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RESEARCH ARTICLE

Sequence and Structural Analysis of Synthetic VP2 Antigenic Protein as a Subunit Vaccine Candidate against Very Virulent Strains of Infectious Bursal Disease Virus

Madiha Fayyaz[1], Muhammad Sarwar Khan[1]*, Faiz Ahmad Joyia[1] and Muhammad Anjum Zia[2]

[1]Centre of Agricultural Biochemistry and Biotechnology (CABB); [2]Department of Biochemistry, University of Agriculture, Faisalabad, Pakistan
*Corresponding author: sarwarkhan_40@hotmail.com

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Infectious bursal disease virus (IBDV) causes immunosuppression in poultry (3 to 6 weeks age), resulting in severe production and economic losses. Current vaccination strategies exploit live attenuated or killed vaccines based on classical virulent IBDV strains, which are proven to be ineffective against variant and/or very virulent (vv) strains - currently circulating in the field throughout the world. Keeping in view the urgent need of clean and cost-effective vaccines of high quality, we have engineered VP2 gene as host protective major antigen. The gene was synthesized to express in chloroplast genome, plastome. Sequence and structural characterization of synthetic gene and corresponding protein were carried out using a variety of molecular biology and bioinformatics tools. In *silico* physico-chemical analysis demonstrated that the synthetic VP2 was an acidic protein of 54KDa. Moreover, it was found to be highly thermo-stable with a computed instability index of 30.38 and hydrophobic in nature with GRAVY index of 0.115. The sequence analyses including phylogeny and multiple sequence alignment with representative classical, attenuated and vv IBDV strains from different regions of the world exhibited antigenic similarity with currently circulating vvIBDV strains and predicted its worldwide effectiveness as a subunit vaccine. The 3D structure prediction using I-TASSER server and surface representation of epitopic loops on VP2 trimer using UCSF Chimera software indicated the potential of synthetic VP2 as an antigenic protein. The generated information has paved the way for the functional characterization of synthetic protein as a subunit vaccine employing transgenic approaches in future.

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INTRODUCTION

Infectious bursal disease (IBD) or Gumboro disease is an infectious disease of poultry caused by infectious bursal disease virus (IBDV) having prevalence across the globe. Usually, 3 to 6 weeks old young poultry birds are more susceptible to this disease. Bursa of Fabricius (BF) is the main lymphoid organ in birds which is severely impaired after clinical manifestation of the disease resulting in transient immunosuppression due to depletion of B lymphocytes. Additionally, negative response of the birds towards vaccination gives chance to various opportunistic pathogens to invade (Muller *et al*., 2012).

Infectious bursal disease virus (IBDV) belongs to genus Avibirnavirus of Birnaviridae family. IBDV is a non-enveloped virus consisting of an icosahedral capsid with a diameter of 50-60nm. Its double-stranded RNA genome comprises of two segments A and B. The 3.2kb segment A comprises of two open reading frames (ORFs) partially overlapping each other. The larger one encodes a polyprotein that cleaves into precursor VP2 (pVP2), VP3 (structural proteins) and VP4 (a serine protease) auto-catalytically. pVP2 is converted into mature VP2 by several proteolytic cleavages at its C-terminus (Zhang *et al*., 2015). The polyprotein is considered as the mediator of IBDV-induced pathogenicity and resulting immunosuppression (Peters *et al*., 2004). VP5 executes the release of viral particles from the host infected cells and is encoded by small ORF. The 2.8kb segment B codes for VP1, which contribute to viral virulence by acting as an RNA dependent RNA polymerase in viral replication and transcription (Ingrao *et al.*, 2013).

Two IBDV serotypes, serotype I and serotype II have been recognized. Strains belonging to serotype I specifically infect the chickens whereas IBDV strains in serotype II have more prevalence in turkeys (Reddy *et al*., 2017). Serotype I of IBDV includes three pathotypes viz classical virulent (cv) viruses, antigenic variants and very virulent (vv) IBDVs (Shabbir *et al*., 2016). Chicken bearing bursal damage due to infection by classical virulent strains showed 20-30% mortality (Lukert and Saif, 2003). Classical virulent strains are also the source of commercially available vaccines against IBDV infection. Antigenic variants emerged through an antigenic shift in early 1980s, which caused rapid bursal atrophy leading mortality rate beyond 50% (Adamu *et al*., 2013). Very virulent IBDV strains appeared in the late 1980s, and the mortality rate reached up to 90% as compared to classical ones (Muller *et al*., 2012; Ingrao *et al*., 2013).

