (3.02Å), Glu^{166} and Tyr^{3} (2.64Å), Lys^{162} and Ser^{7} (2.64Å), Lys^{162} and Leu^{9} (2.43Å), and Asn^{5} and Ser^{89} (3.07Å) (Fig. 2F).

Complex interaction patterns between BoLA class-I alleles and CTL epitopes revealed their structural foundations and intermolecular connections that constitute the basis of their binding affinities. The design of MEV against BVDV relies heavily on understanding of potential epitope-receptor interactions, which is greatly aided by these findings.

Prediction and assessment of linear B-cell epitopes: Apart from choosing CTL epitopes for the development of MEV against BVDV, it is necessary to identify and evaluate linear B-cell epitopes as well. With a specificity threshold of 75%, 16-mer linear B-cell epitopes were predicted using BCPred server, a powerful neural network-based technique. After that, each anticipated epitope was filtered for its toxicity, antigenicity, conservancy, allergenicity and topology. Epitopes meeting the criteria for efficient vaccine production were the target of this careful screening process. Five, six, one and fourteen B-cell epitopes were predicted for the E1, E2, E^{rns} and NS3 proteins, respectively. After evaluating each predicted epitope using stringent criteria (antigenicity, toxicity, allergenicity, conservancy, and topology), only two epitopes were selected: one from E^{rns} protein and one from NS3 protein. With the help of IEDB Analysis Resource, potential epitopes underwent a thorough evaluation based on numerous crucial features, such as β-turn prediction, surface accessibility, flexibility, antigenicity, and hydrophilicity. The study's primary objective was to assess epitopes' immunogenicity and determine whether they were good candidates for vaccine production against BVDV. Chou and Fasman's β-turns prediction method verified the presence of expected B-cell epitopes with β-turns, which are important for hydrophilicity, protein folding, and accessibility of cellular surfaces. The structural soundness of these epitopes was evaluated regarding β-turns.

The range of β-turn prediction values for E^{rns}-protein epitope was 1.011 to -1.276. The results for NS3-protein epitope were as follows: mean=0.973, lowest=0.656, and maximum=1.454. Protein epitopes are widely acknowledged as critical components of an effective immune response to pathogens. Thus, surface-exposed epitopes were identified using Emini surface accessibility tool, with calculated values of accessibility for E^{rns}-protein epitope including mean=1.000, minimum=0.132, and maximum=3.893, and for NS3-protein epitope including mean=1.000, minimum=0.096, and maximum=6.093.

The ability of an epitope to spontaneously interact with immune alleles and produce a strong immunological response is directly related to its adaptability. Karplus and Schulz flexibility analysis technique was used to evaluate epitopes' adaptability. The computed values for E^{rns}-protein epitope ranged from an average of 0.981 to a minimum of 0.905 and a maximum of 1.06. The range for NS3-protein epitope values was also the same: 1.000, 0.893, and 1.113.

The immunogenic potential of epitopes was estimated using Kolaskar and Tongaonkar antigenicity approach. Antigenic scores were estimated as follows: mean=1.012, lowest=0.903, and highest=1.147 for E^{rns}-protein epitope, and mean=1.032, lowest=0.848, and highest=1.247 for the NS3-protein epitope.

Epitope-receptor cell interactions are heavily influenced by hydrophilicity; hence hydrophilicity prediction calculations were also performed. The calculated hydrophilicity values are as follows: for E^{rns}-protein epitope, mean value is 1.705, minimum value is -1.543, and maximum value is 6.329; for NS3-protein epitope, the corresponding values are 1.620, -5.843, and 6.600. Epitopes were omitted from the vaccine if they consistently failed to reach a set criterion. Consequently, only NS3-protein linear B-cell epitope "GLDWPDPGNQQVVETG" aligned with stringent criteria. To prevent BVDV infection, this epitope was chosen as the best possible candidate to be included in the final MEV design (Table 1).

MEV construction, its sequence analysis and immunogenic assessment: For a host-directed immune response to be effective, MEV must have both B- and T-cell epitopes. One B-cell epitope and six CTL epitopes with prominent antigenic profiles, non-allergenicity, non-toxicity, and strong conservation were identified using a careful selection approach to reach this goal (Table 1). CTB was added as an adjuvant to N-terminus of MEV *via* EAAAK linker to overcome the immunogenicity limitations of epitope-based vaccines. Immune responses were boosted and amplified by adding the adjuvant. The EAAAK linker was used to bind the first CTL epitope to the adjuvant, and the GPGPG linker was then used to sequentially attach subsequent epitopes. Not only was the incorporation of the EAAAK linker crucial for efficient attachment of CTL epitope, but it will also aid in stabilizing the construct. In the end, 235 amino acids were included into MEV construct, representing full expression of the adjuvant and epitopes selected for maximum immunogenicity (Fig. 3A).

