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Original Article

Epitope Identification in BEFV Gene for Detecting Effective Points

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Abstract

Background & Objective: The common cause of milk production loss in cattle and water buffalo is bovine ephemeral fever (BEF). Previous cases have been reported in Iran's south regions, with a low mortality rate. As a result, studying BEFV and identifying ideal epitopes for further developing diagnostic Kit is important.
Materials & Methods: To investigate BEFV N protein epitopes, we collected samples, extracted and sequenced DNA, and then used the ExPaSy translate method to deduce the amino acid sequence. Various immunoinformatics techniques were used to analyze physical/chemical properties, secondary structure of protein sequences, membrane topology, antigenic property, and 3D structure. BCPRED and the DiscoTope server, respectively, predicted linear and discontinuous epitopes of BEFV N protein. Finally, the PatchDock server was used to dock peptides and antibodies.
Result: Three linear epitopes and sixteen discontinuous epitopic positions were discovered. Furthermore, molecular docking between epitopes and low-binding-energy antibodies revealed that they have easy access to the immune system.
Conclusion: In this study, bioinformatics techniques were used to predict epitopes of the BEFV N protein for further developing BEFV diagnostic Kits and recombinant vaccines. Furthermore, experimental validation is needed for these epitopes.

Keywords: BEFV, N Protein, Epitopes

Introduction

BEFV is a single-stranded negative-sense RNA arbovirus that belongs to the genus Ephemerovirus in the Rhabdoviridae family (1).

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BEFV has been identified as the causative agent of bovine ephemeral fever. BEF is characterized by a sudden onset of fever, ruminal stasis, anorexia, and lameness. It is also known as 3-day sickness because the clinical manifestation duration is just a few days long. This viral pathogen causes significant economic loss by reducing milk production and causing temporary infertility in bulls (2).

Although the BEF mortality rate is low (1-2%),

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it can reach as high as 80% in extreme outbreaks (3). BEF outbreaks have been recorded in several Asian and Middle Eastern regions in recent years. In 2012, researchers investigated the epidemiology of BEFV in 600 cattle and water buffalo. They came to the conclusion that the average prevalence is 25% (3). As a result, vaccination against BEF is critical in order to significantly minimize the economic losses incurred by BEF.

Eleven functional open reading frames (ORFs) including five structural proteins contained as 3′-N-P-M-G-L-5′, as well as six accessory proteins: GNS, α1, α2, α3, β, γ have been identified in BEFV genome (4). The N gene encodes a 431-amino-acid polypeptide that spans 1328 nucleotides from the transcription initiation consensus sequence (AACAGG) to the conserved transcription termination-poly (A) sequence CATG (A). BEFV N protein as a 52 kDa protein plays a critical role in genome encapsidation. Resulting encapsidated genomic RNA serves as a template for viral replication and transcription. Furthermore, it was discovered that during BEFV genome replication, the N protein enhances BEFV genome resistance to nuclease degradation by binding to nascent negative-sense genome and positive-sense anti-genome RNA (5).

Only recently, the awareness of BEFV prophylaxis gained attention. However, a few BEFV vaccines are now available commercially. In this regard, a recombinant rabies virus (RABV) expressing BEFV glycoprotein (LBNSE-BG) has been generated by W Zheng (6). Moreover, BEFV N protein has immunogenic properties in mice and cattle. In addition, its role in T cell proliferative response induction in cattle has also been proved. All 12 available BEFV N protein MAbs have been mapped to non-conformational sites in the C-terminal half of the protein (7).

Epitopes are defined as regions or segments of an antigen that are recognized by a specific antibody or cellular immunological system. Epitopes can be grouped as follows: continuous and discontinuous. A continuous/linear epitope is a segment of consecutive residues in the primary sequence, while a discontinuous/conformational epitope is a bunch (group) of residues of an antigen that are far apart from each other in the primary sequence but are brought to spatial proximity as a result of polypeptide folding. Conformational epitopes have been found to be the most common B-cell epitope (8). Linear peptide, obtained from the sequence of a given antigen, are able to evoke antibodies that target the peptide as well as the native antigen. (9). The discovery of epitopes on the N protein of BEFV has never been recorded before.

