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RESEARCH ARTICLE

Multi-Epitope Based Vaccine Design and Analysis Against Bovine Viral Diarrhea Virus Using Immunoinformatics Approaches

Maham Javed[1], Maheen Naseer[1], Muhammad Ibrahim[1], Farrukh Jamil[1], Kadir Yeşilbağ[2], Amna Kousar[1] and Muhammad Asif Rasheed[1*]

[1]Department of Biosciences, COMSATS University Islamabad, Sahiwal Campus, Sahiwal, 54000, Pakistan; [2]Department of Virology, Faculty of Veterinary Medicine, Bursa Uludağ University, Bursa, 16000, Türkiye
*Corresponding author: asif.rasheed@cuisahiwal.edu.pk

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ABSTRACT

Bovine viral diarrhea virus (BVDV) is an important cattle virus that causes considerable losses to the farmers in the cattle industry. This paper discusses the development of multi epitope vaccine (MEV) against BVDV using immune-informatics tools. We identified 24 cytotoxic T lymphocyte (CTL) epitopes, 5 helper T lymphocyte (HTL) epitopes and 9 B cell epitopes from viral poly-proteins. These epitopes were chosen due to their strong antigenic activity and non-malignant properties. A particular linker was used, and the adjuvant was incorporated to enhance immunogenicity of the MEV. The 655 amino acids that made up the final MEV vaccine are proven stable, hydrophilic and antigenic. The immune simulation analysis of the vaccine also showed good immune response in the host including the titre values of the antibodies, B-cell and T-cell activities. Conventional vaccines were outperformed by MEV regarding the breadth of recognized antigens because MEV is aimed at universal conserved sequences that span different BVDV genotypes, which significantly simplifies the challenge of the antigen heterogeneity. However, further *In vitro* confirmations are required to validate the computational results. The specific areas of concern include the safety, efficacy and functional feasibility of MEVs which should be tested both in the lab as well as animals. In this research, we present MEV as a more secure and effective approach to current vaccines against BVDV. The vaccine may be considered as a progressive tool for combating BVDV and decreasing the losses in cattle production.

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INTRODUCTION

Bovine Viral Diarrhea Virus (BVDV) was originally identified in US in the 1940s and emerged as a worldwide problem in cattle. The virus transmit through direct contact of affected animals, contaminated equipment, or across the reproductive tracts from an infected cow to her offspring. BVDV is classified as Pestivirus and its genetic material consists of a single-stranded RNA molecule with positive polarity, measuring approximately 12.3 kb. This RNA sequence encodes the information necessary for the synthesis of a poly-protein. This poly-protein is cleaved to generate both structural and nonstructural proteins (Collet *et al.,* 1989).

The infections diagnosed in cattle by BVDV cause various clinical manifestations such as respiratory disease, diarrhea, reproductive disorders and immunosuppression (Peterhans *et al.,* 2010). Three genotypes have been identified namely BVDV-1 (Pestivirus bovis), BVDV-2 (Pestivirus tauri) and BVDV-3 (Pestivirus brazilense, atypical pestivirus) with several sub-genotypes and bio-types of cytopathogenic (CP) and non-cytopathogenic (NCP), respectively. The infected animals show reproductive problems including abortions, stillbirths, and congenital abnormalities (Grooms, 2004). Naturally infected animals and persistently infected (PI) animals born by the infected female cows have been fledged by the NCP biotype during pregnancy are important sources of the virus (Brock, 1995). Furthermore, BVDV has immunosuppressive effects, which if infected, new secondary bacterial and viral infections make disease outcomes worse (Ridpath, 2010).

Vaccines suggested being beneficial in controlling BVDV in cattle, but viral heterogeneity and

immunosuppressive effects of BVDV have remained a concern in development of vaccines (Nejabat *et al.,* 2024). This is because of their high immunogenicity, but they are associated with high risk of fetal infection and possible reversion to virulence (Bolin & Ridpath, 1998). Inactivated vaccines are less risky than live vaccines but the immune response elicited is normally weaker and multiple boosters are therefore needed (Houe, 1995). New advances in immunoinformatics and reverse vaccinology enable the creation of new approaches to vaccine design, epitope-based vaccines, which address the corresponding regions of viral proteins (Lundberg *et al.,* 2013). Moreover, for BVDV vaccination, E2 glycoprotein, NS3 protease and NS5B polymerase proteins are significant as they are immunogenic proteins and have main roles in BVDV disease processes (Chase *et al.,* 2004).

Immunoinformatics allows predicting the cellular epitopes of B cell and T cell with higher antigenicity and immunogenicity. These approaches are essential for the development of MEVs which is created through the consolidation of multiple epitopes to provoke strong immune response comprehensive to variant sub-groups. Specific E2 glycoprotein, NS3 protease, NS5B polymerase, and Erns glycoprotein targets are identified because of their high levels of antigenicity and roles in viral replication and immune system interactions. Among the structural proteins, E2 glycoprotein is the most significant for the production of neutralizing antibodies in humans, while NS3 and NS5B involved in immune control and replication and are potential candidates for a vaccine (Wang *et al.,* 2022).

