Engaging conserved epitopes of spike protein in Avian Infectious Bronchitis Virus (IBV) for expression in the bacterial host

Running title: design of immunogens from conserved epitopes of IBV spike protein

Sanaz Amiri[1], Salman Odooli[2], Najmeh Motamed[3], Bahman Khalesi[4*]

1. Department of Biology, Zarg.C., Islamic Azad University, Zarghan, Iran (sanazamiri13977@gmail.com) ORCID: 0009-0002-8074-0123

2. Department of Microbiology, Shi.C., Islamic Azad University, Shiraz, Iran (S.odooli@iau.ac.ir) ORCID: 0000-0003-2712-4655

3. Department of Poultry Vaccine Research and Production, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension organization, Karaj 3197619751, Iran (Drmotamed95@gmail.com) ORCID: 0000-0003-1658-1033

4. Department of Research and Production of Poultry Viral Vaccine, Razi Vaccine and Serum Research Institute, Agricultural Research, Education and Extension Organization, Karaj 3197619751, Iran (khalesi20022002@yahoo.com) ORCID: 0000-0001-5723-8624

***Corresponding Author:**

Bahman Khalesi

Department of Research and Production of Poultry Viral Vaccine, Razi Vaccine and Serum Research Institute,

Agricultural Research, Education and Extension Organization, Karaj 3197619751, Iran

Tel: +989122978114

Email: khalesi20022002@yahoo.com

Abstract

Introduction: Infectious bronchitis virus (IBV) affects chickens with significant economic losses in the poultry industry. The spike (S) glycoprotein of IBV is an appropriate immunogen for vaccine design. However, its glycosylation and diversity remain to be addressed to design a broadly protective antigen expressed in bacterial hosts. This study employed an *in silico* approach to nominate conserved B-cell epitopes within the S protein as potential targets which could be expressed in *E. coli* hosts. **Material and Methods:** The IBV S protein sequence was retrieved and characterized. Linear B-cell epitopes, N-glycosylation sites, physicochemical properties (flexibility, beta-turn propensity, surface accessibility, and hydrophilicity), and conservation of the sequence were analyzed. **Results:** Less than 30 linear epitopes with a length of > 4 aa were predicted. About 28 residues undergo glycosylation. Beta turns were abundant at the N-terminus of the S sequence. The most flexible and surface accessible residues were G405 and R1157 respectively. The longest hydrophilic region was 1021-1052 residues. The BLAST results showed 100 hits with lengths of 478-1181 amino acids, query coverage of 46%-100% and identity of 62.86%-100%. Six versions of multi-epitope constructs were designed. Conserved B-cell epitopes with favorable physicochemical properties and no N-glycosylation sites were selected. The YGPLQGGCKQSVFK was found to be the most prevalent (89% of sequences) epitope. The first and second constructs were 80-residue and 160-residue NON-IMMUNOGENs, respectively. The third, fourth, fifth and sixth constructs were 216-residue, 228-residue, and 356-residue IMMUNOGENs, respectively. **Conclusion:** Fourth and sixth constructs were nominated as the best broad-spectrum antigens concerning immune responses.

Keywords: B-cell Epitopes; Glycosylation; *In Silico* Vaccinology; Spike Protein; Vaccine Design

1. Introduction

Avian Infectious Bronchitis Virus, which causes huge economic loss in the poultry industry all over the world, belongs to the genus *Gamma Corona* Virus, family *Coronaviridae*. It is an enveloped positive-sense RNA virus with a 27.6 Kbp length genome that affects chickens at all

ages and causes pathogenicity in the respiratory, kidney, and oviduct of chickens, depending on the bird age and the virus strain. IBV outbreaks cause substantial morbidity and mortality in affected flocks (1, 2). The disease exhibits high transmissibility, with morbidity affecting nearly 100% of a flock. Mortality rates vary considerably, ranging from 25–30% in young chicks to as high as 80% under conditions influenced by host immunity, viral strain virulence, and environmental factors (3). The economic repercussions of IBV infections are particularly pronounced in commercial poultry operations. In the United States, where the poultry industry exceeds USD 30 billion in annual revenue, a single IBV outbreak can result in estimated losses of USD 450,000 per week in facilities producing one million broilers (4). Processing plants experience increased condemnation rates of up to 8% in IBV-affected facilities compared to 1% in controlled environments, further exacerbating financial losses. In laying flocks, IBV can reduce egg production by as much as 70%, and affected birds often fail to return to profitable production levels even after recovery. Additionally, IBV-infected commercial flocks suffer an approximate 3% reduction in overall income despite optimal management practices (3).

The host immune response to IBV involves both humoral and cellular components. IgG+ and IgM+ B cells in the trachea surge by day 3 post-infection, peaking at day 4, while T-cell responses (CD3+, CD4+, and CD8+ populations) peak at day 5 (5).

Current IBV control measures primarily rely on live attenuated and inactivated vaccines (6). Live attenuated vaccines elicit robust immune responses but carry risks, including potential reversion to virulence, maternal antibody interference, and recombination with circulating field strains. Inactivated vaccines, while safer, induce comparatively weaker immunity and require priming with live vaccines for optimal efficacy, increasing production costs and logistical challenges (6, 7). A major hurdle in IBV vaccine efficacy is the virus's rapid genetic evolution, driven by high mutation rates and recombination events, which results in the continuous emergence of novel genotypes and serotypes (8). Even minor sequence variations in key antigenic regions can significantly impact vaccine efficacy, necessitating frequent reformulation of vaccine strains (6). Additional factors such as vaccination timing, serotype selection, and the need for revaccination further complicate disease control efforts (9). The spike (S) glycoprotein and nucleocapsid (N) protein of IBV are attractive targets for vaccine design and development (8, 10, 11).

