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Molecular Detection with Phylogenetic Analysis of Bovine Herpesvirus 1.1 in Pneumonic Lungs of Imported African Cattle Associated with Severe Signs of Bovine Respiratory Disease in Sohag Governorate, Egypt

Ahmed M.E. Yousef*[1], & Ahmed M. A. Zaitoun[2]

[1]Infectious Diseases, Faculty of Veterinary Medicine, Sohag University, Sohag, Egypt
[2]Infectious Diseases, Department of Veterinary Medicine, Assiut University, Egypt

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Abstract: *Bovine alphaherpesvirus type 1.1 (BoHV-1)* plays extraordinary roles in the initiation of bovine respiratory disease (BRD) by suppression of lung clearance mechanism of the infected cases. During the period of investigation (30 consecutive months), a total number of 650 cattle were clinically inspected native breed cattle (n= 300) and imported African breed cattle (n= 350). Sixty-three pneumonic lungs samples of the examined diseased cattle with severe signs of BRD were subjected to polymerase chain reaction (PCR) for molecular detection of *BoHV-1*. Forty-eight (76.19%) of 63 pneumonic lung samples were molecularly *BoHV-1* positive. Comparative sequence analyses of glycoprotein C (gC) (OP777904) gene reveal that field *BoHV-1* obtained in the current study shared 99% to 100% identity on both nucleotide (nt) and amino acid **(A.A)** sequences with *BoHV-1* isolates for *BoHV-1* from USA, India, China, Serbia, Cooper strain and commercial vaccines Nasalgen IP and Bovishield gold FP 5 MLV. Comparative sequence analyses of glycoprotein B gene (gB) reveal that field *BoHV-1* obtained in the current study (OQ161992 and OQ161993) shared 99% to 100% identity on both nucleotide (nt) and amino acid **(A.A)** sequences in between and 99 to 100% with *BoHV-1* isolates from Egypt, USA, and commercial available vaccines vaccinal strains..

Keywords: Bovine respiratory disease, *Bovine alphaherpesvirus type 1*, polymerase chain reaction, Phylogenetic analysis, glycoprotein B gene and glycoprotein C gene

INTRODUCTION

Bovine Respiratory Disease (BRD) is a multi-factorial disease involving infectious agents, compromised host immune system, and environmental and managmental factors (Grissett *et al.*, 2015; & Bernal *et al.*, 2023). Four viruses are frequently incriminated as a primary cause of BRD either in single form or mixed form; *BoHV-1, Bovine parainfluenza-3 virus, Bovine respiratory syncytial virus* and *Bovine viral diarrhea virus*. However, *BoHV-1* is more prevalent virus than the others (Fulton *et al.*, 2015; Chien *et al.*, 2022; & Ostler & Jones, 2023).

BoHV-1 plays extraordinary roles in the initiation of BRD by suppression of lung clearance mechanism of the infected cases, allowing the secondary infections (e.g. *Mannheimia haemolytica, Pasteurella multocida*, and *Histophilus somni*) inducing severe pneumonia followed by death (Lovato *et al.*, 2003). Viral genes of *BoHV-1* alone can inhibit specific immune responses in the absence of other viral genes through infection of T lymphocytes of cattle, causing directly and indirectly apoptosis that subsequently lead to suppression of cell-mediated immunity, enhancing establishment of latency and increasing the probability for secondary bacterial infection (Carter *et al.*, 1989; & Bastawecy & Abouzeid, 2016). *BoHV-1* was first described in Germany in the 19th century, being recognized primarily as the cause of infectious pustular vulvovaginitis and balanoposthitis until the mid-1950s when a more virulent strain of the virus *BoHV-1.1* associated with respiratory disease infectious bovine rhinotracheitis emerged in the western United States (Graham, 2013).

BoHV-1 is a member of the Herpesviridae family, subfamily Alphaherpesvirinae, and the genus Varicellovirus. Three subtypes of *BoHV-1* are recognized worldwide: *BoHV-1.1*, *BoHV-1.2a* and *BoHV-1.2b*. The *Bovine herpesvirus* causing meningoencephalitis (previously *BoHV-1.3*) has been classified as *BoHV-5* (Chatterjee *et al.*, 2016). Infected cattle with the *BoHV-1* infection are very likely to become silent carriers after recovery. These animals remain carriers of *BoHV-1* for the rest of their life and treatment with immunosuppressives may reactivate virus replication from its latent status leading to spread of the infection to the rest of the herd (Van Oirschot, 1997; & Preston & Nicholl, 2008).