The present work was focused on MEV constructs to target BVDV using the different bio-immunoinformatics approaches. To ensure that the developed vaccine would only trigger the required immune response, the B-cell and T -cell epitopes present in four BVDV proteins: E2 glycoprotein, NS3 protease, NS5B polymerase, and Erns glycoprotein (Keshri *et al.,* 2023). This approach uses the modern tools in bioinformatics together with molecular biological methods to come up with a safe, efficient and broad-spectrum vaccine candidate for BVDV (Rizzo *et al.,* 2021).

MATERIALS AND METHODS

Retrieval of viral proteome and antigenicity prediction
The National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/) protein database was used to retrieve the whole proteome sequence for BVDV. The sequence of poly-protein of 3898 amino acids was retrieved. The online predictor tool VaxiJen v2.0 (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) was utilized to evaluate the antigenicity of BVDV proteins. A wide variety of properties are included in sequence annotation, including coding regions, invariant domains, variants, references, names, and database cross-references (Pruitt *et al.,* 2005). VaxiJen is unique among platforms since it can predict antigenic protection independently of sequence alignment, overcoming the drawbacks of alignment-dependent methods (Doytchinova & Flower, 2007).

Prediction of cytotoxic T lymphocyte (CTL) epitope: To predict CTL epitopes, NETCTL 1.2 (https://services.healthtech.dtu.dk/service.php?NetCTL-1.2) webserver was used. These predictions were validated by different parameters including assessment of proteasomal C distal cleave utilizing ANN, binding preference, and Transporter Coupled with Antigen Processing (TAP). The threshold of 0.05 was set for the first parameter, 0.15 for the second, and 0.75 for the third parameter. Moreover, VaxiJen v2.0 (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) webserver was used to check whether the predicted CTLs were antigenic or not. Furthermore, we used AllerTOP v. 2.0 (https://www.ddg-pharmfac.net/AllerTOP/) and AllergenFP v. 1.0 (https://ddg-pharmfac.net/AllergenFP/) to determine whether the predicted epitopes were allergenic or not. In addition, the web-based program ToxinPred was used to assess the toxicities of the predicted CTLs. (https://webs.iiitd.edu.in/raghava/toxinpred/multi_submit.php).

Prediction of HTL epitope: Prediction of HTL epitopes is necessary in order to develop the vaccine. By using the default parameters, web-based server NetMHCIIpan 4.0 was used to predict Helper T lymphocyte (HTL) epitopes (https://services.healthtech.dtu.dk/services/NetMHCIIpan-4.0/1-Submission.php). The predicted epitopes were 15 mers long. Lower percentile scores indicate a stronger affinity for MHC class II for epitopes.

Furthermore, these HTL candidates were evaluated using VaxiJen v2.0 (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html), AllerTOP v. 2.0 (https://www.ddg-pharmfac.net/AllerTOP/), AllergenFP v.1.0 (https://ddg-pharmfac.net/AllergenFP/), and ToxinPred (https://webs.iiitd.edu.in/raghava/toxinpred/multi_submit.php) webservers.

Prediction of B cell epitopes: B cells are a subset of lymphocytes that produce antibodies. These antibodies attach to antigens at specific regions called epitopes (El-Manzalawy *et al.,* 2008). All B-cell candidates that fulfilled the expected score, antigenic characteristics, allergic reaction, and toxicity criteria were analyzed using the web tool IEDB server (http://tools.iedb.org/bcell/). Surface easy access, antigenic potential, and hydrophilicity were identified computationally using the IEBD's prediction algorithms.

Vaccine construction: The predicted CTL, HTL, and B cell epitopes were used to build the BVDV vaccine. The best linkers were used to connect them. The primary function of linkers is to promote epitope presentation, and the secondary function is to avoid the development of junctional epitopes (Khan, 2019). Three different types of linkers including AAY, GPGPG, and KK were used to connect the CTL, HTL, and B cell epitopes respectively. In addition, the vaccine's potency was enhanced by adding 140 amino acids lengthy Profilin

(The UniProt database ID: P02584) as an adjuvant at the 5' terminus of the vaccine by using EAAAK linker (Chawla *et al.,* 2023).

Allergenicity and antigenicity of vaccine: To evaluate the allergenicity of the vaccine design and to confirm the absence of allergens, AllerTOP v. 2.0 was used which is an online web server accessible at https://www.ddg-pharmfac.net/AllerTOP/. Along with that, VaxiJen v2.0 webserver was used (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) to verify the vaccine's antigenicity.

Physio-chemical characteristics and predicted solubility: Many physicochemical components of the MEV were tested using the Expasy-ProtParam web server (https://web.expasy.org/protparam/). Vaccine build parameters such as amino acid composition, aliphatic rating of molecular weight, instability index, GRAVY, and half-life were checked to confirm different properties of the MEV.

Tertiary structure prediction, refinement, and validation: The homologous 3D structures of the MEV were checked in protein databank database by using BLAST. When no significant similarity was found, I-TASSER was resorted for modelling. By using simulations and alignment methods, the program identifies 3D atomic models. A model's quality is indicated by its C-score, RMSD, and TM value (Roy *et al.,* 2010).