The spike (S) glycoprotein of IBV plays a pivotal role in viral infectivity and immune recognition (12). The S protein undergoes post-translational cleavage into two subunits: S1, which mediates host cell receptor binding, and S2, which facilitates membrane fusion (13). The S1 subunit contains immunodominant epitopes critical for antigenic neutralization, hemagglutination, and host tropism determination (12). Studies have demonstrated that S1 alone can confer protective immunity, with efficacy correlating with sequence homology between vaccine and challenge strains (14). However, the genetic plasticity of the S1 subunit, driven by frequent mutations and recombination events, poses a major challenge for vaccine design (15). Even minor amino acid variations (2–3%) in the S1 subunit can lead to serotype shifts, underscoring the critical role of a limited number of neutralizing epitopes in immune protection (16). In addition, glycosylation of S protein could be altered where expressed in a prokaryotic host. Hence, design of a novel antigen composed of non-glycosylated protective epitopes is valuable. Such recombinant antigen could be successfully over-expressed in *E. coli*.

Several recombinant proteins and epitope-based antigens expressed in *E. coli* have been reported to elicit protective antibodies in chickens against various viral and bacterial human and avian infections (17-22). Bioinformatics tools are nowadays helpful and powerful in designing novel recombinant antigens and next-generation antigens (23-25).

This study aims to provide novel insights into the molecular determinants of IBV immunogenicity and antigenic variation, with implications for the rational design of next-generation vaccines against IBV.

2. Material and Methods

2.1. Protein Sequence of spike and primary characteristics

A Spike (S) protein reference sequence of infectious bronchitis virus (IBV) was retrieved from the protein database of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/protein/) in September 2024. To find corresponding reviewed sequences in UniProtKB, a protein BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was run on the sequence against the Swiss-Prot database.

Probable signal peptide was predicted by SignalP 6.0 at https://services.healthtech.dtu.dk/services/SignalP-6.0/. Some physicochemical properties of the full-length and mature spike protein were predicted by ProParam at https://web.expasy.org/protparam/. Antigenicity of the sequence was estimated by VaxiJen v2.0 at https://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html. The input data set is a viral protein. The threshold for this model was 0.4.

2.2. Glycosylation analyses

To analyze glycosylation sites of the protein, data available at UniProt about this sequence were surveyed. In addition, N-glycosylated asparagines were predicted by NetNGlyc at https://services.healthtech.dtu.dk/services/NetNGlyc-1.0/. The threshold was set as default (0.5).

2.3. B-cell epitopes and Propensity analyses

Linear B-cell epitopes were predicted by BepiPred 1.0 and BepiPred 2.0 at http://tools.iedb.org/bcell/, as well as BepiPred 3.0 at https://services.healthtech.dtu.dk/. The thresholds were set as the default. The default thresholds for BepiPred 1.0, BepiPred 2.0, and BepiPred 3.0 were 0.35, 0.5, and 0.1512, respectively. Some physicochemical properties (Flexibility, Beta-turn, surface accessibility, and hydrophilicity) involved in B-cell epitope predictions were evaluated by tools available in IEDB at http://tools.iedb.org/bcell/.

2.4. Epitope screening and selection

Peptides with a length of > 4 aa assigned as B-cell epitopes by both BepiPred 1.0 and BepiPred 2.0 were selected to be candidates for the next step. Epitopes containing glycosylated residues were ignored. In addition, the appropriateness of peptides regarding flexibility, accessibility, hydrophilicity, or beta-turn structure was checked.

2.5. Conservancy and antigenicity of epitopes

A BLAST search was run (2 January 2025) on the NP_040831.1 sequence against a clustered non-redundant (experimental) database limited to the Infectious bronchitis virus (taxid:11120). Conservancy of the selected epitopes was evaluated against the sequences obtained from a BLAST search by the Epitope Conservancy Analysis tool at http://tools.iedb.org/conservancy/. The threshold of identity was set as ≥ 70%. Probable antigenicity of the selected epitopes was evaluated by VaxiJen v2.0.

2.6. Epitope validation and construct design

The selected B-cell epitopes served as queries in the IEDB BLAST search to find validated similar B-cell epitopes. For the predicted epitopes, the BLAST was set at 90% similarity. The validated epitopes, along with epitopes with more than 80% prevalence, were selected to be involved in vaccine construct design. If some validated peptides were for avian viruses, the predicted as well as their correspondence in experimental assays were included. The selected epitopes were tandemly fused based on positions in the original protein, spike. The obtained sequence was duplicated if the construct length was less than 100 amino acids. To avoid undesirable disulfide bonds, only 2 Cysteine residues were acceptable, and the addition of this residue in the repeat of the sequence was replaced by Serine (S).

2.7. Primary analyses and construct improvement

ProtParam evaluated some primary physicochemical properties. Antigenicity prediction was performed by VaxiJen 2.0. To enhance the immunogenicity of the construct, a predicted epitope with the highest antigenicity served as a linker between selected epitopes. A dendritic cell binding peptide (SPHLHTSSPWER) named SP was fused to the C terminus of the last construct. Next, the B subunit of cholera toxin (CTB) was added to the N-terminus of the obtained construct. In this regard, a CTB sequence (UniProt ID: P01556) was fused to the N-terminus of the last construct with a flexible linker (GGSG). If C residues were more than 2, those located within the spike-derived sequences were replaced by S. All constructs were evaluated concerning probable antigenicity by VaxiJen.

3. Results

3.1. The Protein Sequence of the spike is variable in length

A reference sequence with the accession number of NP_040831.1 was used as IBV spike for further analyses. The first nine hits found by the protein BLAST run on the query against UniProtKB were spike glycoproteins of avian infectious bronchitis virus. The minimum and maximum lengths of these sequences were 520 and 1162 amino acids, respectively (Table 1).

Table 1. Top BLAST hits (with E-value of 0.0) of IBV spike protein against UniProtKB.