In this study we aim to identify epitopes via immunoinformatics tools. This is the first report of epitope in the N protein of BEFV. However, in *vitro* and in *vivo* experimental studies are required for suggested epitopes verification.

Materials & Methods

Sequence and secondary structure analysis

ExPaSy translate tool (http://expasy.org/tools/dna.html) has been employed to deduce amino acid sequences from DNA sequences of the PCR product (Figure 1) (10). Antigenic characterization of the BEFV N protein was done using VaxiJen v2. 0 online (http://www.ddgpharmfac.net/vaxijen/VaxiJen/VaxiJen.html) as an antigen prediction server (11). It generates the antigenicity of the proteins without involving any alignment and focuses on the physiochemical properties of the selected candidate (12).

Fasta format

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> VIRT-45123:5'3' Frame 1, start_pos=0
MYCTLNKKEIKAVKPTDAIPPQYPKEFFINGNGKKPTLRVPQGKLDLPTV
RELVFGGLERGELVLSHVIRYLYLVGERITEKLEGDWISFGVNIGRRNQE
INVWNFYEVIIEDDQTIDGRRANNVDENDDVWLTLALLAYYRLGRSANQN
HRNNLLIKLNAQIKGYRKDAPNIIDDVAVHGSWVTNSEFCKIAAGFDMFM
NRFKNNKYAHVRFGTVASRYKDAAGLMALGHACDVTGLTIEEILDWIFVS
NVGEDVVKIMEEGNEIDEPYSYMPYMMDMGISNKSPYSSISCPNIYTFLH
LVGTLLTSERSKHARMVSEHNLQNIKMNAFVVSYVKSNKAALTKAFLKSE
DRDYEKRQEEGSDDDEDEDESENDDDFGAMPKSSDPMEWFIFLESNHFIL
PEKVTEFCIRECKKIQNARPNTIGKYLASIV-
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Figure 1. The amino acid sequence of N gene

Physicochemical analysis including molecular weight, negatively charged residues, positively charged residues, theoretical pI, aliphatic index, grand average of hydropathicity (GRAVY), and instability index of BEFV N protein were predicted by ProtParam (https://web.expasy.org/protparam/)(13, 14).

Secondary structure was generated through PDBsum web server (http://www.ebi.ac.uk) as well as SOPMA (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html)(15,16).Additionally, ANOLEA (http://melolab.org/anolea/) and PROCHEK (https://www.ebi.ac.uk/thornton-srv/software/PROCHECK/) were employed for calculating energy at the atomic level in protein structure and Ramachandran plot generation, respectively (17,18). In addition the DiANNA web server (http://clavius.bc.edu/~clotelab/DiANNA/) has been used to predict Disulfide bonds position (19). Furthermore, we use TMHMM (http://www.cbs.dtu.dk/services/TMHMM/) as well as MEMEMBED (http://bioinf.cs.ucl.ac.uk/psipred) with transmembrane topology prediction capability to determine the transmembrane topology of the BEFV N protein (20).

3D structure prediction

Raptor X as an online integrated web server (http://raptorx.uchicago.edu/) was applied for homology modeling of the BEFV N protein in order to predict the conformational of B-cell epitopes in 3D structures (21). Raptor X performed tertiary structure modeling without using any template information and using a powerful in-house deep learning model DeepCNF (Deep Convolutional Neural Fields) Furthermore, MolProbity was used to determine the Z-score as an indicator of overall model quality (22).

Visualization of the generated model was performed using pymol. In addition, 3-dimensional structures of selected continuous B-cell epitopes that are essential for Epitope-antibody docking were generated through PEP-FOLD3 online software (https://mobyle.rpbs.univ-paris-diderot.fr/cgi-bin/portal.py#forms::PEP-FOLD3)(23).

Epitope prediction

In order to ascertain the BEFV N protein B cell linear epitope, BCPRED server (http://ailab-projects1.ist.psu.edu:8080/bcpred/) with 75% specificity and IEDB web server (http://www.iedb.org/) were used (24, 25). Vaxijen online server, ToxinPred (https://webs.iiitd.edu.in/raghava/toxinpred/index.html), and Allergen FP 1.0 (http://ddg-pharmfac.net/AllergenFP/) have been employed to check Antigenicity, toxicity, and allergenicity of selected epitopes, respectively (26, 27). Furthermore, DiscoTope server (http://www.cbs.dtu.dk/services/DiscoTope/) with discotope score threshold of −3.7 has been applied to predict epitopes from the BEFV N protein three-dimensional structures (28).

