RESEARCH ARTICLE

Phylogenetic Analysis and Antigenic Epitope Profiling of Glycoprotein E Gene of Bovine Herpesvirus-1

Diksha Panwar, Aman Kumar*, Neha Singh, Anubha Sharma, Sushila Maan

Abstract

The current study focused on the characterization of the glycoprotein E (gE) gene of Bovine herpesvirus-1 (BoHV-1) isolates and the prediction of antigenic epitopes for peptide-based diagnostics or vaccines. The study utilized a BoHV-1 isolate (BoHV-1/ABT/LUVAS/HSR/2016) stored in the department which was earlier isolated from vaginal swab of aborted buffalo. The nucleotide sequencing of the *gE* gene showed a partial length of 689 bp, exhibiting a 98-99.85% identity with BoHV-1.1 and 1.2 strains from Indian and global sequences available in GenBank database. Phylogenetic analysis clustered the BoHV-1 isolate with BoHV-1.1 and BoHV-1.2 strains. The deduced amino acid sequence revealed characteristic signal sequences, conserved cysteine residues, glycosylation site, and a substitution (L→H) at the 164th position, distinguishing it from other strains. Antigenic epitopes were predicted using various methods, including Kolaskar & Tongaonkar Antigenicity Prediction, Bepipred, ABCpred, and SVMTrip. In total 50 antigenic epitopes were predicted of which the Kolaskar & Tongaonkar method identified 21 antigenic peptides ranging from 6-21 amino acids in length within the 568-mer sequence. In conclusion, the present study provides valuable insights into the phylogenetic relation and peptide profiling of *gE* gene of BoHV-1. Moreover, this study enriches the knowledge related to *gE* which contributes to the prevention and control strategies against BoHV-1.

Key words: Antigenic epitopes, Bovine herpesvirus-1, Glycoprotein E, Peptide-based diagnostics, Phylogenetic analysis.

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Introduction

Bovine herpes virus-1 (BoHV-1), the causative agent for Infectious Bovine Rhinotracheitis (IBR)/pustular vulvovaginitis/balanoposthitis, has nearly cosmopolitan distribution. BoHV-1 generally produces systemic and neurological diseases due to virus's natural predilection for sensorial neurons in the trigeminal ganglia or dorsal root ganglia (Chothe *et al*., 2018). The disease is associated with considerable losses to production caused by increased indirect costs, e.g. decreased milk production, abortion in mid to last trimester, weight loss, and death which altogether affect the health and welfare of animals (Maresca *et al*., 2018). The variability in the clinical signs along with animal declared as seropositive throughout life due to its latency factor (Nandi *et al*., 2009) makes this virus more important from economics and international trade point of view.

The BoHV-1 has three subtypes, *viz*., 1 (BHV-1.1), 2a (BHV-1.2a) and 2b (BHV-1.2b) (Metzler *et al*., 1985) and the genome has ten genes encoding glycoproteins (Muylkens *et al.,* 2007). The *gE* has been categorized under unique short (US) region of the viral genome which is identified as non-essential gene responsible for cell-to-cell transmission of the virus. The *gE* gene has the potential to be utilized as a potent-marker for the vaccine preparation as it is non-essential for virus reproduction, and its deletion does not usually significantly impair virus replication efficiency *in vivo*. For such reasons, the majority of BoHV-1 marker vaccines available worldwide today contain recombinant *gE*-negative viral strains (Weiss *et al*., 2015; Righi *et al*., 2022).

For differentiation of infected from the vaccinated animals (DIVA), it is necessary to have a diagnostic based on the vaccine formulation and in that prospects, *gE* based immunodiagnostics will be more compatible. Although, commercial kits with DIVA strategy for glycoprotein E (*gE*)-deleted vaccines have been developed, yet peptide-based assay may provide a better alternative tool in terms of specificity, ease of use and cost (Pandey *et al*., 2021). With these prospects in mind, the current study was undertaken to analyze phylogenetic relations and predict antigenic epitopes of field strain for the development of peptide-based diagnostics or vaccine candidate against BoHV-1.