Accession	Query Cover (%)	Percent Identity (%)	Sequence Length (aa)	Organism
P11223.1	100	100.00	1162	Avian infectious bronchitis virus (strain Beaudette)
P12651.1	100	96.04	1162	Avian infectious bronchitis virus (strain M41)
P12650.1	98	93.90	1162	Avian infectious bronchitis virus (strain KB8523)
P05135.1	98	86.25	1163	Avian infectious bronchitis virus (strain 6/82)
P12722.1	97	86.58	1154	Avian infectious bronchitis virus (strain D274)
P17662.2	45	78.54	550	Avian infectious bronchitis virus (strain D3896)
P30207.1	44	78.65	520	Avian infectious bronchitis virus (strain UK/142/86)
P30208.1	44	78.85	520	Avian infectious bronchitis virus (strain UK/167/84)
P30206.1	44	77.88	520	Avian infectious bronchitis virus (strain UK/123/82)

3.2. Primary characteristics

A standard secretory signal peptide with a length of 18 amino acids was detected within the IBV spike sequence. This signal peptide was predicted to be transported by the Sec translocon and cleaved between positions 18 and 19 by Signal Peptidase I (Sec/SPI) with a probability of > 97%. Full-length protein of the reference IBV spike sequence composed of 1162 amino acids with a computed molecular weight of 128046.70 Dalton and a theoretical isoelectric point (pI) of 7.71. The mature protein without signal peptide is composed of 1144 amino acids with a computed molecular weight of 126190.29 Da and *p*I of 7.76. The protein sequence was assigned as an immunogen by VaxiJen v2.0 with a probability of 0.4583.

3.3. The spike of IBV is a N-glycosylated protein

Based on the provided data about the glycosylation of IBV spike protein, 28 residues undergo glycosylation. Amongst 8 glycosylated residues validated by experimental studies. NetNGlyc predicted 27 asparagines within the sequence to undergo N-glycosylation (Table 2).

Table 2. Glycosylation Sites on IBV Spike Protein: Experimentally-Determined and Predicted

Residue Number	Sequence Context	Potential (NetNGlyc)	Jury Agreement (NetNGlyc)	Combined Evidence
51	NISS	0.7529	(9/9)	UniRule Annotation, NetNGlyc Prediction
77	NASS	0.6344	(7/9)	UniRule Annotation, NetNGlyc Prediction
103	NFSD	0.7078	(8/9)	UniRule Annotation, NetNGlyc Prediction
144	NLTV	0.7906	(9/9)	UniRule Annotation, NetNGlyc Prediction
163	NLTS	0.6421	(9/9)	UniRule Annotation, NetNGlyc Prediction
178	NETI	0.6101	(6/9)	UniRule Annotation, NetNGlyc Prediction
212	NGTA	0.7300	(9/9)	1 Publication, UniRule Annotation, NetNGlyc Prediction
237	NFSD	0.6116	(8/9)	1 Publication, UniRule Annotation, NetNGlyc Prediction
247	NSSL	0.6270	(8/9)	1 Publication, UniRule Annotation, NetNGlyc Prediction
264	NTTC	0.6341	(8/9)	UniRule Annotation, NetNGlyc Prediction
276	NETG	0.6528	(8/9)	1 Publication, UniRule Annotation, NetNGlyc Prediction
283	NPSG	0.5366	(8/9)	NetNGlyc Prediction
306	NFSF	0.5353	(4/9)	UniRule Annotation, NetNGlyc Prediction
425	NITL	0.6274	(8/9)	UniRule Annotation, NetNGlyc Prediction
447	NVTD	0.7393	(9/9)	UniRule Annotation, NetNGlyc Prediction
513	NETG	0.4644	(5/9)	1 Publication, UniRule Annotation, NetNGlyc Prediction
530	NGTR	0.7148	(9/9)	UniRule Annotation, NetNGlyc Prediction
579	NVTE	0.7121	(9/9)	UniRule Annotation, NetNGlyc Prediction
591	NLTV	0.5724	(8/9)	1 Publication, UniRule Annotation, NetNGlyc Prediction
669	NVST	0.5543	(5/9)	UniRule Annotation, NetNGlyc Prediction
676	NISL	0.6353	(8/9)	UniRule Annotation, NetNGlyc Prediction
683	NPSS	0.6803	(9/9)	NetNGlyc Prediction
714	NCTA	0.3846	(8/9)	NetNGlyc Prediction
947	NVTA	0.6688	(9/9)	UniRule Annotation, NetNGlyc Prediction
960	NASQ	0.5179	(6/9)	UniRule Annotation, NetNGlyc Prediction
979	NGSY	0.5789	(9/9)	UniRule Annotation, NetNGlyc Prediction
1014	NKTV	0.6479	(8/9)	NetNGlyc Prediction
1038	NDTK	0.6340	(8/9)	NetNGlyc Prediction
1051	NYTV	0.5902	(7/9)	1 Publication, UniRule Annotation, NetNGlyc Prediction
1074	NDSL	0.4930	(5/9)	1 Publication, UniRule Annotation, NetNGlyc Prediction

3.4. The protein sequence is not rich in linear B-cell epitopes

Overall, 56 linear epitopes were introduced by BepiPred 1.0. The length of these epitopes was variable from 1 to 26 amino acids. Predicted linear epitopes with a length of > 4 aa were

considered as positive results. Amongst 21 epitopes were longer than a 4-aa peptide. P282 in 277-ETGANPNPSGVQNIQTYQTKTAQSGY-302 epitope flagged as the maximum scored residue (Table 3).