After predicting the structure of MEV, Ramachandran plot was utilized to analyze the results before refinement. The quality of the model was checked using the plot, which indicates the propensity of essential amino acids in favoured and unfavoured locations. The GalaxyREFINE database (https://galaxy.seoklab.org/cgi-bin/submit.cgi?type=REFINE) was used to refine the 3D model generated by I-TASSER (Lee, 2019). After refinement, the model's quality was verified again using Ramachandran plot analysis.

Molecular docking of the MEV with the immune receptor: Vaccine's efficacy depends on its ability to bind to certain immunological receptors in the host. Thus, to anticipate how the MEV might interact with toll like receptor 4 (TLR4), protein-protein docking was employed. Molecular docking and docking refinement were both performed on the web server Auto-Dock and Auto-Dock Vina. The complicated structure of the docked vaccine against TLR was also examined and visualized using Ligplot (Waqas *et al.,* 2023).

Immune Simulation: The online simulation server C-IMMSIM, which is the Immune system simulator (https://150.146.2.1/C-IMMSIM/index.php), was used to create the immunogenic profiles of the MEV construct. For most vaccines on the market today, the optimal time between injections is four weeks. The C-IMMSIM server's most important parameter is the simulation step. Time spent on one simulation phase is 8 hours. In total, the simulation lasted 150 steps throughout the duration of two successive injections, with time steps of 1 and 88, respectively.

RESULTS

Retrieval of viral proteome and antigenicity prediction: The whole proteome sequence of the BVDV polyprotein was retrieved from NCBI. There are a total of eleven proteins, some of which are structural and some of which are non-structural. The polyprotein is composed of structural proteins such as Erns, E1, E2, and core proteins. Once the sequence was retrieved, its antigenicity score was verified to be more than 0.4, indicating that it was an antigen.

Analysis of cytotoxic T lymphocyte (CTL) epitopes prediction: Several CTL epitopes that exhibit significant antigenicity, non-allergenicity, and non-toxicity were discovered through this investigation. It is highly recommended to do experiments to validate these epitopes, especially those with ranks greater than 1.5. It is suggested that epitopes such as CIRPNWWPY, HPEPIQLAY, and LADFEERHY be given priority for immunogenicity testing in the lab. From a total of 115 CTL epitopes, only 24 demonstrated antigenic, non-allergenic, and non-toxic properties as shown in table 1.

Analysis of helper T lymphocyte (HTL) epitope prediction: The results of this study point to several HTL epitopes that show promise as potential research subjects due to their lack of allergenicity, toxicity, and antigenicity. In order to boost immune responses, the peptides with a greater antigenicity score particularly LRDFDAELSELVDYK and YENYSFLNARKLGED may certainly be crucial. In addition to activating B-cells for antibody production and developing protective CD8+ T-cell memory, these epitopes elicit a CD4+ helper response. Only 5 of the 14 HTL epitopes were found to be antigenic, non-allergenic, and non-toxic as shown in table 2.

Analysis of B cell epitopes prediction: One kind of lymphocyte, called a B cell, is responsible for secreting antibodies that attach to antigens. B cell epitopes are the regions that antibodies recognize. Immunity can be induced through the identification of B-cell receptors or released antibodies by B-cell epitopes. An effective immune response may depend on the inclusion of such epitopes in the suggested vaccine. 10 B-cell epitopes were predicted from selected proteins by the IEBD service. It was discovered that 9 of 10 B-cell epitopes were antigenic, non-toxic, and non-allergic as shown in table 3.

Construction of the vaccine: The multi-epitope vaccine was constructed using the 38 candidates that were selected, including 24 CTL, 5 HTL, and 9 B-cell. Attached to the vaccine at its 5' end is an adjuvant called Profilin, which consists of 140 amino acids. By efficiently detaching from other protein locations, the EAAAK linker promotes stability and decreases connections with other places. Adding an adjuvant to a vaccination may make it more immunogenic by stimulating the immune system to mount a more effective adaptive response.

The compatibility of the relations between the epitopes was used to sequentially connect them together.