Table 1: Selected linear B-cell epitope and CTL epitopes from BVDV proteins to design MEV construct

Linear B-cell epitope							
Epitope		Allergenicity	VaxiJen score	Toxicity	Conservancy	Minimum Identity	Topology
GLDWPDPGNQQVVETG		NA	0.7386 (A)	NT	100%	93.75%	Outside
CTL epitopes							
Epitope	MHC-I alleles	Allergenicity	VaxiJen score	Toxicity	Conservancy	Minimum Identity	Topology
AKNDIEGPL	BoLA-2:026:01	NA	1.9117 (A)	NT	98%	77.78%	Outside
TGVPPHLAT	BoLA-3:017:01	NA	0.5948 (A)	NT	99%	77.78%	Outside
WPDPGNQQV	BoLA-2:018:01	NA	0.4618 (A)	NT	100%	100%	Outside
AWPDPGNQQV	BoLA-2:018:01	NA	0.6633 (A)	NT	100%	100%	Outside
TQLEILNNL	BoLA-2:026:01	NA	0.4143 (A)	NT	100%	100%	Outside
DTYENYSFL	BoLA-3:027:01	NA	0.8997 (A)	NT	100%	100%	Outside

Note: Non-Allergen = NA, Antigenic = A, Non- Toxic = NT

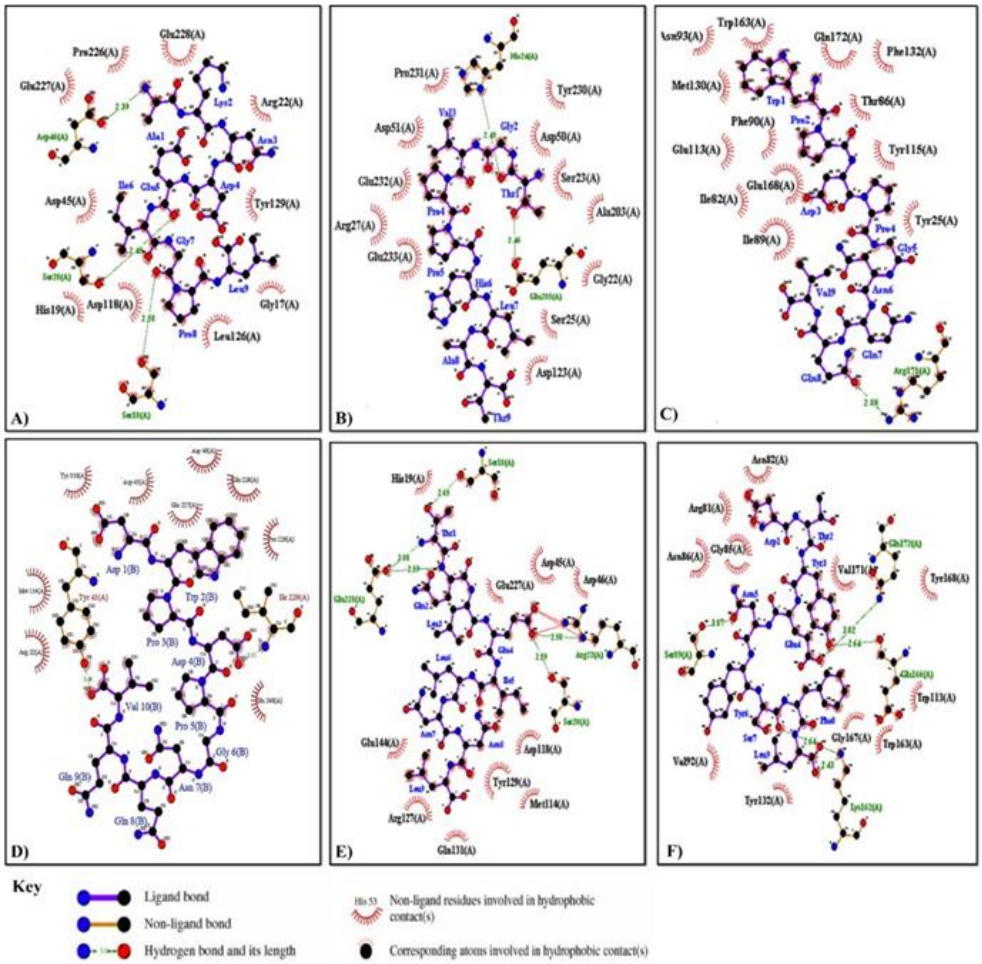


Fig. 2: The binding patterns of the curated CTL epitopes with MHC-I BoLA alleles. A) AKNDIEGPL with BoLA-2:026:01, B) TGVPPHLAT with BoLA-3:017:01, C) WPDPGNQQV with BoLA-2:018:01, D) AWPDPGNQQV with BoLA-2:018:01, E) TQLEILNNL with BoLA-2:026:01, and F) DTYENYSFL with BoLA-3:027:01

The physicochemical features of MEV structure were evaluated, and its secondary structural composition was analyzed. An instability index of 24.14 was obtained, demonstrating the structural integrity of the proposed MEV. The aliphatic index, a measure of thermostability, also appeared at 71.73, lending even more flexibility. The negative GRAVY score of -0.450 suggested the structure is hydrophilic and supported its tendency for interactions with water (Riaz *et al.*, 2023).