Table 3: Predicted peptides by BepiPred 1.0:

No.	Start	End	Peptide	Length	Glycosylation
1	33	42	AFRPPSGWHL	10	-
2	55	64	EFNNAGSSSG	10	-
3	83	92	MTAPSSGMAW	10	-
4	178	183	NETIDV	6	-
5	235	240	TGNFSD	6	237
6	277	302	ETGANPNPSGVQNIQTYQTKTAQSGY	26	283
7	352	359	GPLQGGCK	8	-
8	374	380	SYGGPSL	7	-
9	403	421	KSGGSRIQTATEPPVITQN	19	-
10	488	494	VNPCEDV	7	-
11	512	518	RNETGSQ	7	513
12	559	563	DGSIA	5	-
13	642	646	VGQKE	5	-
14	656	665	STKPAGFNTP	10	-
15	684	688	PSSRR	5	-
16	704	717	VGLPTNDAYKNCTA	14	714
17	926	931	PQNAPN	6	
18	959	964	ANASQY	6	960
19	1026	1030	DFDFN	5	
20	1034	1045	SKWWNDTKHELP	12	1038
21	1138	1146	GKKSSYYTT	9	-

BepiPred 2.0 predicted 34 linear B-cell epitopes, among which 6 peptides were < 4 aa. The shortest epitopes were 1-amino acid, and the longest was a 41-residue peptide. The maximum score (0.652) was rendered to A58 in the 53-SEFNNAGSSS-63 peptide (Table 4).

No epitope was flagged by BepiPred 3.0 when the default threshold (0.1512) was adjusted.

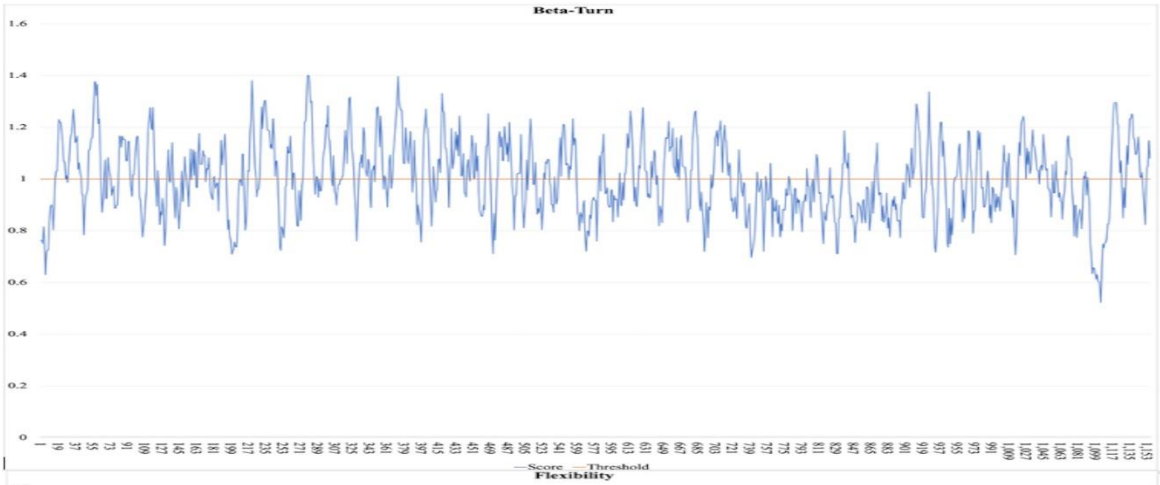
Table 4: Predicted peptides by BepiPred 2.0:

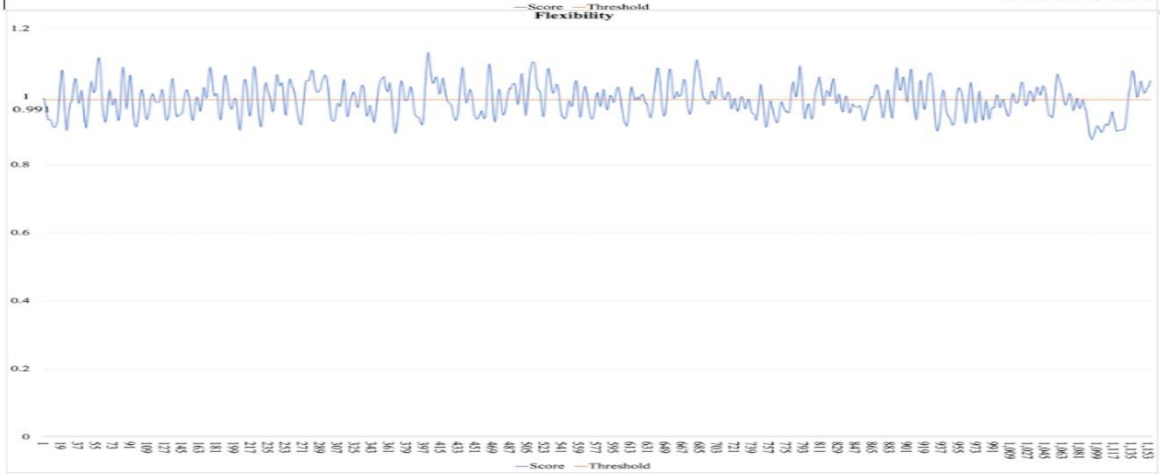
No.	Start	End	Peptide	Length	Glycosylation
1	33	42	AFRPPSGWHL	10	-
2	54	63	SEFNNAGSSS	10	-
3	86	95	PSSGMAWSSS	10	-
4	119	127	GCPLTGMLQ	9	-
5	149	153	VAKYP	5	-