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Materials and Methods

DNA Extraction

Nine-days old viable SPF eggs (Indovax®) were inoculated via Chorio-allantoic membrane (CAM) route with the MDBK adapted field isolates of BoHV-1 (BoHV-1/ABT/ LUVAS/ HSR/2016) from aborted buffalo (Samrath *et al.,* 2016). After incubation at 37°C in the incubator with 50-55 % relative humidity for 72 hr, the CAM was retrieved and processed for DNA extraction using Invitrogen PureLink™ genomic DNA mini kit as per manufacturer's recommendations. Quantity and qualitative examination of extracted nucleic acid were done by Nano drop 2000 (Thermo Fischer Scientific).

PCR and Sequencing

The self-designed primer set BoHV-1-gE-F-5'-ATGCAGCCGACAGCACCGA-3' and BoHV-1-gE-R-5'-AGGGACAATCACCCGCTGTACT-3' was used for amplification of full length *gE* of BoHV-1. The 25 µL reaction mixture prepared for amplification of *gE* gene consisted 12.5 µL 2X Phusion® High-Fidelity PCR w/HF Buffer (New England Biolabs), 1 µL of each forward and reverse primers along with 1 µL DMSO, 3.5 µL (75.6 ng/µL) DNA template and nuclease free water to make up the volume 25 µL. Amplified PCR products were gel electrophoresed in 1% agarose stained with ethidium bromide (0.5 µg/mL) along with 1 Kb molecular weight marker (Thermo Fisher Scientific). PCR amplified products were visualized using gel documentation system. The PCR products were purified using a Gel extraction kit (Qiagen, Germany) and then sequenced commercially in both the directions using Sanger's method of sequencing using *gE*-forward and reverse primers. The sequence obtained was trimmed using BioEdit 7.2 (Hall, 1999) to remove the overlapping or unresolved nucleotides (nt) and then subjected to BLAST analysis to confirm the identity of the virus.

Phylogenetic Analysis

The ClustalW algorithm in the MEGA 11.0 program was used to align the nucleotide (nt) sequences of *gE* with other sequences retrieved from the NCBI database. The evolutionary relationship was deduced by constructing a phylogenetic tree using the Neighbour joining method, in MEGA 11.0 (Kumar *et al*., 2018; Tamura *et al*., 2021). After nucleotide alignment, the deduced amino acid sequences were captured and further processed. BioEdit was used to generate the dot plot for the nucleotides within the coding region (Hall, 1999).

Antigenic Epitope Prediction

The web-based Immune Epitope Database Analysis Resource (IEDB) tool was employed for the prediction of B-cell epitope for translated ORF region of the *gE* gene (accessed at http://tools.immuneepitope.org/tools/bcell/iedb input). The methods used by the tool included Kolaskar and Tongaonkar Antigenicity Prediction (Kolaskar and Tongaonkar, 1990), Bepipred and Bepipred 2.0 (Larsen *et al*., 2006; Jespersen *et al*., 2017) and stand alone servers ABCpred (Saha and Raghava, 2006) and SVMTrip (Yao *et al*., 2012). The peptides with scores above the threshold for each method were selected as the most suitable candidates and too short or too larger epitopes were excluded.

Molecular Modeling and Protein Structure Assessment

The homology-modeling server SWISS-MODEL (Waterhouse *et al*., 2018) and Phyre2 web server (Kelley *et al*., 2015), was used for the modeling of *gE* protein. A three-dimensional model for *gE* protein was created utilizing the list of 50 templates. The geometrical properties of the modeled protein structures were evaluated using qualitative model energy analysis (QMEAN). The models with the greatest QMEAN values were selected. For structural validation, the Ramachandran plot for the models was generated using MolProbity (Gooch, 2010).

Results and Discussion

Analysis of Nucleic Acid Sequence of Recombinant Plasmid

The nucleotide sequencing for *gE* amplified product yielded a partial length of 689 bp. The homology study using BLAST analysis revealed 98-99.85% identity with BoHV-1.1 & 1.2 strain of Indian and global sequences available in Genbank data base (Table 1). The Neighbor-joining phylogenetic tree