To determine whether the construct could be used as a vaccine, the immunogenic profile needed to be assessed. The chimeric vaccine scored an antigenicity of 0.6000, proving its ability to stimulate an immune response. At the same time, it was found that vaccine's structure did not cause any allergic reactions or toxicity in the host body.

The construct was also evaluated for its solubility, and a SOLpro score of 0.974515 was given, indicating that it dissolves well in solution. SOPMA and PSIPRED were used in tandem to deduce MEV's secondary structure. The results of SOPMA analysis showed that 44.18 percent of MEV residues were folded into random coils, while 30.1 percent were folded into alpha helices, 18.8 percent were folded into extended strands, and 6.3 percent were folded into beta turns. Concurrently, PSIPRED validated the construct's amino acid sequence, which was found to be 58.23% coils (145 amino acids), 28.91% alpha helices (72 amino acids), and 12.85% β-strands (32 amino acids) (Fig. 3B). This structural analysis provided validity to the strategic combination of B- and CTL-epitopes in MEV construct, hence augmenting the MEV's potential as a highly effective MEV against BVDV.

Tertiary structure prediction, refinement, and validation: To predict tertiary structure of chimeric vaccine, numerous computational methods, including as IntFOLD,

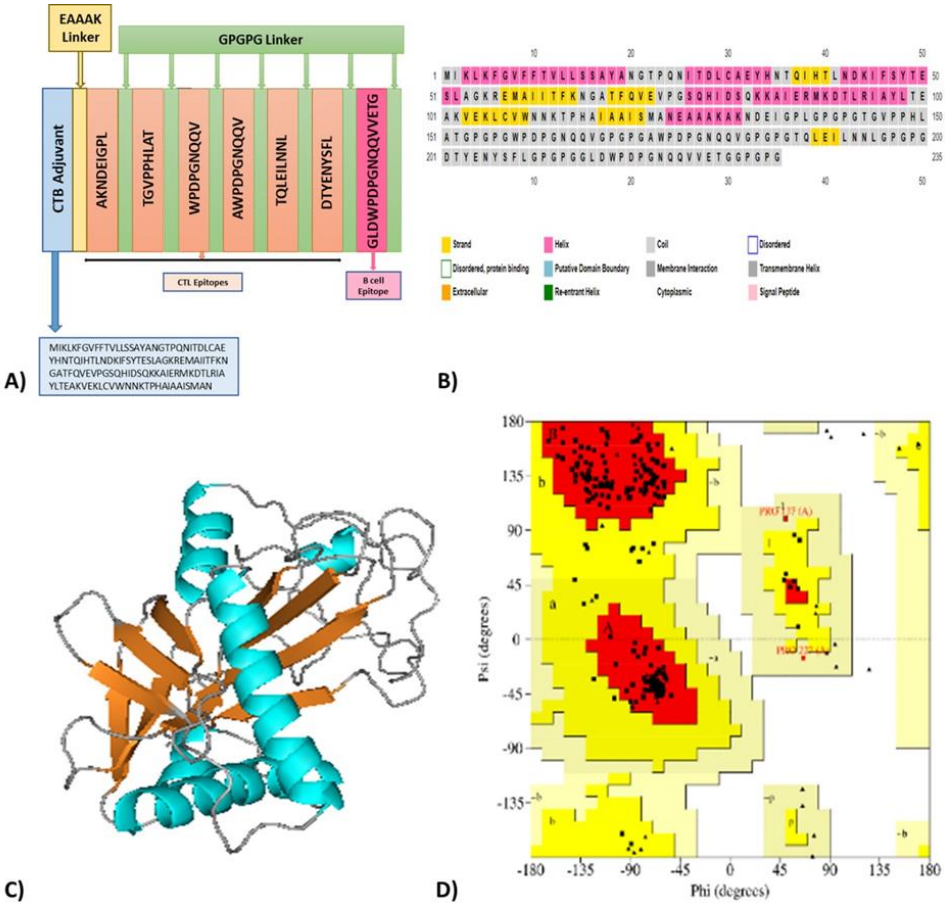


Fig. 3: Design and structural analysis of chimeric vaccine against BVD. A) Physical map of MEV construct B) Secondary structural parameters of MEV, C) Predicted 3D structure of MEV, and D) Ramachandran plot validating the stereo chemical properties of structure.