Table 1: CTL Epitopes Prediction of retrieved sequence

No.	Peptide	Position	Antigenicity	Score	Allergenicity	Toxicity
1	LSEVLLLSL	551-559	Antigen	0.4858	Non-Allergen	Non-Toxic
2	CIRPNWWPY	620-628	Antigen	2.6214	Non-Allergen	Non-Toxic
3	LLITGVQGY	685-693	Antigen	0.5985	Non-Allergen	Non-Toxic
4	CITGDQLHY	881-889	Antigen	0.8591	Non-Allergen	Non-Toxic
5	KVDLAGLLL	1270-1278	Antigen	0.4590	Non-Allergen	Non-Toxic
6	VSSKWQLIY	1373-1381	Antigen	0.7136	Non-Allergen	Non-Toxic
7	YLTVDFMYY	1384-1392	Antigen	0.9980	Non-Allergen	Non-Toxic
8	LADFEERHY	1516-1524	Antigen	1.6712	Non-Allergen	Non-Toxic
9	LTDEAEYGV	1684-1692	Antigen	0.6322	Non-Allergen	Non-Toxic
10	KLRAAMVEY	1896-1904	Antigen	0.7746	Non-Allergen	Non-Toxic
11	FLDIAGLKI	1973-1981	Antigen	0.8166	Non-Allergen	Non-Toxic
12	ATGSKDYHY	2111-2119	Antigen	1.4896	Non-Allergen	Non-Toxic
13	HPEPIQLAY	2184-2192	Antigen	1.8338	Non-Allergen	Non-Toxic
14	ATEDEDLAV	2236-2244	Antigen	0.8898	Non-Allergen	Non-Toxic
15	LEDTTHLQY	2311-2319	Antigen	1.1499	Non-Allergen	Non-Toxic
16	ISALATYTY	2479-2487	Antigen	0.4616	Non-Allergen	Non-Toxic
17	GVNYKVTKY	2775-2783	Antigen	0.5294	Non-Allergen	Non-Toxic
18	KIGLETGNY	2993-3001	Antigen	1.1804	Non-Allergen	Non-Toxic
19	KTARNINFY	3038-3046	Antigen	1.2519	Non-Allergen	Non-Toxic
20	EVAVKRSKY	3134-3142	Antigen	1.1443	Non-Allergen	Non-Toxic
21	YSEVTWEQL	3383-3391	Antigen	1.0294	Non-Allergen	Non-Toxic
22	VAFSFLLMY	3724-3732	Antigen	1.1181	Non-Allergen	Non-Toxic
23	TAPSSQTTY	3752-3760	Antigen	0.6107	Non-Allergen	Non-Toxic
24	VSSKTGQPY	3832-3840	Antigen	0.8010	Non-Allergen	Non-Toxic

Table 2: HTL Epitope Prediction of retrieved sequence

No.	Peptide	Position	Antigenicity	Score	Allergenicity	Toxicity
1	QSPYVVVATNAIESG	2032-2046	Antigen	0.5530	Non-Allergen	Non-Toxic
2	AQRYGIEDGINVTKS	2124-2138	Antigen	0.5859	Non-Allergen	Non-Toxic
3	YENYSFLNARKLGED	2215-2229	Antigen	0.8498	Non-Allergen	Non-Toxic
4	LRDFDAELSELVDYK	3068-3082	Antigen	0.8971	Non-Allergen	Non-Toxic
5	RTGFEKLANLNLSMS	3783-3797	Antigen	0.7800	Non-Allergen	Non-Toxic

Table 3: B-cell Epitopes Prediction of retrieved sequence

No.	Position	Peptide	Length	Antigenicity	Score	Allergenicity	Toxicity
1	1612-1623	TAFFGIMPRGTT	12	Antigen	0.7122	Non-Allergen	Non-Toxic
2	2089-2101	QAQRRGRVGRVKP	13	Antigen	0.9151	Non-Allergen	Non-Toxic
3	2247-2261	LGLDWPDPGNQQVVE	15	Antigen	0.6984	Non-Allergen	Non-Toxic
4	2457-2469	KPSFPGDSETQQE	13	Antigen	0.4621	Non-Allergen	Non-Toxic
5	2645-2657	GWEAKELSERTAG	13	Antigen	0.9599	Non-Allergen	Non-Toxic
6	2901-2910	KNNLEEKDIP	10	Antigen	0.7971	Non-Allergen	Non-Toxic
7	2979-2989	EKGGPSTTNSQ	11	Antigen	0.9377	Non-Allergen	Non-Toxic
8	3185-3194	QESKKQMTLT	10	Antigen	0.7157	Non-Allergen	Non-Toxic
9	3393-3402	AGINRKGAAG	10	Antigen	1.1122	Non-Allergen	Non-Toxic

The CTL, HTL, and B cell epitopes were joined by three distinct kinds of linkers including KK, GPGPG, and AAY linkers. The primary objective in developing multi-epitope vaccines is to inhibit the synthesis of junctional epitopes. They also help with epitope presentation and vaccination. There was a total of 655 amino acids in the finished vaccine construct as shown in figure 1.

Allergenicity and antigenicity of vaccine: The potential vaccination was tested for allergenicity to study any expected side effects when administered to the body. With an antigenic characteristics score of 0.7135, the projected result indicated that the MEV candidate does not include any allergens. We learnt that MEV has a higher antigenicity score than the sequence we obtained from NCBI after comparing their antigenicity.

Analysis of physiochemical properties: Our vaccine offers a number of noteworthy characteristics. A molecular weight of 71651.26 is associated with its 655 amino acids. This material has an isoelectric point (pI) of 8.88. An Aliphatic index of 73.74 showed that the vaccine is stable across a range of temperatures. According to the Expasy-ProtParam website, the suggested vaccination has a half-life of 30 hours in mammalian reticulocytes (In vitro), more than 20 hours in yeast (In vivo), and more than 10 hours in E. coli (In vivo). A GRAVY score of -0.333 indicates that the vaccine construct has a high affinity for water. Vaccine stability is indicated by an instability index of 39.70. The VaxiJen v2.0 server's prediction of the vaccine's antibodies score further verifies that it does not cause allergic reactions. This proves the vaccination can stimulate a sufficient immune response and is immunogenic. The physiochemical properties of the MEV are shown in table 4.