Table 1: BLAST analysis showing significant Sequence alignment

Description	Total Score	Query Cover	Per. Ident	Accession
Bovine alphaherpesvirus 1 isolate C33	1277	100%	99.94%	MH751901.1
Bovine alphaherpesvirus 1 isolate Pyramid IBR MLV vaccine	1267	100%	99.85%	MH724205.1
Bovine herpesvirus type 1.1 strain Abu-Hammad truncated glycoprotein E (*gE*)	1262	100%	99.71%	EU850282.1
Bovine herpesvirus type 1.2 strain SM023, complete genome	1256	100%	99.56%	KM258882.1
Bovine herpesvirus type 1.2 strain B589, complete genome	1251	100%	99.42%	KM258881.1
Bovine herpesvirus type 1.2 strain K22, complete genome	1245	100%	99.27%	KM258880.1
Bovine herpesvirus type 1.2, complete genome	1240	100%	99.13%	OP035381.1
Bovine herpesvirus 1 glycoprotein E gene, complete cds	1223	100%	98.69%	AF133121.1

was constructed using MEGA 11.0 (Saitou and Nei., 1987) after multiple sequence alignment with different genotypes BoHV-1 and BoHV-5 (Fig.1). The present BoHV-1 was clustered with BoHV-1.1 and BoHV-1.2. The deduced aminoacid sequence revealed the presence of signal sequences, conserved cysteine residue, and glycosylation site along with substitution of leucine from histidine (L→H) at 164th position as in BoHV-1.1 and BoHV-1.2 sequences (Fig. 2). Chowdhury *et al.* (2000) also analyzed the *gE* of BoHV-1 and found similar signal sequences and N-linked glycosylation site and conserved cysteine residues as observed in the present study. However, above mutation at 164th position was not observed earlier. Further analysis of sequencing is required to eliminate the possibility of machine-based mutation.

The sequence translated from our strain was 229-mer in length. However, for antigenic epitope prediction the sequence obtained after alignment with different retrieved sequences using BioEdit was of 568-mer length (consensus sequence) which was used for further analysis. According to the method of Kolaskar and Tongaonkar, the 568 amino acid sequence contained 21 antigenic peptides ranging from 6-21 amino acid length. Table 2 lists the peptide lengths, their sequences, and their position along the entire length of the sequence. Fig. 3 illustrates the anticipated peptides of BoHV-1 *gE* based on antigenic propensity (y-axis) and sequence position (x-axis). The average antigenic propensity value was 1.044, with minimum and maximumranging from 0.887 to 1.245, respectively. This method uses the physicochemical characteristics of amino acid residues that frequently appear in experimentally determined antigenic epitopes. According to earlier studies, this approach has an experimental accuracy of 75% (Kolaskar and Tongaonkar, 1990; Mishra *et al*., 2017).

The results obtained using various other tools namely Bepipred Linear Epitope Prediction or BepiPred, BepiPred 2.0 Linear Epitope Prediction, ABCpred, and SVMTrip are summarized in Table 2. Using BepiPred and BepiPred 2.0, residues with a score above the cut-off value (0.35 and 0.5 as the default value, respectively) were predicted to be part of the epitope (Fig. 4 A and B). The BepiPred 1.0 and 2.0 predicted 26 and 14 antigenic peptides, respectively, of which 21 were excluded as they were either too small or too large epitopes. Bepipred 2.0 predicts B-cell epitopes from a protein sequence using a random forest algorithm trained on epitopes and non-epitope amino acids that it identifies from crystal structures, whereas BepiPred predicts the location of B-cell epitopes using a hidden Markov model and a propensity scale method (Larsen *et al*., 2006; Jespersen *et al*., 2017). Elhag *et al*. (2020) also utilised these models for successful prediction of antigenic epitopes for proposing a vaccine against *Pseudomonas aeruginosa*. The trained