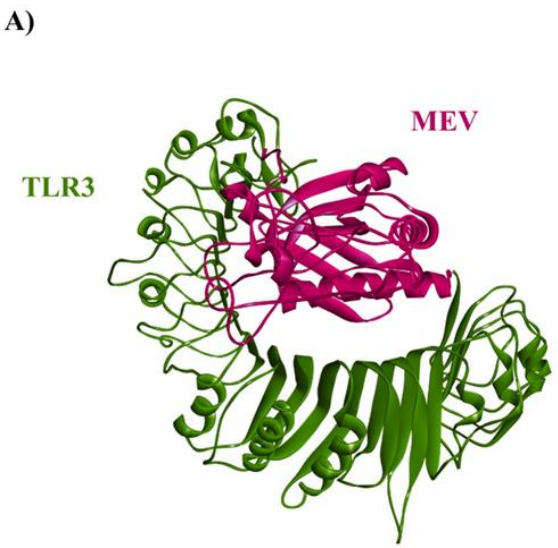
I-TASSER, Robetta and RaptorX servers were utilized. The GalaxyRefine and SAVES servers carried out the subsequent refining and validating the anticipated structures.

With 91.3% of amino acids located in the preferred region and 8.7% located in the prohibited region, the Robetta server-provided 3D model displayed a promising profile (Fig. 3D). Additional testing was conducted using VERIFY3D and ERRAT assessment methods. By confirming that a significant proportion i.e., 84.74% of amino acids attained an averaged 0.20 3D-ID score, VERIFY3D proved the accuracy of the proposed model. Likewise, ERRAT predicted a quality factor of 90, further demonstrating the reliability of the anticipated model. Therefore, the model obtained from the Robetta server achieved an acceptable standard of quality, as determined by the cumulative assessment of the tertiary structure (Fig. 3C). The designed construct's reliability as a possible MEV against BVDV was ensured by a painstaking and rigorous methodology, as shown by the careful prediction, refining, and rigorous validation of the tertiary structure.

Discontinuous epitopes in MEV: It was demonstrated that the MEV construct contains six distinct discontinuous epitopes with varying numbers of residues (14, 50, 65, 5, 3, and 14). Potentially useful epitopes were those with solvent accessibility values higher than the threshold value of 0.5. Scores of solvent accessibilities of these epitopes were 0.783, 0.714, 0.663, 0.574, 0.529, and 0.783, suggesting potential immunogenicity and antigenic relevance.

Binding affinity analysis of MEV with bovine TLR3: Docking analysis figured out potential binding sites of bovine TLR3 engaged by the vaccine protein, MEV (Fig. 4A). A total of 32 MEV residues in an interfaced area of 1040.3Å were found to stably interact with 33 residues of bovine TLR3 that covered 1057.9Å interfaced region (Fig. 4B). MEV+TLR3 complex revealed the presence of five hydrogen bonds and two salt bridges. Tyr^{18}, Glu^{242}, Thr^{215} and Gly^{245} of MEV were discovered to form stable hydrogen bonds with Tyr^{300}, Lys^{268}, Tyr^{233} and Gln^{236} of bovine TLR3. Salt bridges were also generated between Glu^{242}, and Glu^{32} of MEV and Lys^{268} and Lys^{276} of bovine TLR3, further strengthening the interaction network (Fig. 4C). A total of 213 non-bonded connections within the complex were computed. In conclusion, protein-protein interaction study characterized a network of hydrogen bonds, salt bridges, and non-bonded contacts between MEV and bovine TLR3, all of which contribute to the complex's stability and affinity. These results shed light on the possible pathways by which MEV triggers an immunological response in the host.

Analysis of docked complex's binding affinity: For biological complex, the study of binding energetics is indispensable as the Gibbs free energy or binding free energy (ΔG) influences the likelihood of interactions under given biological conditions. Thus, the binding affinity of MEV with TLR3 was ascertained at 25˚C. A total of ΔG values of -12.9 kcal mol^{-1} for the MEV+TLR3 complex was calculated, where the negative ΔG value suggested the energetically viable docking. Table 2 shows the complex K_d values, interfacial contacts (ICs), and non-interacting surfaces (NIS).



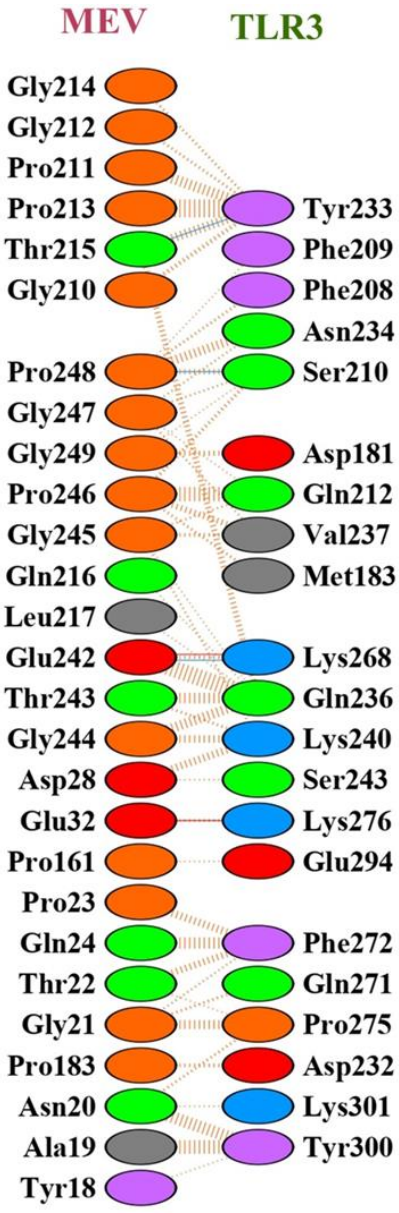


Fig. 4: Interaction analysis of the docked complex (MEV + bovine TLR3). A) Visualization of the docked complex via Discovery Studio, B) Pictorial representation of the interacted amino acid residues obtained from PDBsum, C) Tables indicating the number of hydrogen bonds and salt bridges formed in the docked complex.