Table 4: Physiochemical Properties of multi-epitope vaccine

No.	Parameters	Results	Remarks
1	Number of amino acids	655	Suitable
2	Molecular weight	71651.26	Average
3	Theoretical pI	8.88	Significantly
4	Ext. coefficient	129180	Basic
5	Estimated half-life	30 hours (mammalian reticulocytes, in vitro). >20 hours (yeast, in vivo). >10 hours (Escherichia coli, in vivo).	Satisfactory
6	instability index	39.70	Stable
7	Aliphatic index	73.74	Thermostable
8	Grand average of hydropathicity	-0.333	Hydrophilic

Fig. 1: The MEV construct has an adjuvant (blue), linkers (pink, red, cyan, and dark green), CTL (yellow), HTL (light green), and B-cell (grey) epitopes.

The MEV candidate's tertiary structure, refinement, and validation: A number of three-dimensional tertiary structures were predicted by I-TASSER for the MEV, with confidence values (C-scores) ranging from -3.75 to -1.60 and z-scores between 1.15 and 2.98. Additional study was conducted on the structure associated with the C-score of -1.60. A root-mean-square deviation (RMSD) of 11.8±4.5 Å and a predicted TM score of 0.52±0.15. The 3D structure of MEV is shown in figure 2A. To generate high-quality models, the Galaxy REFINE server improved loop fineness and the energy function, which resulted in better model quality. The validity of the structure was predicted before refinement using the Ramachandran Plot which showed 50.9% amino acids in the recommended zone, 38.8% were in the authorized range, and 6.0% were outliers as shown in figure 2B. Upon refining, 72.9% of amino acids were located inside the preferred region, 22.7% in the permitted region, and 2.160% as outliers as shown in figure 2C.

MEV's molecular interaction with TLR4: The docking result is shown in figure 3. The ligand in the provided image represents a heterocyclic compound, characterized by interconnected rings composed of carbon, nitrogen, and other atoms. The carbon atoms, depicted as black circles, form the backbone of the ligand, while nitrogen atoms (blue circles) and oxygen atoms (red circles) indicate polar groups capable of forming hydrogen bonds. A chlorine atom, represented by a green circle, contributes to the hydrophobic properties of the ligand. Bonds between these atoms are depicted as solid lines, with double bonds shown as two parallel lines, indicating regions of conjugation or aromaticity. The hydrogen bond is illustrated by green dashed line connecting the ligand and Asp500(A), the distance being 3.12 Å. This interaction may well entail the oxygen atom of Asp500(A), which sets the ligand firmly within the binding site. The surrounding red arcs represent the hydrophobic interplay between the ligand and different amino acid residues like Leu527(A), Ile326(A), and Ala328(A) and Ala420(A). These residues build a hydrophobic pocket for which the ligand bonding is explained by van der Waals forces. Furthermore, other 'charge-bearing' amino acid residues such as polar residues like Tyr325(A) and Lys531(A) although does not form strong bond with the ligand but can form weak bonds with the ligand.