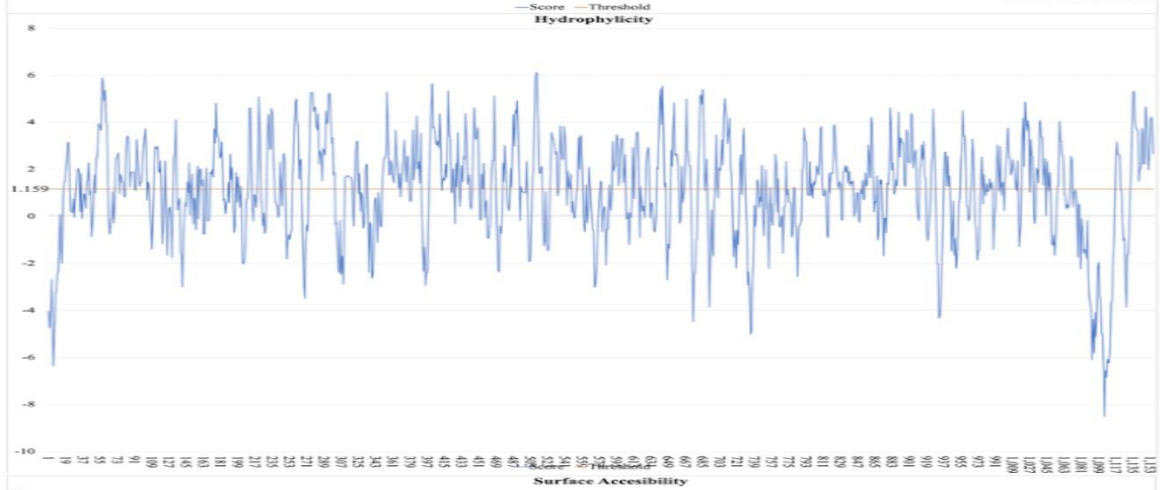
6	222	226	DGSPR	5	-
7	236	252	GNFSDGFYPFTNSSLVK	17	237, 247
8	278	287	TGANPNPSGV	10	283
9	315	341	YKESNFMYGSYHPSCKFRLETINNGLW	27	-
10	351	364	YGPLQGGCKQSVFK	14	-
11	375	392	YGGPSLCKGVYSGELDHN	18	-
12	408	427	RIQTATEPPVITQNNYNNIT	20	425
13	449	460	TDSAVSYNYLAD	12	-
14	515	523	TGSQLLENQ	9	-
15	532	549	TRRFRRSITENVANCPYV	18	-
16	565	578	IVPKQLEQFVAPLF	14	-
17	589	595	SFNLTVT	7	591
18	644	649	QKEDME	6	-
19	657	673	TKPAGFNTPVLSNVSTG	17	669
20	682	690	TNPSSRRKR	9	683
21	701	711	VESVGLPTNDA	11	-
22	858	865	DAIQANAQ	8	-
23	892	900	SQQRELATQ	9	-
24	906	917	VKSQSIRYSFCG	12	-
25	986	997	ARDMYMPRAITA	12	-
26	1013	1053	VNKTVITTFVDNDDFDFNDELSKWWNDTKHELPDFDKFNYT	41	1014, 1038, 1051
27	1081	1085	EKLSI	5	-
28	1129	1159	GIMPLMSKCGKKSSYYTTFDNDVVTEQYRPK	31	-

3.5. Propensity analyses

Beta turns were condensed at the N-terminus of the spike sequence. The maximum scored residues in this regard were P282 and N283 with a 1.401 score. The most flexible residue was G405 with a score of 1.131. E514 was the most hydrophilic residue with a score of 6.129. The most surface accessible residue was R1157 with a score of 9.192. The longest hydrophilic peptide was 1021-FVDNDDFDFNDELSKWWNDTKHELPDFDKFNY-1052 (Figure 1).


Beta-Turn
Score
Threshold


Flexibility
Score
Threshold


Hydrophylicity
Score
Threshold

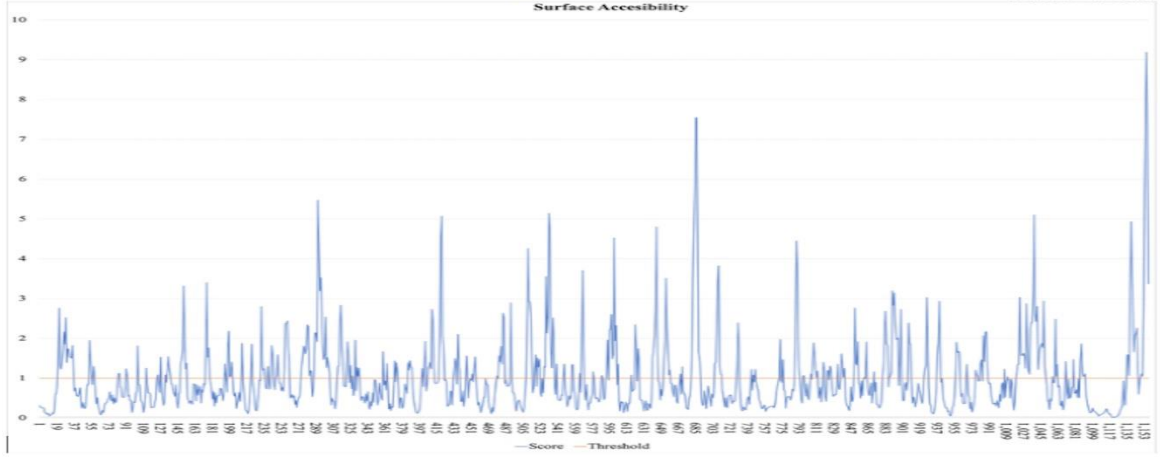

Surface Accesibility
Score
Threshold

Figure 1. Physicochemical properties analysis of the IBV spike protein. Four properties presented are in favor of B-cell epitope propensity. Graphs are named by the corresponding property. The X-axes represent the amino acid number while the Y-axes denote the score of the predicted property. (A) Beta-Turn, (B) Flexibility, (C) Hydrophilicity, and (D) Surface Accessibility scores are plotted against the amino acid residue position. The orange line represents the default threshold for each prediction method. Regions with scores above the threshold are considered likely to possess the corresponding property and are potential B-cell epitope candidates.

Based on the aforementioned criteria for B-cell epitope selection, 11 peptides derived from the IBV spike protein were chosen for further analysis. These selected epitopes represent regions predicted to exhibit favorable characteristics for B-cell recognition and antibody binding.