C)

Hydrogen Bonds

TLR3	Bond Length (Å)	MEV
TYR-300	1.67	TYR-18
LYS-268	2.29	GLU-242
TYR-233	1.34	THR-215
TYR-233	1.26	THR-215
GLN-236	1.86	GLY-245

Salt Bridges

TLR3	Bond Length (Å)	MEV
LYS-268	2.29	GLU-242
Lys-276	1.34	Glu-32

Table 2: Binding energy (ΔG) calculations, dissociation constant (K_d), interfacial contacts (ICs), and non-interacting surfaces (NIC) of MEV+TLR3 using PRODIGY webserver.

Docked complex	ΔG (kcal/M)	K_d (M)	Interfacial Contacts (ICs)						Non-Interacting Surfaces (NIS)	
			Charged-charged	Charged-polar	Charged-apolar	Polar-polar	Polar-apolar	Apolar-apolar	Charged (%)	Apolar (%)
MEV+TLR3	-12.9	3.4e-10	4	10	15	8	30	33	24.23	36.43

Molecular dynamics and simulation analysis: The stability of the complex revealed the kinetics of the interaction between bovine TLR3 and active residues of MEV. The complex's trajectory analysis showed a high degree of stability, with very small changes throughout the duration of simulation.

The study of RMSD for measuring structural stability identified significant patterns. Both the chimeric vaccine and vaccine-receptor docked complex showed small variations i.e., from 3.5 to 5.1 Å and 3.8 to 5.2 Å. An increase in RMSD was seen up to 5 ns, after which the docked complex almost remains stabilized with very small variations. Additionally, the RMSD variation was less than 4, demonstrating that the vaccine protein and the docked complex were stable under the experimentally optimized conditions (Fig. 5A).

The analysis of RMSF which measures the typical residue deviation across time, added new information. Significant residue-level changes were seen in vaccine protein with an average of 2.2 Å compared to a smaller average fluctuation of 1.8 Å in docked complex with bovine TLR3. This discrepancy provided strong evidence that interaction of vaccine with the bovine receptor improved its stability, a feature often seen in larger and more complex vaccine proteins (Fig. 5B, 5C).

RoG analysis revealed various patterns when evaluating the compactness of the complex. The RoG value of the unbound vaccine was 15.4 Å, while RoG value of the bound vaccine-receptor complex was 14.9 Å. These results reaffirmed the compact binding of vaccine with the receptor thus contributing to complex's stability (Fig. 5D).