Epitope-antibody docking analysis

PatchDock server (https://bioinfo3d.cs.tau.ac.il/PatchDock/php.php) has been implemented in present study (29). Furthermore, 6E8E has been utilized as a target receptor for selected epitopes.

Result

The ProtParam results showed that the complete amino acid sequence of the BEFV N protein comprises 431 amino acids and has a molecular weight of 49.311 kDa. The number of negatively charged residues (Asp+Glu) and positively charged residues (Arg + Lys) were 65 and 52, respectively. The formula of the protein that denotes Carbon (C), Hydrogen (H), Nitrogen (N), Oxygen (O), and Sulphur (S) is $C_{2195}H_{3412}N_{592}O_{663}S_{19}$. There are total numbers of 19710 atoms in the protein while the extinction coefficient of the protein is 148960. The theoretical PI and aliphatic index of the BEFV N protein were 5.32 and 83.23, respectively. The GRAVY and instability index were -0.436 and 44.9, respectively. The estimated half-life of this protein is 30 hrs in mammalian raticulocytes, >20 hrs in yeast and > 10 hrs in Escherichia coli. The BEFV N protein physico-chemical properties are given in Table 1.

Percentages of the secondary structures by "SOPMA" as well as (and) PSIPRED are given in Table 2. The results revealed that the proportion of random coils, β strand, and α helices accounted for 45, 6, and 49% of the secondary structure, respectively (Figure 2). In addition, three disulfide positions including 3 – 233, 190 – 292, and 408 – 412 have been predicted via "DiANNA" web server (30). The BEFV N protein antigenic property with a score of 0.5229 was identified by the VaxiJen 2.0 server.