3.6. Conservancy and antigenicity of the sequence and selected epitopes

The BLAST results showed 100 hits (E-value: 0.0) with a length of 478-1181 amino acids. Query coverage and identity were 46%-100% and 62.86%-100%, respectively. The total scores were 912-2400. None of the selected epitopes was found in all of the 100 sequences (100 hits of BLAST). The shortest (VGQKEDME) and longest (GIMPLMSKCGKKSSYYTTFDNDVVTEQYRPK) selected epitopes shared ≥ 70% sequence identity with 62% of the spike IBV sequences. The minimum prevalence of epitope was assigned for SEFNNAGSSSG, which was found in 11% of the spike IBV sequences (with ≥ 70% identity). The most prevalent epitope was YGPLQGGCKQSVFK, matched with 89% of the sequences (Table 5).

Table 5: Conservancy and antigenicity of the selected B-cell Epitopes from IBV Spike Protein

Start	End	Sequence	Length	Percent matches (%)	Minimum identity	VaxiJen 2.0 antigenicity
33	42	AFRPPSGWHL	10	84	30.00%	**-0.1683**
54	64	SEFNNAGSSSG	11	11	27.27%	**0.3816**
83	95	MTAPSSGMAWSSS	13	32	30.77%	**0.0072**
351	364	YGPLQGGCKQSVFK	14	89	28.57%	**-0.2917**
374	392	SYGGPSLCKGVYSGELDHN	19	26	26.32%	**0.3564**
403	421	KSGGSRIQTATEPPVITQN	19	59	26.32%	**0.0822**
515	523	TGSQLLENQ	9	67	44.44%	**0.2337**
642	649	VGQKEDME	8	62	37.50%	**1.1888**
656	665	STKPAGFNTP	10	67	30.00%	**0.0801**

701	711	VESVGLPTNDA	11	36	27.27%	**0.3565**
1129	1159	GIMPLMSKCGKKSSYYTTFDNDVVTEQYRPK	31	62	16.13%	**0.4975**

3.7. Validated B-cell epitopes and obtained constructs

Among the selected linear B-cell epitopes predicted by BepiPred 1.0 or BepiPred 2.0, 2 peptides matched to hits validated in positive B-cell epitope assays. All these epitopes were predicted by BepiPred 2.0 (Table 6).

Table 6. Validated B-cell epitopes. B-cell epitopes matched to hits validated in positive B-cell epitope assays.

Predicted epitope	Position	Server/tool	Glycosylation	Matched hits	Antigen	Organism
VESVGLPTNDA	701-711	BepiPred 2.0		PRRRSFIEDLLFTSVESVGL	Spike glycoprotein (UniProt:P11223)	Avian coronavirus
GIMPLMSKCGKKSSYYTTFDNDVVTEQYRPK	1129-1159	BepiPred 2.0	-	EKDFLSFDNDVF	Replicase polyprotein 1ab (UniProt:K9N7C7)	Middle East respiratory syndrome-related coronavirus (MERS coronavirus)
				KDFLSFDNDVFI	Replicase polyprotein 1ab (UniProt:K9N7C7)	Middle East respiratory syndrome-related coronavirus (MERS coronavirus)
				LSFDNDVFIKKY	Replicase polyprotein 1ab (UniProt:K9N7C7)	Middle East respiratory syndrome-related coronavirus (MERS coronavirus)

The peptides involved in vaccine construct design were as follows:

AFRPPSGWHL, YGPLQGGCKQSVFK, PRRRSFIEDLLFTSVESVGLPTNDA, and GIMPLMSKCGKKSSYYTTFDNDVVTEQYRPK (Table 7).

Table 7. The selected epitopes and properties.

Sequence	Position	BepiPred 1.0 score	BepiPred 2.0 score	VaxiJen 2.0 antigenicity	Percent matches (%)	Minimum identity
AFRPPSGWHL	33-42	0.94	0.55	**-0.1683**	84	30.00%
YGPLQGGCKQSVFK	351-364	0.61	0.54	**-0.2917**	89	28.57%
PRRRSFIEDLLFTSVESVGLPTNDA	Validated B-cell epitopes	-	-	-	-	-
GIMPLMSKCGKKSSYYTTFDNDVVTEQYRPK	1129-1159	-	0.55	**0.4975**	62	16.13%

The primary construct consisted of the tandem fusion of these peptides (Table 8). This construct was an 80-residue peptide with a molecular weight of 9033.33 Da and a theoretical *p*I of 9.30.

VaxiJen 2.0 predicted the peptide as a NON-IMMUNOGEN with a score of 0.3290 as a viral antigen. The second construct was obtained by duplicating the primary construct and replacing additional C residues (Table 8). This construct was a 160-residue protein with a molecular weight of 18016.52 Da and a theoretical *p*I of 9.57. VaxiJen 2.0 predicted the peptide as a NON-IMMUNOGEN with a score of 0.3463 as a viral antigen. The third construct in which an epitope served as a linker (Table 8) was a 216-residue protein with a molecular weight of 24435.53 Da and a theoretical *p*I of 5.17. VaxiJen 2.0 predicted the peptide as an IMMUNOGEN with a score of 0.6029 as a viral antigen. The fourth construct, in which a dendritic cell binding peptide was fused to the C-terminus of the previous construct (Table 8) was a 228-residue protein with a molecular weight of 25851.06 Da and a theoretical *p*I of 5.39. VaxiJen 2.0 predicted the protein as an IMMUNOGEN with a score of 0.5940 as a viral antigen. The next construct sequence in which the CTB sequence was fused to the N-terminus via a flexible linker (GGSG) (Table 8) was a 356-residue protein with a molecular weight of 40048.46 Da and a theoretical *p*I of 6.28. VaxiJen 2.0 predicted the protein as an IMMUNOGEN with a score of 0.5712 as a viral antigen. The last construct obtained via replacement of additional C residues (Table 8). VaxiJen 2.0 predicted the protein as an IMMUNOGEN with a score of 0.5882 as a viral antigen.