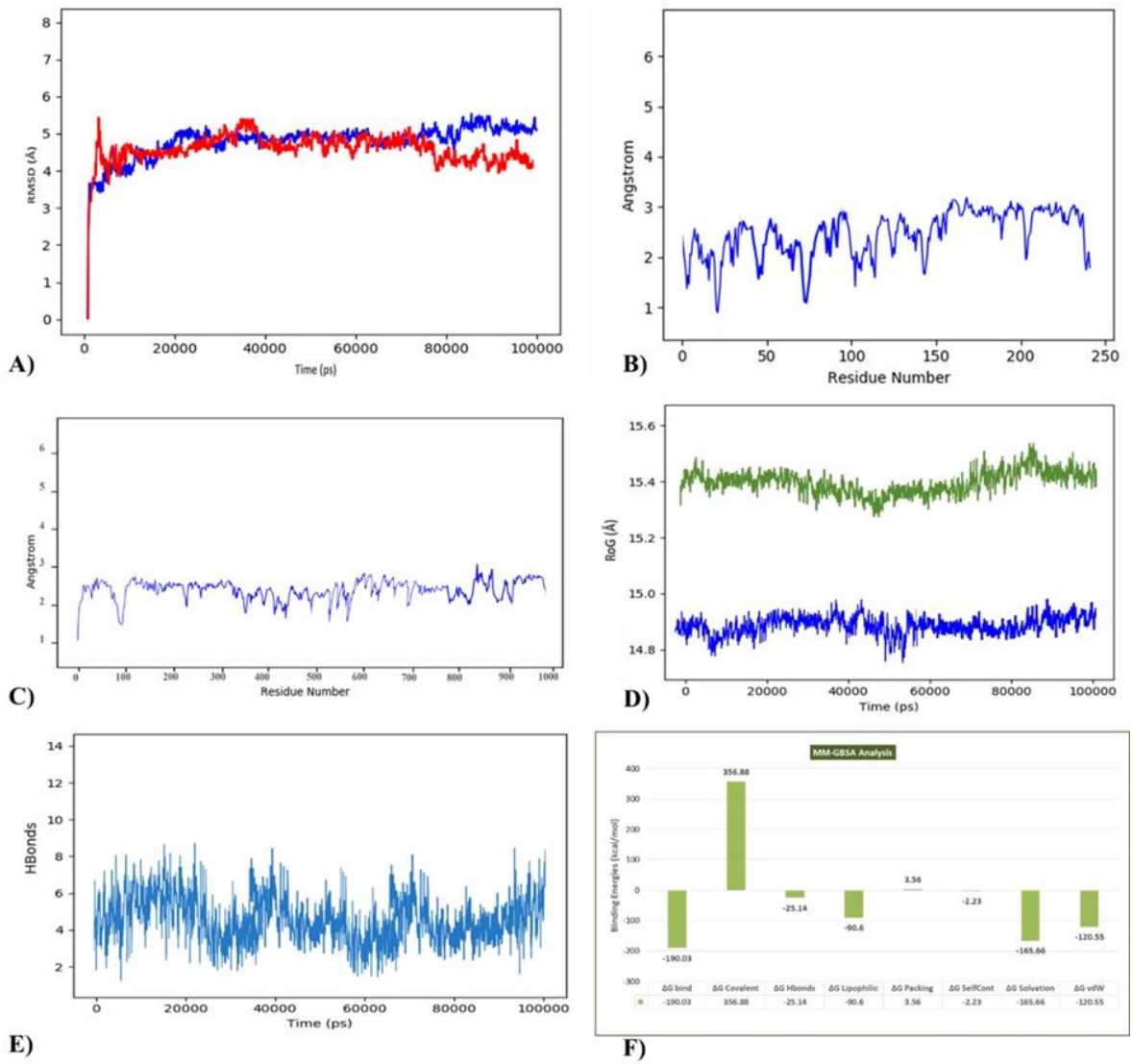


Fig. 5: Molecular Dynamics Simulation of MEV and bovine TLR3 complex at 100 ns. A) RMSD plot specifying the stability of MEV and MEV+TLR3 at 100 ns, B) RMSF plot showcasing flexibility of MEV, C) RMSF plot indicating molecular dynamics in MEV+TLR3 complex, D) RoG indicating compactness of the docked complex, E) Plot presenting hydrogen bonds in the docked complex, F) Graphical representation of MM-GBSA analysis.

The complex's binding affinity was revealed by studying its hydrogen bonds. There is strong attraction between the vaccine protein and active residues of bovine receptor, as evidenced by the detection of fourteen hydrogen bonds between these two molecules (Fig. 5E). Finally, the kinetics of the complex was measured by means of a binding energy analysis performed with MM-GBSA. The docked complex has a binding energy of -190.03 kcal/mol, according to the calculations. Hydrogen bonds and van der Waals (vdW) interactions both contributed to the negative binding energy, confirming high stability and robust interaction of the vaccine-receptor complex (Fig. 5F).

In conclusion, there is strong evidence that MEV interacts with bovine TLR3 receptor in a stable and energetically favorable manner, as shown by the findings of MD simulations, which consistently validate the docking study.

Simulated immunological responses: MEV is a promising new approach to protect cattle from BVDV and immune simulation is an essential tool for evaluating and verifying its efficacy as a comprehensive defense strategy. During immunological simulation investigations, increased immunoglobulin levels (IgM, IgG+IgM, IgG1, IgG2, and IgG1+IgG2) following antigen delivery demonstrated that MEV generated robust secondary and tertiary immunity (Fig. 6A). The presence of multiple long-lived B cell isotypes was indicative of isotype switching and the formation of memory cells (Fig. 6B, 6C and 6D), contributing evidence to MEV's capacity to elicit an immune response. HTL and CTL populations both experienced enhanced immune responses after vaccination following cytotoxic T cell pre-activation (Fig. 6E and 6F). Fig. 6G, 6H and 6I show how the immune system was strengthened by the increased activity of macrophages, dendritic cells, and Natural Killer (NK) cells. The strength of the immunological response was shown by increased levels of interferon gamma (IFN-) and interleukin-2 (IL-2) (Fig. 6J). These findings from the immunological simulation experiment supported the potential of MEV as a potent candidate against BVDV.