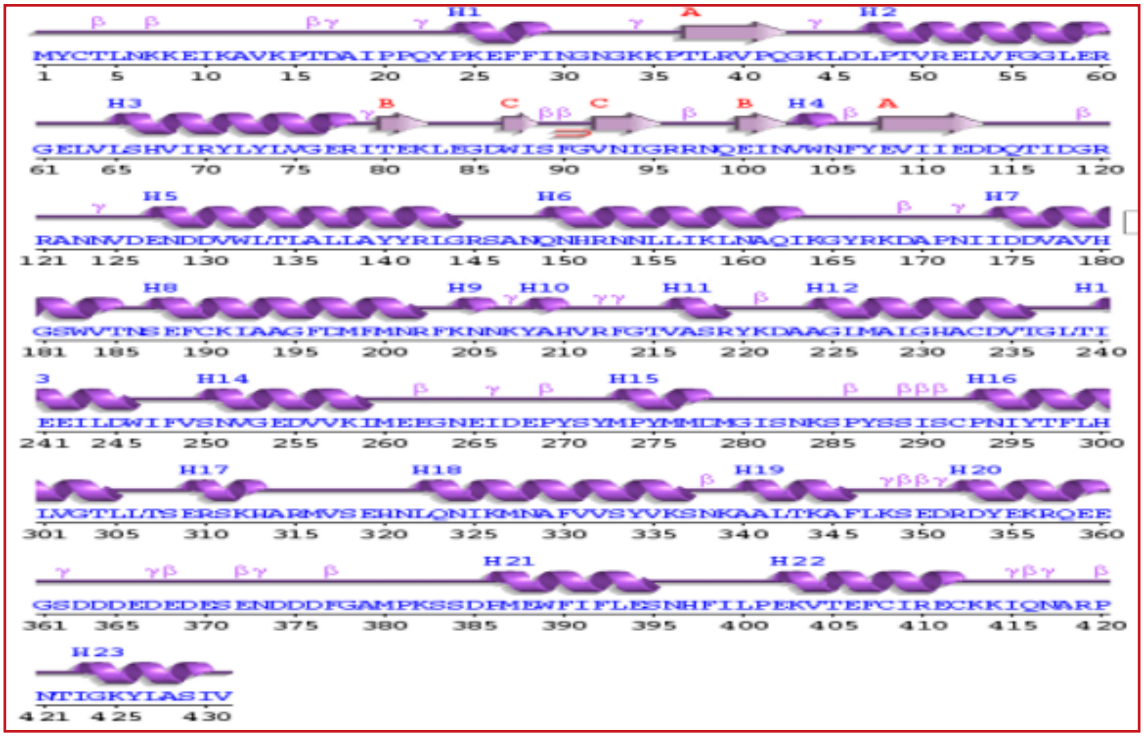

Figure 2. Secondary structure of the BEFV N protein, Helices are labeled as H1, H2; Beta turn as β; Gamma turn as χ and Beta hairpin

The results of TMHMM and MEMEMBED revealed that BEFV N protein is located (found) in the extracellular space. According to the ANOLEA analysis, 11.37 amino acids have a high energy level.

We also generated a 3D structure of the BEFV N protein using the RaptorX online server. The Ramachandran plot of tertiary structure showed that 87.68 percent of BEFV N protein amino acid residues were in the most preferred regions, 11.6 percent in the additional permitted region, 0.3 percent in the generously allowed regions, and 0.5 percent in the disallowed region.

Furthermore, in order to evaluate the quality of the model, MolProbity was used to compute Z - Score. Z - Score of predicted structure was −0.25 that represents a higher quality of the model. The results of the predicted 3D structure of BEFV N protein are shown.

A total of 16 discontinuous B-cell epitopes were predicted using a DiscoTope server.

The longest epitope is 22 amino acids long and starts and ends at 350 and 371 amino acids, respectively (Table 3).

Moreover, BCPRED server results revealed a start position of 12 meric B-cell epitopes of 12, 94, 117, 146, 203, 231, 279, 354, 408, 258, 166, 44, 182, 382, and 369 for BEFV N protein (Table 4). However, toxicity, and allergenicity analysis of predicted epitopes confirmed that the eleven epitopes are of non-allergen, and non-toxic. The 12mer epitopes availing antigenic scores beyond the threshold value 1.0 secures antigenic characteristics. In that consequence (as a result) 8 out of 11 12meric epitopes were excluded. Three 12mers

AVKPTDAIPPQY, FKNNKYAHVRFG, and MGISNKSPYSSI having antigenic scores of 1.1489, 1.3076 and 1.0888 respectively proved to secure antigenic propensity. The selected epitopes AVKPTDAIPPQY, FKNNKYAHVRFG and MGISNKSPYSSI are submitted to PEP-FOLD3 online software. Pymol has been implemented to visualize the selected PDB files of the targeted epitopes. Linear and discontinuous B-cell epitopes are shown in 3D structure using the pymol.

PDB files of AVKPTDAIPPQY, FKNNKYAHVRFG, and MGISNKSPYSSI epitopes as well as 6E8E are submitted to PatchDock server. Epitopes along with their score, area of interface and ACE value are listed in Table 5. Additionally, Pymol has been applied to visualize PDB files of docking complex.

Table 1. Physico-chemical properties of the selected sequences

Sequence	Length	Molecular weight	Extinction coefficient	Theoretical PI	Aliphatic index	GRAVY	Instability index	Instability classifies
BEFV N Protein	431	49.311 kDa	148960	5.32	83.23	-0.436	44.9	unstable

Table 2. Percentages of the secondary structure for BEFV N protein

Sequence	Alpha helix	Beta strand	Coil
BEFV N protein	49%	6%	45%

Table 3. Discontinuous epitopes predicted from the 3D structure of the BEFV N protein using DiscoTop

Region	Epitope	Position
1	KPTDAIPPQ	14-22
2	NGN	30-32
3	K	82
4	E	84
5	RNQE	97-100
6	ANQNH	147-151
7	G	165

8	RKDA	167-170
9	KN	204-205
10	K	207
11	E	268
12	ISN	281-283
13	S	318
14	H	320
15	EDRDYEKRQEEGSDDDEDEDES	350-371
16	DDDF	374-377