VP2 is an external capsid protein and is regarded as major host-protective antigen as it bearsconformational epitopes - antigenic determinants that elicit neutralizing antibodies. These antigenic determinants also contribute to serotype specificity (Reddy *et al*., 2017). The antigenicity of VP2 protein is conformation-dependent because loss of cross-reactivity of neutralizing antibodies has been depicted in Western blots against denatured VP2 (Schnitzler *et al*., 1993). A 145 amino acid (aa) long hyper-variable region (HVR) of VP2 protein is documented as the major antigenic site (Shabbir *et al*., 2016). This hyper-variable region contributes towards molecular typing of diverse IBDV isolates as it varies greatly among the pathotypes of serotype I and also between two IBDV serotypes.

The X-ray structure revealed that VP2 folds into trimers, comprising of base, shell and projection domains as three major domains (Letzel *et al*., 2007). The base and the shell domains comprise of conserved N- and C-termini of the protein, respectively. However, the hyper-variable region (206 to 350 aa) of VP2 form the projection domain. HVR of VP2 consists of two major hydrophillic regions A and B. Region A spans 212 to 224 aa whereas region B comprises 314 to 325 aa (Alkie and Rautenschlein, 2016). A P_{BC} loop (aa 219-224) was found to form within region A while the loop P_{HI} (aa 316-324) was constituted by region B. These two loops have a role in neutralization of virus and also in antigenic structure variation. Two minor hydrophilic peaks were found to constitute the loop P_{DE} (249 to 254 aa), and the loop P_{FG} (279 to 284 aa). Amino acid 253 and 284 in Loop P_{DE} and $P_{FG,}$ respectively, play important roles in cell tropism and pathogenicity in chickens (Qi *et al*., 2009;Coulibaly *et al*., 2010).

Current vaccination programs against infectious bursal disease (IBD) exploit live attenuated or killed pathogens-based vaccines using classical virulent strains. These vaccines have not been found effective in controlling the antigenic variants and/or very virulent (vv) IBDV strains, currently circulating in the field and causing production and economic havoc in the poultry industry worldwide. In this study, we presented the sequence characterization of a synthetic VP2 protein using different bioinformatics tools. This sequence analysis has proven the synthetic VP2 protein as a potential field-strain-matched recombinant subunit vaccine candidate against infectious bursal disease when expressed in plants.

MATERIALS AND METHODS

Analysis of DNA sequence encoding VP2 protein: For the development of field strain matched subunit vaccine, KS strain (accession number L42284) was selected from GenBank database for coding sequence of *VP2*. KS strain belongs to vvIBDV pathotype as vvIBDVs are presently more prevalent in the field worldwide. Engineering of *VP2* gene sequence was executed, with codon optimization, followed by commercial synthesis to be expressed in chloroplasts for the development of recombinant subunit vaccine. Diagnostic restriction analysis was used for confirmation of synthetic *VP2* clone in pUC57 cloning vector. Analysis of the protein was carried out essentially as discussed by Qamar and Khan (2017).

Synthetic *VP2* gene sequence was translated using Justbio tool (http://www.justbio.com/index.php?page+translator). Physico-chemical properties of synthetic VP2 protein were determined using Protparam - a tool of ExPASY server (https://web.expasy.org/protparam/). MUSCLE v3.2 software (Edgar, 2004; https://www.ebi.ac.uk/Tools/msa/muscle/) was used for execution of multiple sequence alignment to find out amino acid residues involved in antigenicity of VP2 protein and to figure out phylogeny.