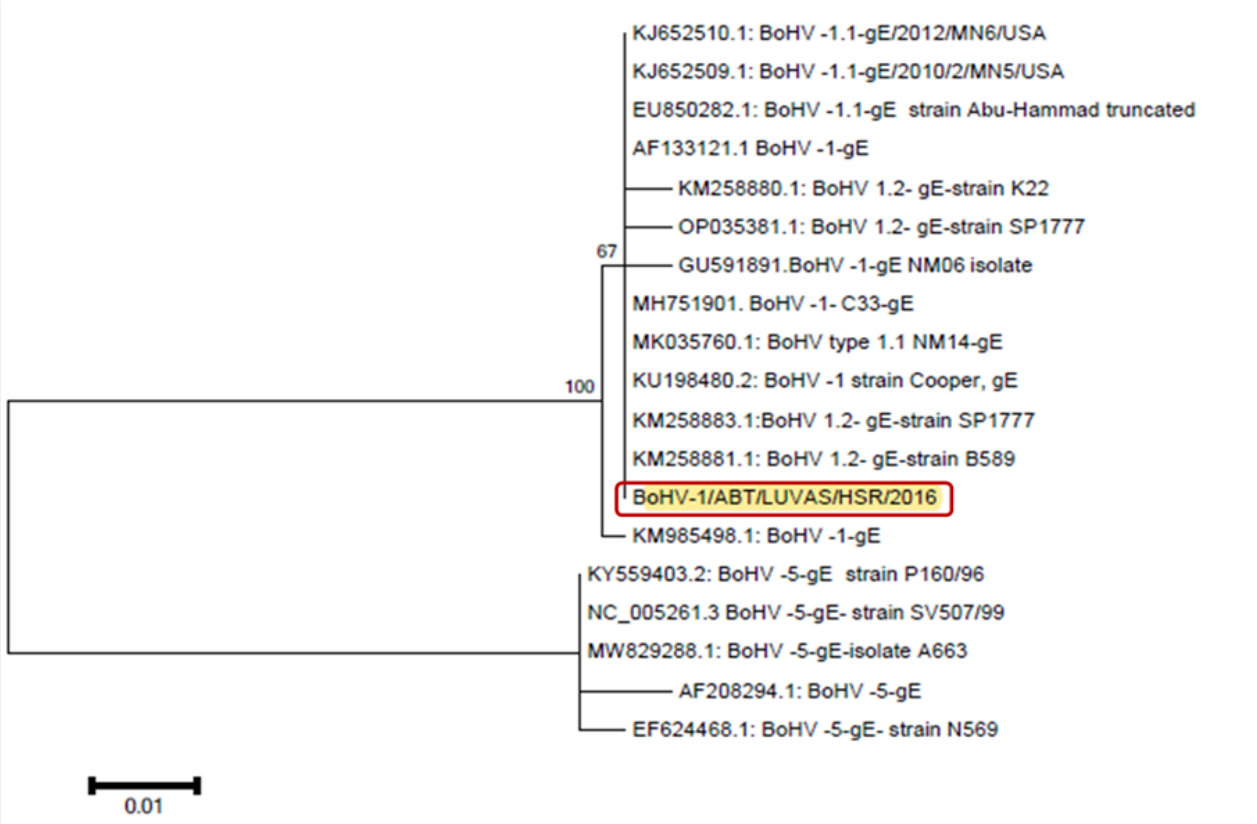

Fig. 1: Phylogenetic analysis using the *gE* gene›s nucleotide sequences. The phylogenetic relationship was established using MEGA 11.0 software with the neighbour joining method. Texas lists the GenBank accession numbers of the compared strains. Each tree node displays the bootstrap values (%) and the current study isolate is highlighted in yellow.