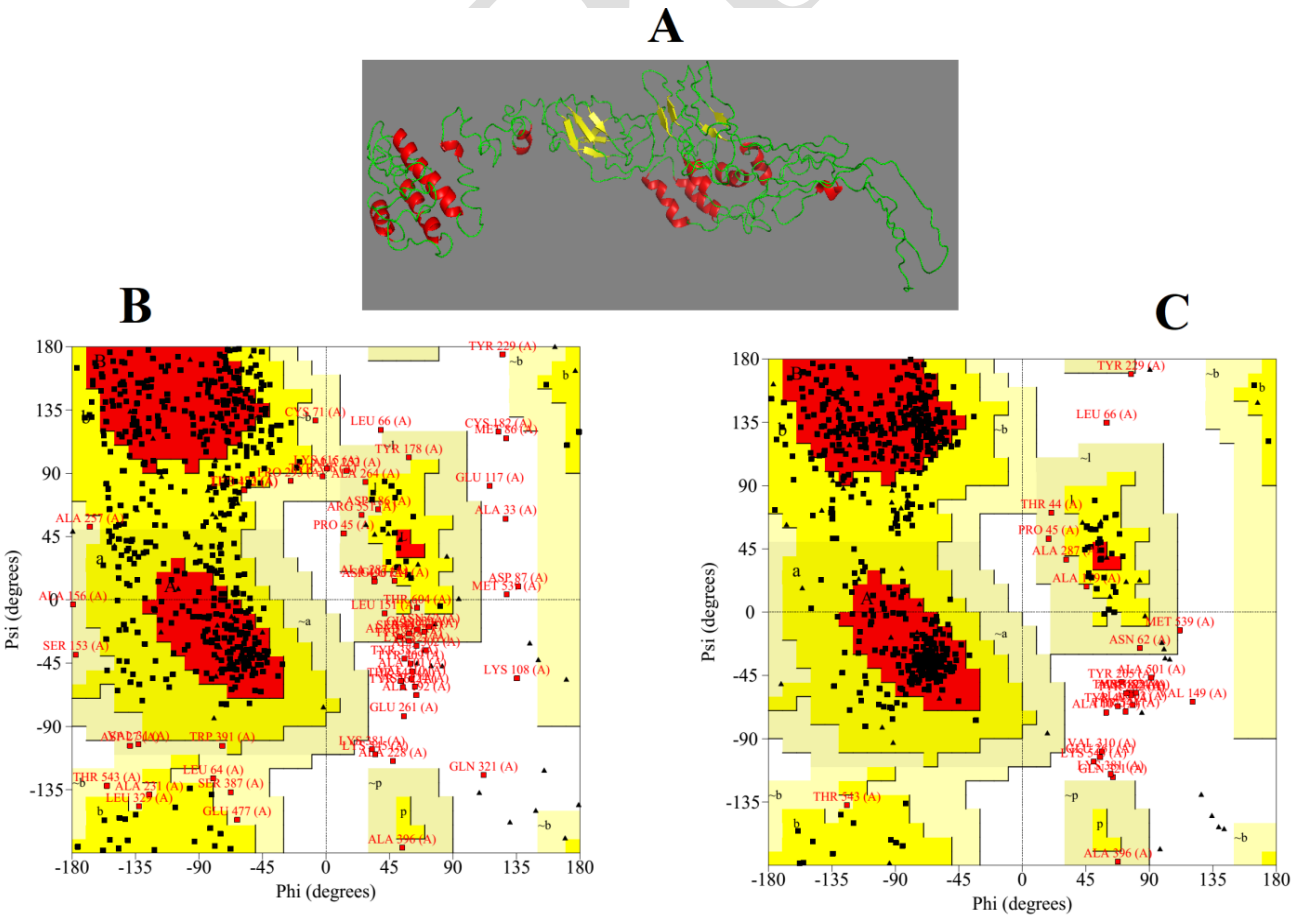

Fig. 2: Ribbon diagram of chimera multi-epitope vaccine construct predicted structure model (A). Before refinement, the model had 50.9% residues in the favourable region (B). After refinement, the number of residues in favourable regions increased significantly *i.e.*, 72.9% (C).

Immune Simulation: An In-silico simulation was performed using the C-IMMSIM immune service to evaluate the immunogenic characteristics of the multi-epitope vaccine. The details of the simulation results are shown in figure 4. Early in the timeline, the antigen count (black line) peaks at 700,000 counts per mL (figure 4A). IgM and IgG antibodies titers that climb continuously with time, reaching a scale of about 10,000 arbitrary units. While this is happening, the populations of both B-cells and TH cells are growing. The second dose of vaccine sets off the secondary immune response, which increases antibodies titers significantly. Both B-cell populations and TH-cell population showed significant peak again. Titers marking a rapid and strong rise in antibody levels.

Throughout both stages, the number of functional B-cells grows, and memory B-cells keep the vaccine's antigenic imprint in the time between doses. The levels of active TH cells grow significantly between days 5 and 10 and then stay steady until booster injection of MEV. Moreover, there is an up-regulation of cytokines and interleukins after vaccination (figure 4B) with an even greater up-regulation after the second dose. The increasing Simpson index over time, as shown in the insert plot, demonstrates the production of IL-2 dominant clones that are specific to epitopes. In addition to a robust main reaction, the data showed that the engineered vaccination also causes a secondary response that is both quicker and more powerful. This provides more evidence that two booster doses of the multi-epitope vaccination may successfully induce long-term protection in cattle.

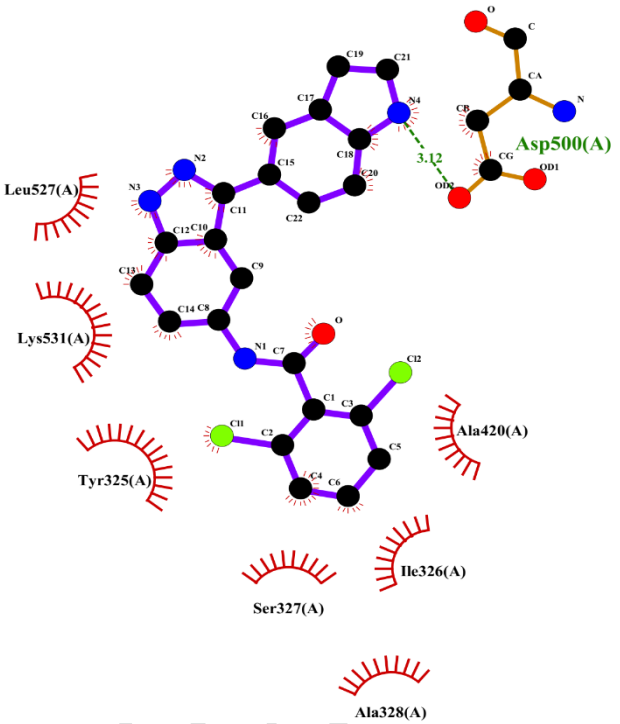

Fig. 3: The docking result of MEV and TLR4 is shown in the figure. The hydrogen bond is illustrated by green dashed line connecting the ligand and Asp500(A), the distance being 3.12 Å. The surrounding red arcs represent the hydrophobic interplay between the ligand and different amino acid residues like Leu527(A), Ile326(A), and Ala328(A) and Ala420(A).