4. Discussion

This study employed an *in silico* approach to assign potential B-cell epitopes within the IBV spike (S) protein that could serve as targets for a broadly protective vaccine. Our analysis revealed significant sequence variability in the S protein across different IBV strains, consistent with previous findings that highlight the high mutation rate of the S1 gene and its impact on genotype, antigenicity, and pathogenicity (26, 27). This variability underscores the challenges associated with vaccine design strategies that often fail to provide cross-protection against multiple IBV serotypes and genotypes (26, 28). The reviewed sequences found by BLAST search indicated that the spike sequence length is variable in various strains. Hence, some epitopes could not be found in shorter sequences at all. In order to overcome this shortage, we employed a strategy covering a broad spectrum of strains. A large sequence was considered to avoid missing any potential epitope.

The predicted N-glycosylation sites on the IBV spike protein may also play a significant role in vaccine design. Glycosylation influences protein folding, stability, infectivity, antigenicity, and receptor binding (29). These modifications are essential for proper protein folding and maturation, and they can also impact the accessibility of epitopes to neutralizing antibodies. Glycans can enhance immune responses by targeting immune cell receptors or serve as an immune evasion strategy through the formation of a "glycan shield" that masks viral proteins from immune recognition (30-33). Therefore, preserving native glycosylation patterns in vaccine candidates may be critical for eliciting effective antibody responses (34). This criterion is important when DNA or mRNA platforms of a vaccine are employed. The current study aims to select epitopes to design a construct appropriate for expression in *E. coli* hosts. Since spike is a glycoprotein, its expression in prokaryotic hosts could alter the glycosylation pattern. Glycosylated epitopes could escape from antibodies raised to the same epitope without glycosylation. So, those B-cell epitopes with no N-glycosylation post-translational modification are of interest to be selected. These epitopes could be retained in a context rather than the native protein (native glycosylation pattern). Theoretically, epitopes with no glycosylation could be recognized by specific antibodies in native and other contexts. In the current study, those epitopes appropriate for expression in the *E. coli* host, which has a different pattern of glycosylation, are considered. Hence, those linear B-cell epitopes to be glycosylated were excluded. To avoid any false negatives, experimentally determined and predicted glycosylation sites on IBV spike protein were considered.

Accuracy of linear B-cell epitope prediction could be relied on the employed tools and approach. BepiPred 1.0 and BepiPred 2.0 are robust tools to predict B-cell epitopes. In addition, a combination of BepiPred results with properties involved in B-cell epitope predictions (flexibility, hydrophilicity, and surface accessibility) could enhance the accuracy of B-cell epitope prediction. This approach has been used in recent studies to select B-cell epitopes of interest and antigen designs (23, 24). In a study, average BepiPred 1.0 results, along with average scores of flexibility, hydrophilicity, and surface accessibility, served to evaluate designed constructs in regard to B-cell epitope density (35). In this study, an external loop (loop 3) was nominated as the best-exposed peptide. This peptide was predicted as a linear epitope by BepiPred 1.0 and some other B-cell epitope predictors. Subsequent experimental studies validated this peptide as a protective region, Omp34, triggering specific antibodies against *Acinetobacter baumannii* (20, 36-38). In another

study, BepiPred 1.0 was among the most successful tools in recognizing top-ranking experimental epitopes. Some neutralizing epitopes were partially predicted only by this linear B-cell epitope predictor (25).

Although the maximum scored residues concerning to the beta turn (P282 and N283) were within B-cell epitopes predicted by BepiPred 1.0 and BepiPred 2.0 (277-ETGANPNPSGVQNIQTYQTKTAQSGY-302 and 278-TGANPNPSGV-287, respectively), N283 is predicted to be glycosylated. Hence, these peptides were excluded from the B-cell epitope selection. The most surface accessible residue was R1157 with a score of 9.192. The longest hydrophilic peptide (1021-FVDNDDFDFNDELSKWWNDTKHELPDFDKFNY-1052) encompasses amino acids anticipated to undergo glycosylation. The most hydrophilic residue (E514) was flanked by an epitope predicted by BepiPred 2.0. However, this epitope was not appropriate concerning antigenicity and conservancy. A predicted B-cell epitope (KSGGSRIQTATEPPVITQN) encompassed the most flexible residue (G405). However, this epitope was not appropriate concerning antigenicity and conservancy.

The identification of conserved B-cell epitopes is crucial for developing vaccines that can elicit broadly neutralizing antibody responses against diverse IBV strains. The high prevalence of the YGPLQGGCKQSVFK epitope (89% of the sequences) suggests that this region may be a promising target for vaccine design. However, the fact that none of the selected epitopes were 100% conserved underscores the need for a multi-epitope vaccine approach that can target multiple regions of the S protein to overcome viral escape mechanisms (8). Successful multi-epitope vaccines against human metapneumovirus (hMPV) and IBV have demonstrated that this strategy could induce strong immunity and provide up to 80% protection in challenged chickens (8, 10, 11).

To enhance the validity of epitope selections and immunogen probability, two measures were considered. First, the involvement of an epitope with the highest antigen probability (estimated by VaxiJen). Second, involvement of predicted epitopes harboring identical 5- or 6-meric peptides with experimentally validated B-cell epitopes, one of which was a similar experimental epitope of IBV. This approach had been used in previous studies (25, 35). The largest number of B-cell epitopes are distributed in 5-10 amino acids length-wise (39). Hence, 5- or 6-meric identical

peptides within validated B-cell epitopes are sufficient to elevate the validity of predicted B-cell epitopes.