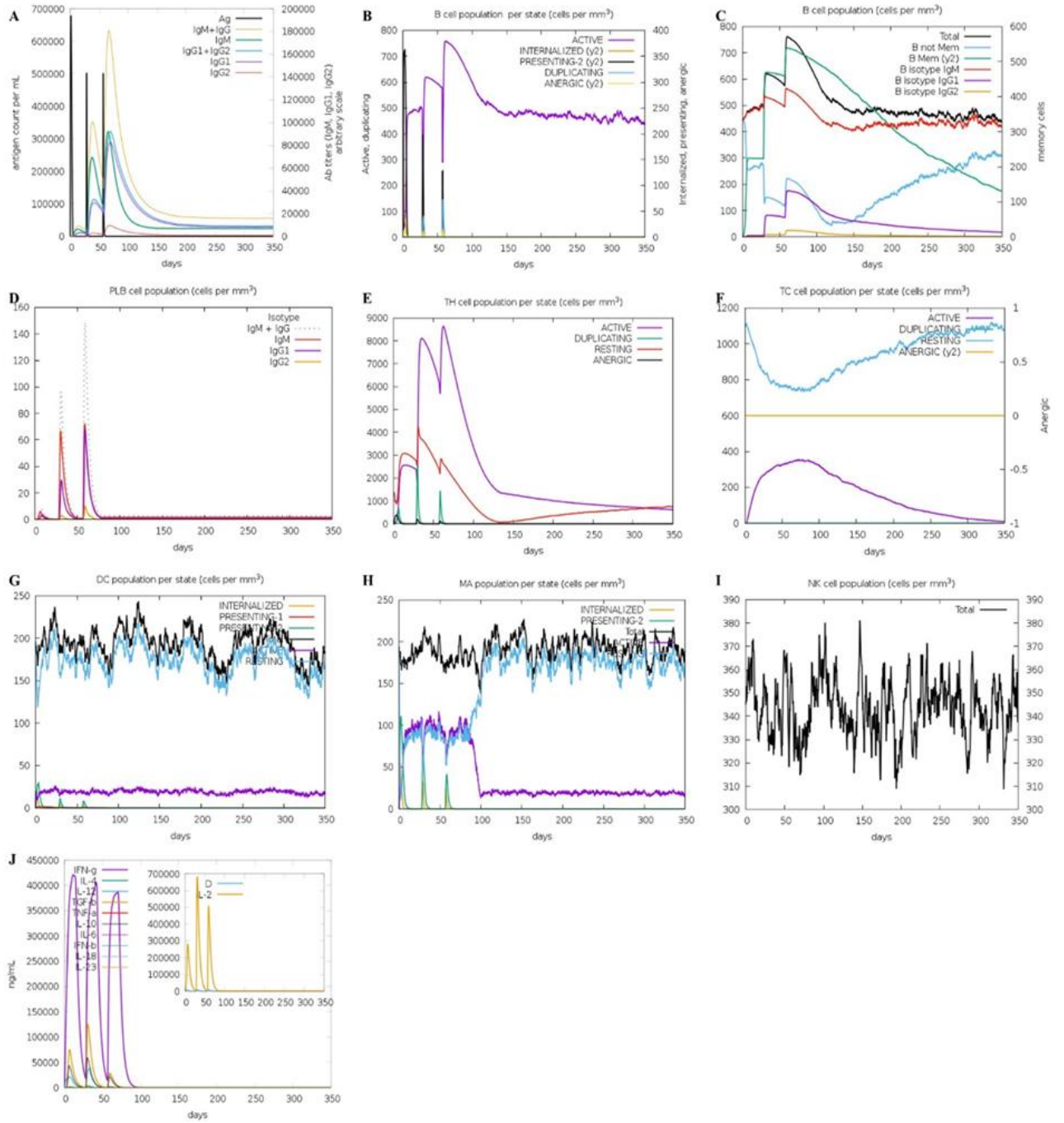


Fig. 6: Immune Responses Induced by MEV Antigen Exposure. A) Antigen-induced Immunoglobulins production, B) Heterogeneous activation of plasma B-cells, C) Memory B cells and production of active B isotypes, D) Activation of plasma B cells, E) Profiling of helper T cells generation, F) Dynamics of antigen-induced cytotoxic T cell population, G) Spatial and temporal distribution analysis of Dendritic Cell (DC) populations, H) Proliferation of Macrophages, I) Antigen-induced NK cells, J) Interferons- and cytokines-induced immunomodulatory signals.

DISCUSSION

Vaccines are crucial in preventing infectious diseases, but their effectiveness can be impacted by factors like antigen choice, administration route, dosage schedule, storage, animal health, genetics, herd immunity and emerging variants (Endale *et al*., 2022). Livestock in Pakistan contributes significantly to GDP.

The sector faces significant losses due to transmissible diseases, with BVD being a chronic threat Ain *et al*., 2023). Current vaccines, including MLV and KV, have drawbacks like limited protection duration, cold storage requirement, cost issues, and challenges in antigenically diverse populations (Kirthi *et al*., 2023).

The study explores a novel immunoinformatic methodology for developing an epitope-based vaccine targeting BVD, leveraging advancements in reverse vaccinology and immunoinformatic techniques (Parihar *et al*., 2022). The BVDV-1b genotype was chosen due to its importance in Asian epidemics, including Pakistan (Rivas *et al*., 2022), and China's prevalence due to shared border and animal traffic, which can spread illness. This approach presents a promising solution to BVD vaccination challenges.