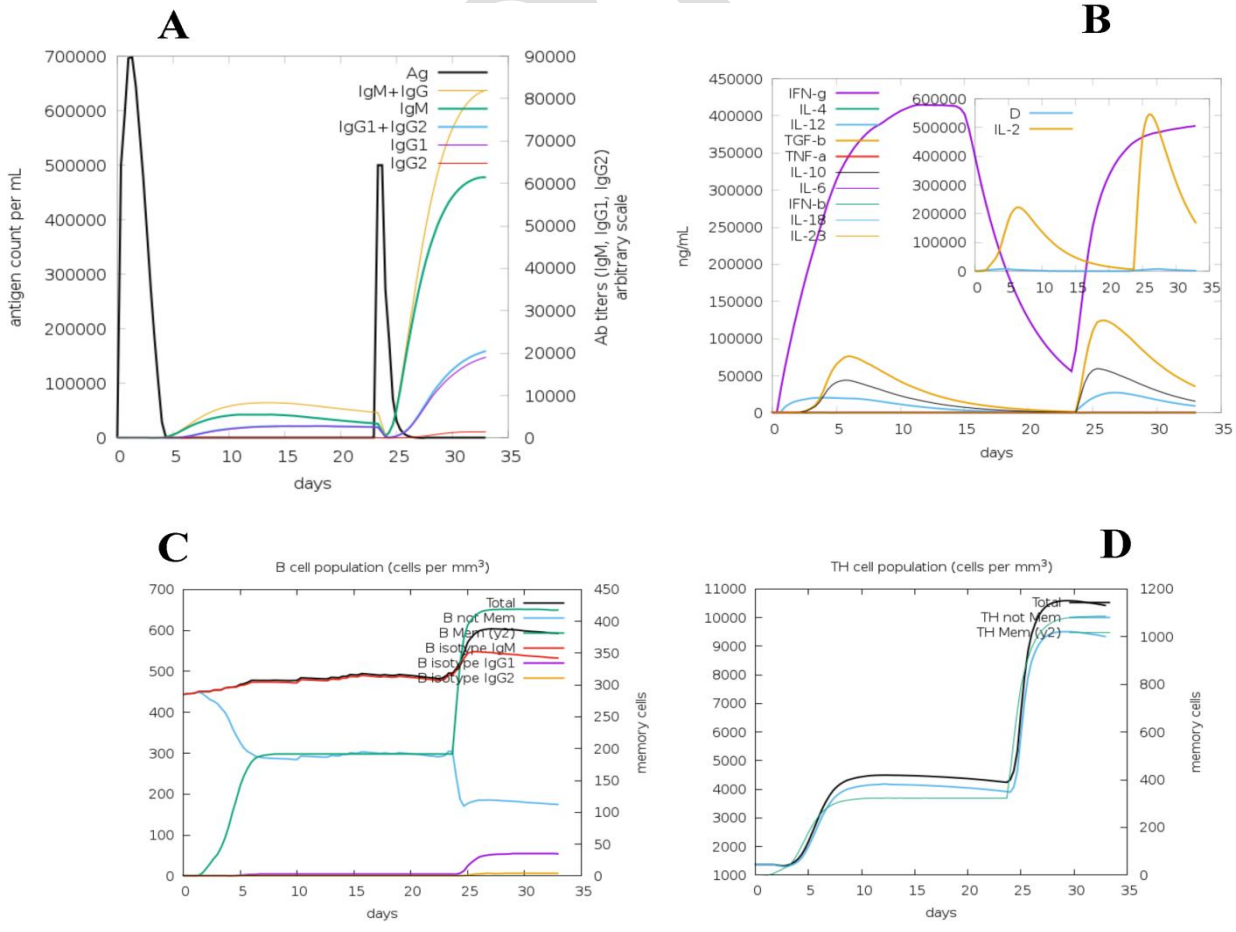

Fig. 4: Immune simulation analysis to evaluate the immunogenic response of multi-epitope vaccine. Two injections of MEV were given which increased IgM and IgG antibodies titers continuously with time (A). There is an up-regulation of cytokines and interleukins after vaccination (B) with an even greater up-regulation after the second dose. Populations of both B-cells (C) and TH cells (D) increased significantly.

DISCUSSION

An innovative multi-epitope vaccine (MEV) targeting bovine viral diarrhea virus presents a potential advancement over traditional modified live vaccines (MLVs) and killed/inactivated vaccines widely used in the cattle industry. BVDV is an important infectious agent for cattle, characterized by reproductive disorders, immunosuppression, respiratory distress, and gastrointestinal infections (Ridpath, 2010). Studies have shown significant economic losses caused by BVDV is commonly due to systemic infections as well as some limitations in traditional vaccines that need new strategies such as epitope-based vaccine design (Houe, 1995).