Nucleotide BLAST suite of BLAST engine (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch) was used for choosing *VP2* gene sequences homologous to sequence of synthetic *VP2* gene, required for phylogenetic analysis. For Phylogenetic tree construction, MEGA version 7.0 (Kumar *et al*., 2016; downloaded from http://www.megasoftware.net/) was used and the tree was constructed using complete *VP2* gene sequences employing Tamura-Nei model based maximum-likelihood method. Further, bootstrap method was used with 100 replicates to estimate topological accuracy. For construction of phylogenetic tree, Serotype I IBDV sequences homologous to synthetic *VP2* gene were selected from GenBank database with accession numbers as D6948 (AF240686), 3529/92 (KC189836), 89163 (HG974563), UK661 (X92760), UPM94/273 (AF527039), NB (EU595667), SDH1 (AY323952), SH/92 (AF533670), 78/ABIC (JX424077), GZ/96 (AY598356), mb (DQ927040), UPM97/61 (AF247006), Gx (AY444873), SH99 (LM651365), OKYM (D49706), Harbin-1 (EF517528), Ventri-IBDV-Plus (KJ547670), TASIK (AF322444), MG-7 (JF811919), 19th CEF-adapted (EU595669), 02015.1 (AJ879932), 94432 (AM167550), SA-KZN95 (KF241548), PO7 (AY665672), HZ (DQ825652), 16th CEF-adapted (EU595668), LD-847-04 (JF965438), HK46 (AF051838), DG12 (JX948746), OKYMT (D83985), Chinju (AF508176), 100056 (KU234528), 21stCEF-adapted (EU595670), HuB-1 (KF569805), Cro-Ig/02 (EU184685), SH95 (AY134874), Cro-Po/00(EU184687), gep5 (AF443294), HN(KT884486), SD10LY01 (KF569803), ZZ-11 (JX682711), CAHFS_K669 (JN585293), 2009CAH495-SESW (JF907703), JBN2011 (MF188862), RPM14/ABT/MVC/India (KU578102), BD 3/99 (AF362776), KNU08010

(HQ224883), CAHFS-785-SESW (JF907702), B-SD-RZ (GQ166972), VRDC-IBDV-SZ (KJ547671), QL (JX682709), KSH (AF165151), EDE14/ABT/MVC/India (KU558697), T09 (AY099456), KZC-104 (AB368968), NKL14/ABT/MVC/India (KU578098), SK53 (KJ198843), KMRG-48 (AB368970), PY12 (KX223749), 7741-SEGA-SESW (JQ403646), KK1 (AF165150), India/BGE15/ABT/MVC/2015 (KT870148), G202 (FJ842491), HuN11 (LM651367), GHUT-12 (DQ778035), IBD13HeB01 (KP676467), HLJ-0504 (GQ451330), 2/Kaduna. NG/2009 (JX424048), UPM04/ 190 (KU958716), BGE14/ABT2/MVC/2015 (KT884452), 80/Kaduna. NG/2011 (JX424079), THI14/ABT/MVC/ India (KU578104), YS07 (FJ695138), HeB10XS02 (KF569801), MDI14/ABT/MVC/India (KU558699), 88180 (AM111353), GX-NN-L (JX134485), VRDC-IBDV-WZ (KJ547673), VCN14/ABT/MVC/India (KU578100), A-BH83 (JF811920), VRDC-IBDV-EZ (KJ547672), IBDV77/Georgiavaccine (JX424076), HPR-2 (EF418036), STC (D00499), VRDC-IBDV-NZ (KJ547674), Irwin Moulthrop (AY029166), BDG23 (FJ842492), Cro-Pa/98 (EU184689), KK54 (KJ198844), D78 (AF499929), JNeto-BR (AY780423), JD1 (AF321055), CS-2-35 (EF418033) and IBDV strains from serotype II viz OH (M66722) and 23/82 (AF362773) to be used as outgroups.

Structural analysis of VP2 protein: SOPMA server (Geourjonand Deleage, 1995) was employed for prediction of secondary structure of VP2 protein whereas prediction of synthetic VP2 protein 3D structure was executed using I-TASSER server (http://zhanglab.ccmb.med.umich.edu/I-TASSER/download/). For structural evaluation and visualization of predicted 3D structure of VP2 protein, Chimera software package (http://www.cgl.ucsf.edu/chimera) was used.

RESULTS

Sequence analysis of *VP2* gene: For the synthesis of *VP2* gene, a vvIBDV strain KS with accession number L42284 was selected for its *VP2* gene sequence after a GenBank search. *VP2* gene expression cassette (*Prrn: VP2: TpsbA*) was synthesized commercially and cloned at *Eco*RV restriction site in pUC57 cloning vector. For multiplication, expression cassette was transformed into *E. coli* using heat shock method and the clone was confirmed through restriction analysis (Fig. 1). Presence of synthetic *VP2* gene cassette was exactly represented by restriction with *Eco*RV enzyme (Fig. 1A, lane 2). However, an approximately 2 kb fragment (Fig. 1B, lane 2) resulted from restriction with *Eco*RI+*Hin*dIII, showed the synthetic gene cassette together with multiple cloning sites (MCS) of pUC57. The orientation of the synthetic VP2 expression cassette in pUC57 was confirmed as 5'→3' through restriction with *Xba*I alone and double digestion with *Xba*I and *Bam*HI (Fig. 1B, lanes 3 & 4).