For the design of the primary construct, validated epitopes and the most conserved epitopes were considered and fused tandemly with no artificial linker. Linker repetitions could abrogate the immune response to linker-specific antibodies (23). A multi-epitope vaccine against *Staphylococcus aureus* based on B-cell epitopes in which seven repeats of the GPGPG served as a linker, elicited antibodies that failed to recognize the original target antigens and develop protection (40). In contrast, it has been demonstrated that a construct consisting of tandemly fusion of a B-cell epitope-containing peptide (5X repeats) of Omp34 could elicit antibodies recognizing the original antigen in *A. baumannii* (20, 38). The designed antigens with a molecular weight of 10 kDa could ensure their immunogenicity with regard to size. Hence, to accommodate this criterion, the second antigen consisted of a double primary construct was designed. This antigen is appropriate in terms of size; however, its antigen probability was still not acceptable. Since more than two cysteine residues could potentially result in two or more disulfide bonds, additional cysteines were replaced by S amino acids. Serine and alanine are the most conservative substitutions for cysteine (41). In a study, substituting cysteine with serine resulted in enhanced immunogenicity of two cysteine-containing peptides of the influenza virus (41). In another study, replacement of either Cys21 or Cys124 of hepatitis B surface antigen (HBsAg) with serine resulted in diminished binding to antibodies. However, simultaneously substituting both cysteine residues restored the wild-type protein behavior (42). Cysteine to serine substitution improved the production of a recombinant antigen for human Chagas disease without losing its antigenicity (43). In a recent study, cysteine to serine substitutions in IL-18 reduced its aggregation and enhanced its IFN-γ-inducing activity (44). Based on these previous studies, the cysteine to serine substitution in the repeat of the cysteine-containing epitope of construct 2 could enhance production of the recombinant antigen, reduce its aggregation in overexpression conditions, and even enhance its immunogenicity as predicted by VaxiJen. If the substitutions resulted in diminished binding to antibodies, the first repeat of the epitope could compensate for this adverse effect.

In construct v.3, a non-glycosylated predicted epitope with the highest antigen probability served as a linker to fuse other epitopes. This strategy enhanced the antigenicity of the designed construct such that nominated as an immunogen by VaxiJen. In addition, the use of 7 repeats of a predicted

epitope instead of artificial linkers (such as GPGPG, that employed in the previous study (40)) could enhance epitope density. High epitope density is a successful strategy in vaccine design employed in various previous studies (20, 38, 45-48). High epitope density could break IgG-mediated immune suppression against a specific epitope (45). High epitope density could also bring about protectivity to a given epitope (48). This strategy enhances antigenicity, immunogenicity, as well as mucosal and systemic immune responses (46, 47). In the 4th version of the designed construct, a chicken dendritic cell binding peptide was fused to the C-terminus of the antigen sequence. The peptide, which was fused to the protective antigen VP2, resulted in significantly higher *in vitro* expression of DC markers as well as cytokines IFN-γ, IL-12, TNF-α, IL-1β, IL-6, and CXCLi1 than the VP2-control peptide. To orally deliver the antigen, *Lactobacillus saerimneri* M11 was used as a chicken-borne bacterium. This designed vaccine elicited mucosal and humoral immune responses with higher protective efficacy than the VP2 group (49). The 5th version of the designed antigen harbored the B subunit of cholera toxin (CTB) added to the N-terminus of the construct with a flexible linker (GGSG). In a previous study, a short peptide of the Ata antigen of *A. baumannii* fused to the C-terminus of CTB with a GGSG linker showed protection against lethal doses of *A. baumannii* challenges (50). Two cysteines existed within the CTB sequence. Hence, in the 6th version of the designed construct, the two cysteine residues of the 5th version of the construct were replaced by S. This modification could result in the production of specific antibodies unable to recognize the original epitope, including cysteine residues.

As a limitation of the current study, pure in silico analyses should be validated by further experimental investigations. So, it could be suggested to experimentally evaluate the 4th and 6th versions of the designed construct to select the best candidate concerning protective immune responses. In addition, to the best of our knowledge, no specialized tool was available to predict epitopes in chicken and analyze the chicken immune responses against the designed constructs. Hence, further research is needed to translate these findings into effective vaccines against IBV outbreaks and reduce economic losses in the poultry industry.

Conclusion

This study aimed to identify conserved, antigenic, and validated B-cell epitopes within the IBV spike protein using a combined *in silico* approach. Although our analysis revealed significant sequence variability in the IBV S protein, several conserved B-cell epitopes with favorable physicochemical properties, and N-glycosylation patterns were predicted. Non-glycosylated epitopes were involved in designing a construct appropriate to be recombinantly expressed in a bacterial host such as *E. coli*. Achieving this milestone could decrease the cost and labor of vaccine production. These findings suggest that a multi-epitope vaccine approach targeting these conserved regions may be a promising strategy for eliciting broadly neutralizing antibody responses against diverse IBV strains. Six versions of multi-epitope vaccine candidates were designed and analyzed. However, further research is needed to experimentally validate the immunogenicity of these constructs and assess their ability to elicit protective immunity in animal models. This research contributes to develop novel and broadly protective IBV vaccines, offering hope for a more sustainable and resilient poultry industry.

Acknowledgment

The authors wish to thank Razi Vaccine and Serum Research Institute for supporting the conduct of this research.

Ethics
Not Applicable
Conflict of interests:
The authors have no relevant financial or non-financial interests to disclose.

Availability of data and material

The authors confirm that the data supporting the findings of this study are available within the article and supplementary materials.

Funding:
The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.
Author contributions:

Conceptualization: S. A., S. O., S. A., N. M., B. K.; **Methodology:** S. A., S. O., S. A., N. M., B. K.; **Formal analysis:** S. A., S. O., S. A., N. M., B. K.; **Investigation:** S. A., S. O., S. A., N. M., B. K.; **Supervision**: B. K.; **Writing - Original Draft:** S. A., S. O., S. A., N. M., B. K.; **Writing - Review & Editing**, S. A., S. O., S. A., N. M., B. K.

Declaration of AI Use

The authors confirm that no generative AI tools were used in the design, execution, or interpretation of this study. All experimental data, statistical analyses, and manuscript preparation were conducted through traditional human-led methods. AI-based software was employed only for routine tasks such as language editing and all outputs were critically reviewed and validated by the authors for accuracy and clarity.

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