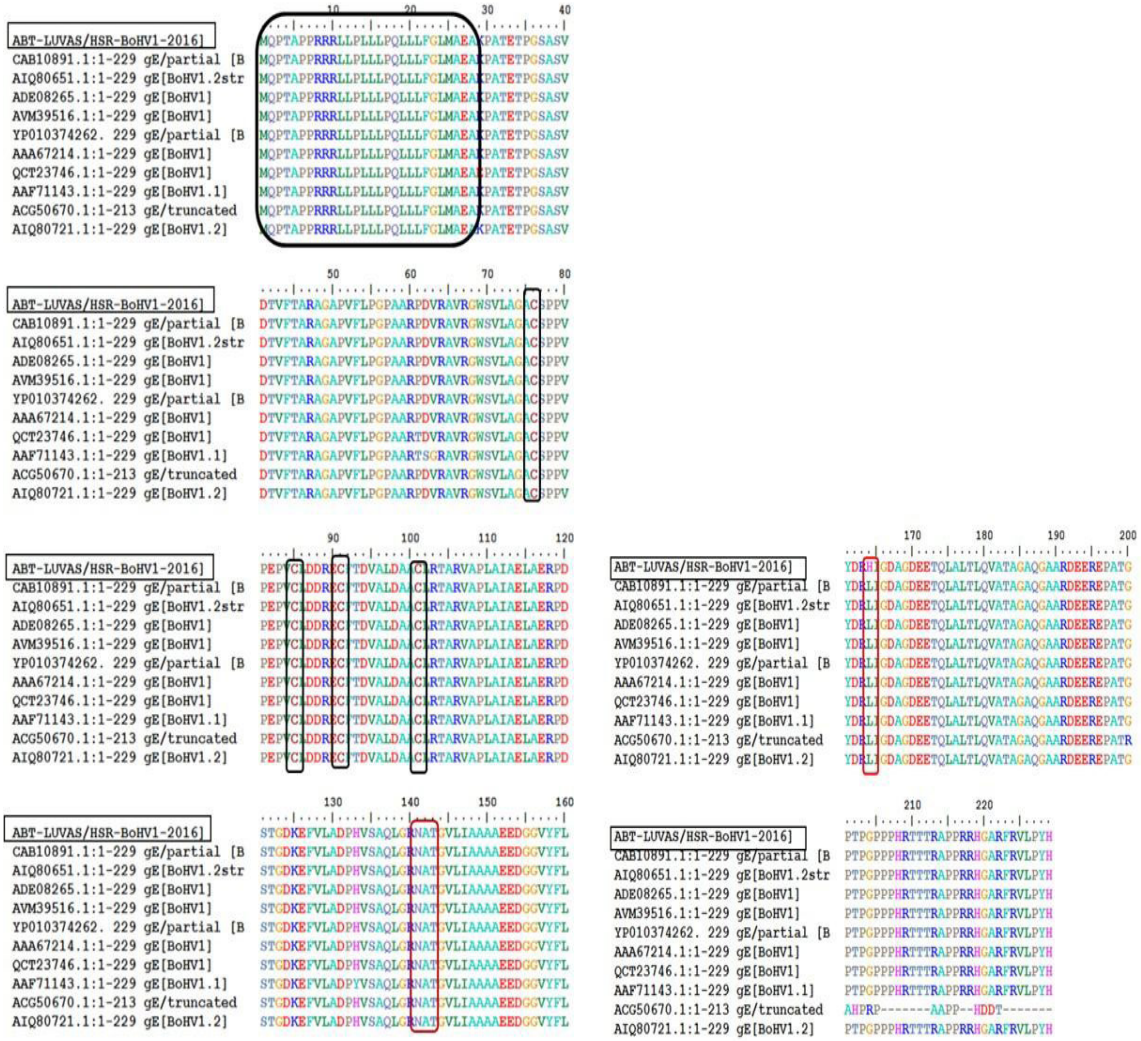

Fig. 2: Dot plot of coding region of current study isolates with other strains from NCBI constructed using BioEdit version 7.0.

recurrent neural network result was used by the ABCpred to rank the anticipated B-cell epitopes. The probability of a peptide being an epitope increases with the peptide score in ABCpred. Antigenic epitope prediction by ABCpred is accurate up to 65.93% (Saha and Raghava, 2006). The ABCpred server yielded 32 antigenic peptides with score above threshold, of which top five were selected and had a score of more than 0.90. To improve prediction performance, Tri-peptide similarity and Propensity scores (SVMTriP) were combined with Support Vector Machine (SVM). Two 16-mer peptide sequences namely YFLRTAGDCALIRIYE and WDYSLVVTS DRLVRAV, with score of 1.00 and 0.984, respectively, were predicted using SVMTriP. In total, 50 antigenic epitope peptides were predicted by combining various prediction tools and server. By considering the maximum accuracy the peptides predicted by Kolaskar and Tongaonkar method may be considered first for the synthesis or while designing a peptide based diagnostic assay or vaccine followed by peptides predicted using ABCpred, SVMTrip and Bepipred. The synthetic peptides may be further utilized for diagnosis as they are more robust,precise, and cheaper than antibodies commonly used in diagnostic tests (Pandey *et al*., 2021).

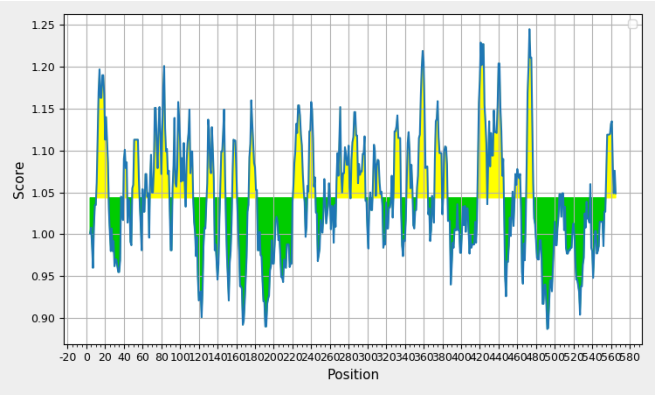

Fig. 3: Graphical representation of the results predicted by Kolaskar and Tongaonkar Antigenicity method (Threshold (Th) = 1.044). The area in yellow depicts the score above the threshold and the green depicts the score below the threshold value.