Protparam - a tool of ExPASY server (https://web.expasy.org/protparam/) was employed to analyze the physico-chemical properties of synthetic *VP2* gene sequence after being translated into protein sequence. Synthetic VP2 protein was characterized as acidic in nature with a molecular weight of 54151.46 Daltons after calculated isoelectric point (pI) of 5.17. The calculated isoelectric point may help to purify VP2 protein from crude extract using 2 dimensional-polyacrylamide gel electrophoresis (2D-PAGE) through isoelectric focusing. Measured extinction co-efficient of 40925 contributes towards determination of VP2 concentration. The instability index assists in estimation of the stability of query protein in a test tube. A stable protein has an instability index of less than 40 (Guruprasad *et al*., 1990). Direct relation between a protein's instability index (AI) and mole fraction of valine, alanine, leucine and isoleucine (Val, Ala, Leu and Ile) of that protein, may be considered as a helpful factor for increase in globular proteins thermostability (Susan and Balaii, 2005). The computed instability index of 30.38 and aliphatic index of 96.79 indicated the highly thermostable nature of synthetic protein. Being highly thermostable, we expect that our synthetic protein, if expressed in some edible plant parts, would work effectively as an oral vaccine. The calculated Grand average of hydropathicity (GRAVY) index (0.115) for VP2 protein depicted the membraneous (hydrophobic) nature of the protein.

Determination of evolutionary relationships through phylogenetic analysis is a crucial step in several research fields such as revealing lateral gene transfer, prediction of gene function, discovery of new species and comparative genomics (Dereeper *et al*., 2008). Complete synthetic *VP2* gene sequence was aligned with 96 closely related sequences along with the sequence of KS strain (the parent strain for synthetic *VP2* gene sequence) for phylogenetic analysis. These 96 homologous sequences were obtained from GenBank database exhibiting 97-99% homology with the sequence of synthetic *VP2* gene. MUSCLE v3.2 (https://www.ebi.ac.uk/Tools/msa/muscle) an online tool was used for multiple sequence alignment. The selected strains were of various geographic origins and represented different pathotypes of IBDV. For the construction of phylogenetic tree, Tamura-Neimodelbased maximum-likelihood method was employed using MEGA v7.0 software (Kumar *et al*., 2016; Fig. 2). Clustering of synthetic *VP2* gene was observed in the same clade as of the parent KS strain. Following determination of amino acid (aa) sequence of synthetic *VP2* gene hyper variable region (HVR), it was aligned with HVR sequences of representative vvIBDVs of various geographic origin (BD from Bangladesh, RPM14/ABT/MVC from India, MG-7 from Brazil, KS from Israel, SH95 from China, UK661 from UK., OKYM from Japan, 2009CAH495-SESW from USA., MM125 from Pakistan and SDH1 from Iran) together with D78 and mb vaccine strains. 145 amino acid residues constitute the aligned HVR, covering position of VP2 protein sequence from 206 to 350 (Fig. 3). Exact matching of hyper-variable region of synthetic VP2 protein to characteristic pattern of representative vvIBDV strains together with the parent KS strain was observed. Characteristic serine-rich heptapeptide "SWSASGS" of vvIBDVs covering amino acids 326-332 following major hydrophilic region B (MaHR B), was also found in synthetic VP2 protein. This serine-rich heptapeptide is known to indicate the virulence of virus strains.

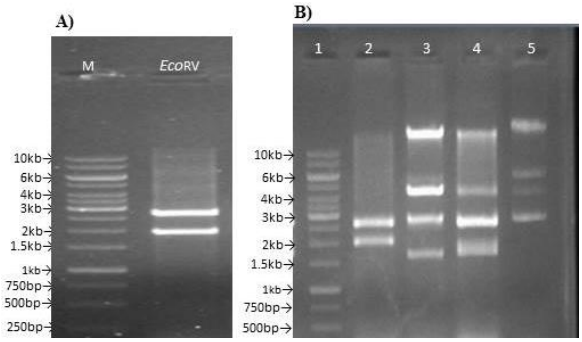

Fig. 1: Clone confirmation of *VP2* gene expression cassette by diagnostic restriction analysis. A) Clone confirmation with *Eco*RV. B) Confirmation of clone and its orientation with different enzymes. Lane 1 Marker DNA, Lane 2 *Eco*RI+*Hin*dIII, Lane 3 *Xba*I, Lane 4 *Bam*HI+*Xba*I, Lane 5 undigested.