Based on their central roles in viral attachment to host cell receptors, viral replication, and immunogenicity, four essential proteins (E1, E2, E^{rns}, and NS3) from the BVDV were chosen for the present study. It was crucial to evaluate the immunogenic properties of these chosen proteins to facilitate effective vaccine design. Therefore, the antigenic and allergic properties of all four proteins were thoroughly analyzed with the goal of finding highly antigenic and non-allergenic candidates. Antigen-specific B-cells, CTLs, or HTLs should be activated by a successful vaccine. To initiate a humoral response, naive B cells must be exposed to and recognized by antigens. Naive T cells need to have peptide antigens coupled to MHC molecules on APCs to initiate the cellular response. The immune system must be able recognize the difference between healthy and infected cells so that it can activate CTLs (Lymphocytes + CD8) and kill off foreign particles by attaching a peptide to MHC-I proteins. Epitopes possessing notable antigenicity, devoid of allergenicity, toxicity, and displaying a substantial level of conservation were selected for subsequent investigations. Following that, a collection of six CTL epitopes (AKNDEIGPL, TGVPPHLAT, WPDPGNQQV, AWPDPGNQQV, TQLEILNNL, and DTYENYSFL) and one linear B-cell epitope (GLDWPDPGNQQVVETG) was chosen from BVDV proteins to design MEV. The molecular interactions between the chosen CTL epitopes and their corresponding BoLA class-I alleles were examined to assess their effectiveness in eliciting the anticipated immunological responses. The epitopes "TQLEILNNL" and "DTYENYSFL" derived from the antigenic proteins of BVDV illustrated notable stability in their interactions with receptor proteins. These interactions were characterized by the formation of five hydrogen bonds with promising bond lengths, suggesting the potential for effective immunological activation. Since the sequencing of the bovine MHC II locus has not been completed, and the bovine genome project is still ongoing, only BoLA MHC-I alleles have been loaded into the web servers for analysis (Garcia *et al*., 2023).

Finally, the MEV construct was created by employing EAAAK and GPGPG linkers to combine the immunogenic CTL- and B-cell epitopes and CTB as an adjuvant. Because of its high binding potential for GM1 ganglioside receptors, the N-terminus of MEV was modified by adding CTB adjuvant to make it a more effective antibacterial agent. Mucous epithelial cells have GM1 receptors that will contribute to a stronger immunological response. High antigenicity, immunogenicity, biosafety, and lack of allergenicity were observed in the final vaccine. It was found to be soluble, thermally stable, and hydrophilic upon investigating its physico-chemical properties. Since there was no existing template to use as a basis for the proposed MEV, comparative structural modelling was done and selected the best predicted structure after assessing through ERRAT, Verify3D and the Ramachandran plot.

To stimulate an immunological response, the candidate vaccine protein must maintain a constant contact with immune receptors. Bovine TLR3 is reported to be the primary sensor of BVD infection that triggers the innate immune response. The chimeric vaccine protein bound to bovine TLR3 efficiently with the least amount of energy required. In addition, MD modelling of MEV+TLR3 demonstrated the structural stability and compactness of both the unbound vaccine and the vaccine-receptor complex using RMSD, RMSF, and RoG analyses. Vaccine having antigens that stimulate both B- and CTL-type immune responses is expected to be very effective. Important for BVD immunological modelling, MEV also elicited robust macrophage activity and durable CTL-mediated immune responses. After primary immunization, the number of helper T cells also dramatically increased. Active immunoglobulins, IFN-, and IL-2 were all present in high enough quantities to provide additional assurance of broad resistance to BVD infection.

A potential paradigm change towards the prevention of viral and infectious diseases is suggested by MEVs. However, unlike traditional vaccines, MEVs use immunogenic B- and T-cell epitopes from a wide variety of target proteins to decrease the possibility of autoimmune reactions. Immuno-informatics in MEV development has been demonstrated by numerous research groups to be capable of producing highly effective vaccines against a variety of diseases (ul Qamar *et al*., 2021; Abdulabbas *et al*., 2023; Salahlou *et al*., 2024). Vaccines that make strategic use of mucosal adjuvants provide an extra line of defense against infection. This MEV is a lighthouse for more efficient and specific disease preventive strategies, and it is an appropriate option for accelerating the development of vaccines to combat BVD.

Conclusion: In conclusion, MEV against BVD is now feasible because of the novel reverse vaccinology approach used in this study. This study uses immunoinformatic and computational studies to discover surface-exposed immunogenic peptides, with the goal of developing an effective vaccine that can induce long-lasting adaptive immunity in animals. While bioinformatics study has provided a solid groundwork, *in vivo* testing of these epitopes is still an essential next step. This study not only represents a major step forward in vaccinology but also provides a view into the future of disease prevention, where novel vaccine candidates and cutting-edge approaches may completely alter current practices.

Authors contribution: MSK conceived and designed the project, and the research work is conducted by RR and SZ under the supervision of MSK. The manuscript is prepared by RR and critically reviewed by MSK.

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