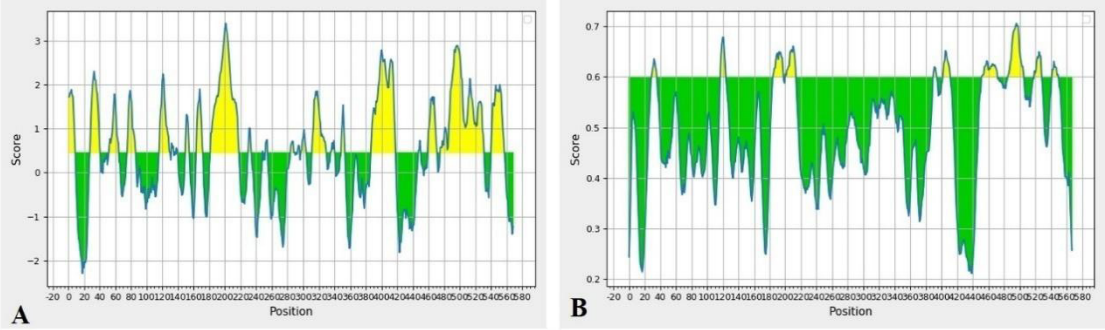

Fig. 4: The results predicted by A) Bepipred (Th = 0.350) and B) Bepipred 2.0 (Th = 0.50). The area in yellow depicts the score above the threshold and green depicts the score below the threshold value.

Table 2: B-Cell epitope prediction results using Bepipred, Bepipred 2.0, SVMTrip, and ABCPred

No.	Start	End	Peptide	Length
			Bepipred	
1.	1	8	MQPTAPPR	8
2.	28	40	AKPATETPGSASV	13
3.	52	64	VFLPGPAARPDVR	13
4.	75	86	ACSPPVPEPVCL	12
5.	116	129	AERPDSTGDKEFVA	14
6.	164	171	GDAGDEET	8
7.	182	217	AGAQGAARDEEREPATGPTPGPPPHRTTTRAPPRRH	36
8.	311	329	EQCPDPAGWPHECEGAAYA	19
9.	387	418	DHTRPEAAAADAPEPGPPLTSEPAGAPTGPAP	32
10.	459	470	GPVYTSPTNEPL	12
11.	540	556	PLEDDAAPARTPAAPDY	17
			Bepipred 2.0	
12.	26	40	AEAKPATETPGSASV	15
13.	57	64	PAARPDVR	8
14.	116	129	AERPDSTGDKEFVA	14
15.	182	217	AGAQGAARDEEREPATGPTPGPPPHRTTTRAPPRRH	36
16.	281	291	PEAPACLHPAD	11
17.	310	344	YEQCPDPAGWPHECEGAAYAAPVAHLRPANNSVDL	35
18.	387	416	DHTRPEAAAADAPEPGPPLTSEPAGAPTGP	30
19.	538	555	RDPLEDDAAPARTPAAPD	18
			SVMTrip	
20.	260	275	YFLRTAGDCALIRIYE	16
21.	370	385	WDYSLVVTSDRLVRAV	16
			Kolaskar and Tongaonkar Antigenicity method	
22.	11	23	LLPLLLPQLLLFG	13
23.	49	56	GAPVFLPG	8
24.	68	88	GWSVLAGACSPPVPEPVCLDD	21
25.	92	105	FTDVALDAACLRTA	14
26.	107	113	VAPLAIA	7
27.	129	136	ADPHVSAQ	8
28.	143	148	GVLIAA	6

29.	174	182	ALTLQVATA	9
30.	221	232	FRVLPYHSHVYT	12
31.	236	244	SFLLSVRLQ	9
32.	267	281	DCALIRIYETCIFHP	15
33.	283	297	APACLHPADAQCSFA	15
34.	306	315	YSRLYEQCPD	10
35.	328	335	YAAPVAHL	8
36.	342	348	VDLVFDD	7
37.	353	364	ASGLYVFVLQYN	12
38.	372	379	YSLVVTSD	8
39.	418	427	PWLVVLVGAL	10
40.	429	445	LAGLVGIAALAVRVCAR	17
41.	458	463	FGPVYT	6
42.	470	477	LDVVVPVS	8