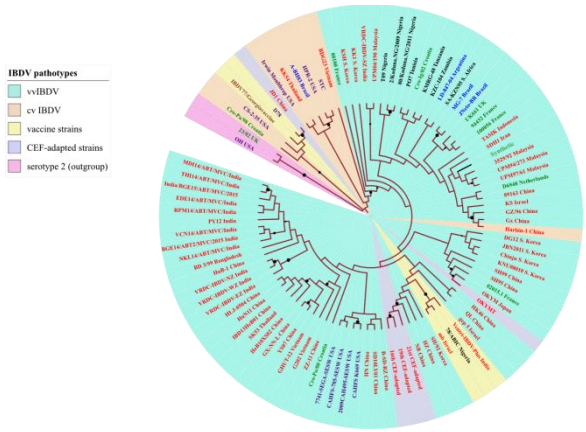

Fig. 2: Phylogenetic tree based on full-length synthetic *VP2* gene and its homologous sequences through BLAST analysis. Synthetic *VP2* gene sequence was denoted with yellow green letters while the rest of sequence labels were denoted with colors according to origin of strain (red labelled strains from Asia, green from Europe, purple from North America, blue from South America and black from Africa). Strain pathotypes were differentiated with background colors. Black circles of different sizes on various branches indicate the bootstrap vales of ≥70% similarity.

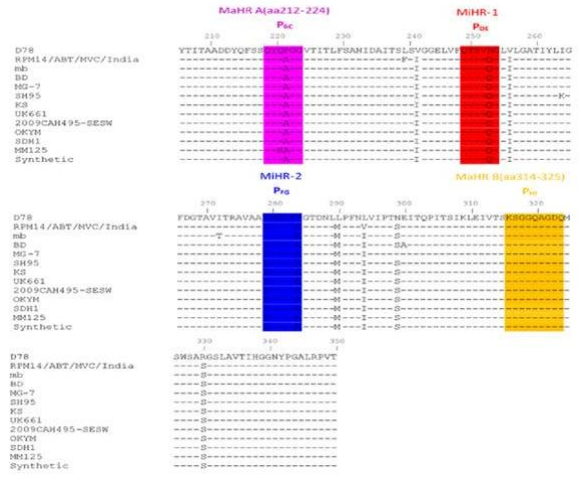

Fig. 3: Comparison of amino acid sequence of hyper variable region (aa 206-350) of synthetic *VP2* protein with those of representative strains. The representative strains include vvIBDVs of different geographic origins along with two vaccine strains viz. D78 and mb and their sequences were taken from GenBank database (see Materials and Methods for accession numbers; strain MM125 has accession number KU321593). Two major (MaHR A and B) and two minor (MiHR 1 and 2) hydrophilic regions are shown. Loops P_{BC}, P_{DE}, P_{FG} and P_{HI} within these regions are boxed in majenta, red, blue and orange colors, respectively. Amino acids are numbered as described by Mundt and Muller (1995).

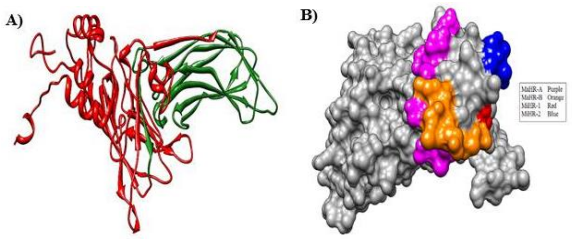

Fig. 4: Structure modeling of synthetic *VP2* gene encoded protein. A) Predicted 3D structure of the synthetic *VP2* gene encoded protein. B) Surface representation of major and minor hydrophilic regions (epitopic sites) on a VP2 trimer depicting the surface loops in colors as presented in multiple sequence alignment.