Table 4. Continuous predicted epitope from BCPRED server

Start position	End position	Epitope	Score	Antigenicity score	Allergenicity	Toxicity
117	128	IDGRRANNVDEN	0.997	1.0508	allergen	Non-toxic
369	380	DESENDDDFGAM	0.981	0.9106	Non-allergen	Non-toxic
408	419	CIRECKKIQNAR	0.979	0.6013	allergen	toxin
354	365	YEKRQEEGSDDD	0.928	0.8565	allergen	Non-toxic
258	269	KIMEEGNEIDEP	0.901	-0.0511	Non-allergen	Non-toxic
166	177	YRKDAPNIIDDV	0.868	0.2118	allergen	Non-toxic
44	55	KLDLPTVRELVF	0.765	0.2138	Non-allergen	Non-toxic
146	157	SANQNHRNNLLI	0.755	0.7761	Non-allergen	Non-toxic
231	242	HACDVTGLTIEE	0.738	0.8906	Non-allergen	Non-toxic
12	23	AVKPTDAIPPQY	0.699	1.1489	Non-allergen	Non-toxic
203	214	FKNNKYAHVRFG	0.698	1.3076	Non-allergen	Non-toxic
94	105	IGRRNQEINVWN	0.533	0.8916	Non-allergen	Non-toxic
182	193	SWVTNSEFCKIA	0.447	0.4140	Non-allergen	Non-toxic
382	393	KSSDPMEWFIFL	0.446	0.5195	Non-allergen	Non-toxic
279	290	MGISNKSPYSSI	0.364	1.0888	Non-allergen	Non-toxic

Table 5. Representation of geometric shape complimentary score, area of interaction and ACE value of the epitope receptor docking complexes. The significantly low ACE value of docking complexes indicated elevated reactivity between epitope and receptor

Epitope	Target receptor	Score	Area	ACE value
AVKPTDAIPPQY	6E8E*	8340	1125.10	-370.21
FKNNKYAHVRFG	6E8E	8250	1140.30	-280.87
MGISNKSPYSSI	6E8E	8412	1190.20	-336.82

* Bovine Antibody.

Discussion

BEF as a vector-borne viral disease in cattle and water buffalo have been documented across temperate climatic zones of Asia, Africa, and Middle-East. This inflammatory disorder is characterized by unexpected onset of high fever, stiffness, nasal discharge, dyspnea, and significant reduction in milk production. BEFV with single-stranded RNA genome is the causative agent of BEF. This negative-sense strand encodes several non-structural proteins including a nucleoprotein (N), a surface glycoprotein (G), a polymerase-associated protein (P), a large RNA-dependent RNA polymerase (L), and a matrix protein (M) that play a crucial role in protective neutralizing antibody induction. Therefore, these antigenic proteins are suitable for epitope mapping and induce immunization against BEF (31, 32).

In this experimental study basic sequence properties were firstly analyzed in order to obtain better understanding of BEFV N protein structure and function. We, via bioinformatics analyses, disclosed that BEFV N protein is a hydrophilic (GRAVY) and unstable (instability index) protein, without transmembrane helices, and the protein sequences localized outside the membrane. Moreover, disulfide bonds as an integral factor in determining the functional linkages and stability of proteins were analyzed using "DiANNA" web server. Six cysteine amino acids making three disulfide bonds in BEFV N protein contributed to protein stability. Amino acid 190, 233, 408, and 412 were located on helix structure, 292 on coil structure, and 7 was on strand structure.

Conclusion

Finally, in the present study, epitopes prediction of the BEFV N protein was conducted through numerous online tools. Only 3 linear B-cell epitopes that fulfil all criteria including non-allergen, antigenic property, and non-toxicity have been found. Additionally, through IEDB web server, it was approved that these regions have the best potential in terms of surface accessibility, hydrophilicity, and flexibility. Moreover, epitope-antibody docking of selected epitopes confirms that the epitopes will induce immune system and specific immunogenicity generation. Furthermore, via 3D structure of the BEFV N protein, sixteen discontinuous epitopic locations have also been identified. We can conclude that these epitopes/antigenic determinants are important in provoking the desired humoral immune response against BEFV N protein. Therefore, these epitopes are highly useful for developing diagnostic kits as well as recombinant vaccines. Further experimental research is required to confirm these epitopes.

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Conflict of Interest

The authors declare no conflict of interest.

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