ABCPred

	Rank	Sequence	Start position	Score
43.	1	PATGPTPGPP-PHRTTT	195	0.94
44.	2	GVLIAAAAEEDG-GVYF	143	0.93
45.	3	PAG-WPHECEGAAYAAP	316	0.92
46.	3	YETCIFHPEAPA-CLHP	274	0.92
47.	3	SASIDWYFLRTAG-DCA	254	0.92
48.	4	DVALDAACLR-TARVAP	94	0.91
49.	4	PGSASVDTVFTA-RAGA	35	0.91
50.	5	HRTTTRAPPRRH-GARF	206	0.90

Homology modeling of BoHV-1 *gE* protein using the SWISS Model and Phyre2 server revealed a maximum identity of 36.3% for the current study protein with Protein Data Bank in Europe (PDBe): 2giy chain A (x-ray diffraction 1.78 Å). A total of 177 residues (31% of sequence) were modeled with 100.0% confidence by the single highest scoring template, *i.e.,* PDBe: 2giy chain A (Fig. 5A). Sequence identity above 30% is a relatively good predictor of the expected accuracy of a model (Fiser, 2010). A Ramachandran plot was generated with 1.27% outliers (Fig. 5B). However, outliers do not necessarily infer errors in the model, they may be genuine, unusual, and of biological interest (Chen and He, 2020). Thus, the lower value for outliers in current study may not imply any serious concern. This indicates that the structure was of high quality (Table 3). The QMEAN score was –1.78. The Ramachandran plot showed that the modeled structure had an 89.87% favourable zone, which served as validation for the modeled structure (Fig. 5B). The MolProbity score was 1.67, which is a combined protein quality score and indicates the crystallographic resolution at which a good-quality model is anticipated (Gooch, 2010). The lower number for MolProbity score indicates a good quality model of protein structure.

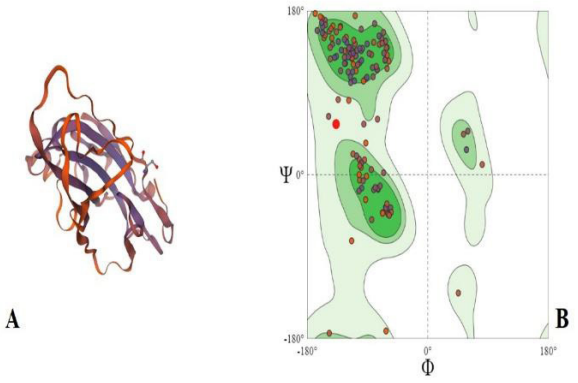

Fig. 5:A) 3-D structure and B) Ramachandran plot of BoHV-1 *gE* protein

Table 3: Results of the Ramachandran analysis using MolProbity

MolProbity score	1.67	
Ramachandran favoured	89.87%	
Clash Score	3.30	
Ramachandran outliers	1.27%	(A322 ARG, A354 PRO)
Rotamer outliers	0.0%	
C-beta deviations	0	
Bad bonds	0/1303	
Bad angles	14/1789	A249 PHE, (A319 PRO-A320 ALA), (A323 TRP-A324 PRO), (A316 ARG-A317 PRO), A391 ASP, A347 ASP, A325 HIS, A370 HIS, (A285 ALA-A286 PRO), A338 HIS, A250 PHE, (A234 THR-A235 PRO), A276 TYR, A282 HIS
Cis prolines	1/147	(A319 PRO-A320 ALA)

Conclusions

The present study reported the isolation, phylogenetic characterization and peptide profiling of field strain of BoHV-1 based on *gE* sequence. The antigenic epitope-based peptides appear to be appealing candidates for diagnostic, preventive, and therapeutic vaccinations. Additionally, the 3-D structure of *gE* protein validated in the study may be utilized further for molecular docking or protein interactions studies.Moreover, studies on molecular and antigenic characterization of more field isolates from different clinical signs will be helpful in understanding of molecular epidemiology for better diagnostic assays of vaccine candidate.

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