Structural analysis: Prediction of a protein structure is not only critical for analysis of its function but also for other downstream applications i.e. perception of complex association between structure and its sequence is mandatory for designing an enzyme and/or a drug. But, 3-dimensional (3D) structure of a protein is not solely reliant on its amino acid sequence. Accuracy of predicted secondary structure is crucial for precise prediction of a protein structure and function as well (Faraggi *et al*., 2012). A protein's secondary structure is the local conformation of its polypeptide backbone. Mostly, secondary structure plays the role of a bridge to link a protein's primary structure with its tertiary structure. Structural as well as functional analyses of proteins are dependent on their secondary structure in several ways (Wang *et al*., 2016). SOPMA software was used for secondary structure prediction of synthetic VP2 protein because of its 4% extra prediction power relative to other tools with 73.2% success rate (Geourjon and Deleage, 1995). Secondary structure predicted for synthetic VP2 protein revealed 25.24% alpha helices, 13.31% beta turns, 27.20% beta sheets and 34.25% random coils.

I-TASSER server (Yang *et al*., 2015; http://zhanglab.ccmb.med.umich.edu/I-TASSER/download/) was used for the prediction of 3D structure of synthetic VP2 protein through iterative assembly of structure (Fig. 4A). UCSF Chimera software was used for highlighting the epitopic regions on the surface of synthetic VP2 trimer. These epitopic regions were represented in multiple sequence alignment of hyper-variable region of representative homologous strains with that of synthetic VP2 protein (Fig. 4B).

DISCUSSION

Current vaccination programs against infectious bursal disease (IBD) exploit live attenuated or killed pathogens based on classical virulent strains of infectious bursal disease virus (IBDV). These vaccines have not been found effective in controlling the antigenic variant and/or very virulent (vv) IBDV strains currently circulating in the field and causing production and economic havoc in the poultry industry worldwide. Keeping in view the current scenario and dire need of field strain matched vaccine, a vvIBDV strain KS having accession no. L42284 was selected for its *VP2* gene sequence to be used as a template for the commercial synthesis of *VP2* gene, from GenBank database with the aim to develop recombinant subunit vaccine through chloroplast transformation.

Phylogenetic analysis of full-length synthetic *VP2* gene sequence with closely related sequences selected from GenBank database revealed the clustering of syntheic VP2 gene within the same clade as of vvIBDV KS strain - the parent strain of synthetic *VP2* gene. Furthermore, close relationship among synthetic *VP2* gene and vvIBDV strains representing different geographic areas of the world signified the current project for development of recombinant VP2 subunit vaccine matching the vvIBDV field strains as circulating worldwide (Jackwood, 2016).

Multiple sequence alignment of synthetic VP2 hyper-variable region (HVR) with those of vvIBDV strains representing various regions of the world together with two vaccine strains revealed the same epitopic sites in HVR of synthetic *VP2* gene as documented in the HVR of vvIBDVs from various continents of the world. HVR consists of neutralizing antigens and is a major domain in VP2 protein (Shabbir *et al*., 2016). The synthetic VP2 hyper variable region, 145 amino acids (aa) in length covering positions 206-350 of the protein, exposed aa residue A at position 222, I at 242, 256, 294 and S at 299. According to Jackwood and Sommer-Wagner (2007), this pattern characterize the strains of vvIBDV pathotype. Additionally, 326-SWSASGS-332, a characteristic serine-rich heptapeptide sequence following the major hydrophillic region B (MaHR B) and amino acid residue Q at 253 position have major contribution in virulence and cell tropism (Qi *et al*., 2009). Nevertheless, VP2 protien alone is not involved in virulence but other IBDV proteins viz viral proteins 1, 3, 4 and 5 might also contribute towards pathogenesis (Qin and Zheng, 2017). Moreover, surface representation of epitopic regions on synthetic VP2 trimer predicted its potential as an antigenic protein and/or a subunit vaccine candidate.

Conclusions: The sequence and structure analysis of synthetic VP2 protein elucidates its effectiveness as a subunit vaccine as compared to conventional killed or live-attenuated vaccines due to similarity in antigenic structure with those of circulating vvIBDV strains in many geographic areas across the world.

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Authors contribution: This manuscript is based on PhD thesis of first author. MSK conceived and designed the project. All the research work was executed by MF. Draft of the manuscript was prepared by MF. It was reviewed and critically analyzed by MSK, FAJ and MAZ. All the authors approved the final version of manuscript.

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