The MEV approach has several distinct advantages over conventional vaccines. One of the main advantages is the vaccine safety in target animal species. Unlike MLVs, which are associated with a higher risk of virus shedding from the vaccines and potential complications in pregnant animals, MEV is constructed with non-infectious materials to ensure that it poses no risk for post-vaccination infections or wide range of adverse effects (Peterhans *et al.,* 2010). This makes it safe for use in all the target animal populations, including calves and pregnant animals (Bolin & Ridpath, 1998). Killed vaccines, widely used in many territories, even when safe, generally induce weak immune responses because of their inability to better activation of cellular immunity (Chase *et al.,* 2004). MEVs, by incorporating highly antigenic CTL, HTL, and B-cell epitopes, is expected to overcome this limitation by stimulating both humoral and cellular immune responses.

Another significant advantage of MEV is its ability to deal with high levels of antigenic diversity exist in BVDV sub-genotypes (Yesilbag *et al.,* 2017). There are 3 main genotypes of the virus, officially classified as distinct virus species, and about 30 clusters of sub-genotypes existed under these genoptypes (Alpay & Yesilbag, 2015). Traditional vaccines are often relied on specific strains of the virus (BVDV-1a strains in particular), which may not provide adequate protection against genetically diverse field viruses (Lundberg *et al.,* 2013). On the other hand, MEV consists of selectively conserved epitopes in multiple BVDV genes to ensure maximum coverage and efficacy. The inclusion of high-scoring antigen epitopes in the MEV construct, such as the CTL epitope CIRPNWWPY with an antigenicity score of 2.62 and the HTL epitope LRDFDAELSELVDYK with a score of 0.89, highlights the ability to induce and establish strong immunity (Wang *et al.,* 2022).

MEV exhibits significant benefits in immune stimulation. Computational immune simulations showed that MEVs induce strong primary and secondary immune responses, characterized by increased antibody titers and activation of B-cells and T-cells well (Sanchez-Trincado *et al.,* 2017). These simulations suggest that MEVs can induce a prolonged immune response that is rapid and robust upon subsequent antigen application (Kumar Prajapatia, 2018). In contrast, although MLV is known to mimic natural diseases and spread with immune memory yet they need careful handling and risk adverse effects (Grooms, 2004). Killed vaccines, although safe in some respects, often have strong humoral immunity, may limit its effectiveness in combating viral infections i.e. BVDV (Chase *et al.,* 2004).

MEV offers practical advantages in terms of robustness and results (Wang *et al.,* 2022). In addition to cutting down on production time and expenses, the computerized design technique guarantees physicochemical and heat stability, as predicted by the GRAVY score (-0.333) and aliphatic index (73.74) (Lee, 2019). Cold storage requires complexity, making it difficult to manufacture killed vaccines in areas where limited resources are available. It also requires repeated manufacturing and chemical reactions, which can compromise antigenicity and immunogenicity (Yesilbag *et al.,* 2017).

However, there are some shortcomings of the MEV approach in managing an organization. Thus, relying on computational tools, which give valuable estimates for the efficiency of the vaccine in targeting the causative agents of chronic diseases. Theoretical studies predict that MEV should be tested *In vitro* and *In vivo* to estimate efficiency (potency), stability, and resistance (Jyotirmayee, 2022). In addition, the quality and cost, as well as the mass production, should be compared to the existing vaccines for using products effectively in the livestock industry (Jyotirmayee, 2022).

The tertiary structure of the MEV protein contains a highly distinct binding cavity into which the ligand is inserted. The ligand becomes stabilized and acquires distinctive opportunities for interactions with the target residues. An Inter-molecular interaction of hydrogen bond exist with 3.12 Å showing that it is indeed the specific interaction that is important for the ligand's binding affinity. Furthermore, the hydrophobic residues form a complementary non polar region that further enhances the binding of ligand.

Traditional vaccines, such as MLV and killed vaccines have been the cornerstone of BVDV control programs for decades. Despite the shared risks, MLV confers robust, long-lasting immune responses due to replication of live attenuated virus in vaccines, while killed vaccines provide robust protection and efficacy. But both vaccine technology has limitations: BVDV MLVs are mostly not suitable for pregnant animals, and killed vaccines require booster doses to generate adequate immunity. Comparing the conventional killed vaccines, despite non-experimented yet, MEV vaccine formulated in the present study may better suits protection in the field conditions.

Conclusions: The development of a multi-epitope vaccine (MEV) against bovine virus diarrhea virus (BVDV) offers a promising alternative to overcome the limitations of traditional vaccines including both MLV and killed ones. Using computational immunoinformatics tools, MEV incorporates conserved CTL, HTL, and B-cell epitopes, awaited to result in protection and long-lasting stability. Broad coverage of BVDV genotypes is to be ensured by that approach. Unlike in modified live vaccines, MEV eliminates risks of recurrent infections and complications in pregnant animals. Compared to killed vaccines, it is expected to improve the immune response by effectively stimulating humoral and cellular immunity. Immuno-informatics and immune-simulation show that the vaccine can provide strong, long-lasting immunity, and the

designed vaccine has the features of stability, scalability and bring high-cost performance. Those findings provide the solid foundation for further assessment of their *In vitro* and *In vivo* potential in T lymphocytes activation and protection against BVDV in the different tissue culture studies and animal disease models.

Author's Contribution: MJ wrote the manuscript and performed analysis. MN and AK performed analysis. KY, MI and FJ reviewed the manuscript. MAR conceived the idea and reviewed the manuscript. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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