The General Organization of Vet. Services registers multiple vaccines against the major pathogens implicated in BRD for use in Egypt. However, despite prophylactic vaccination, BRD still conservatively estimated to cost the Egyptian economy losses especially

***Corresponding Author:** Ahmed M.E. Yousef

in Upper Egypt. Therefore, the current work was carried-out to reveal-up the followings: Role of *BoHV.1* infection in the diseased cattle with various signs of BRD with descriptions of the clinical and necropsy findings. Molecular detection of *BoHV1* genomes (glycoprotein C and glycoprotein B genes) in deep pulmonary tissues of the necropsied cases by conventional PCR using species-specific primers. DNA sequencing of the molecularly detected *BHV-1* strains with molecular comparison with between them and a vaccinal strain illustrated in Genbank.

MATERIAL AND METHODS

Ethical Approval

The Faculty of Veterinary Medicine at Sohag University followed all experimental protocols that were approved by the committee's consideration of animal research.

Animal and Areas of Study

During the period of investigation, from July 2021 to April 2023, a total number of 650 cattle were clinically inspected. These animals were belonged to Four areas in Egypt; Sohag, Assiut, and Cairo governorates (native breed cattle), and Abu-Simbel (African breed, imported cattle). The clinically inspected cattle included two groups. Group I was native breed cattle (n= 300) located in different areas of Sohag, Assiut, and Cairo governorates. The Second group was the African breed cattle (n= 350) that were imported from African countries such as Ethiopia, Djibouti, and Sudan to compensate the shortage of meat production in Egypt.

Clinical Examination

History taking, species, age, sex, and breed of animals were recorded. General physical examination including the respiratory system and the dehydration state of diseased animals was carried out as described by (Smith, 2015).

Samples

Tissue specimens were taken from pneumonic lungs after gross necropsy for animal's emergency slaughtered inside and/or outside slaughterhouse or recently succumbed animals with severe respiratory signs was carried out as described by (King *et al.*, 2014). Pneumonic lungs were taken in a clean, dry and sterile polyethylene bags under a septic condition for virological investigation. Samples were identified to show the serial numbers, animal species, breed, age, sex, date of sampling and other observations. All samples were put into tank double wall ice box and brought to Laboratory of Infectious Diseases and the Central Laboratory, Faculty of Veterinary Medicine, Sohag University, Egypt with minimal delay.

Conventional PCR

Extraction of *BoHV-1* nucleic acid from tissue specimens of pneumonic lungs was done by using a commercial extraction kit (QIAamp® DNA Mini Kit 50, Qiagen, Hilden, Germany, catalog No.: 51304) according to manufacturing kit instructions. The extracted DNA was stored in -20^{o}c till used.

PCR Amplification

The extracted DNA was subjected to PCR using species-specific primers that coded for gB gene as previously described by Fuchs *et al.* (1999) (Table, 1) and gC gene as previously described by Van Engelenburg *et al.* (1993) (Table, 2). PCR was performed with HotStarTaq Master Mix Kit for both gB and gC gene in a total of 50 µL containing 25 µL HotStarTaq Master Mix (Qiagen, Germany, catalog no.: 203443) with (2.5 units HotStarTaq DNA Polymerase 1 x PCR Buffer 200 µM of each dNTP), 1 µL of each forward and reverse primer (10 Pmol/µl), 17 µL of RNase-Free Water, and 6 µL of DNA template. PCR for gB was performed under the following amplification conditions: initial activation step of 15 min at 95°C, 35 cycles consisting of 1 min at 95°C, 1min at 58°C, 1 min at 72°C and 1 cycle for 10 min at 72°C (Albayrak *et al.*, 2020) while PCR for gC was performed under the following amplification conditions: initial activation step of 15 min at 95°C, 38 cycles consisting of 1 min at 96°C, 1min at 60°C, 1 min at 72°C and 1 cycle for 10 min at 72°C (Intisar *et al.*, 2009).

Identification of PCR products

Five microliters of each PCR product with 1 µL loading dye (DNA blue gel loading dye buffer (6x), Thermo Scientific™, U.S.A., Cat. no.: R0611) was loaded carefully in each well and 6 µL DNA Ladder (Gelpilot 100 bp DNA molecular weight plus ladder (100)**,** Qiagen, Germany, catalog no.: 239045) in 1.5% agarose gel (TopVision Agarose**,** Thermo Scientific™, U.S.A., Cat. no.: R0491) for gB gene and 2% agarose gel with DNA ladder (Gelpilot 50 bp DNA molecular weight ladder**,** Qiagen, Germany, catalog no.: 239025) for gC gene stained with ethidium bromide (Ultrapure™ Ethidium bromide (10 mg/ml), Thermo Scientific™, U.S.A., Cat. no.: 15585011) and visualized by the Transilluminator (UVP UV, White & Blue Light Transilluminator Analytik Jena®, California, U.S.A.) and UVP MultiDoc-It Imaging System (Analytik Jena®, Upland, California, U.S.A., Model: 97-0192-04) running at 100 V for 40 minutes in Tris-Acetate-EDTA Buffer (1x) (Thermo Scientific™, U.S.A., Cat. no.: B49) to detect the 478 bp product for gB gene and the 173 bp of gC gene of *BoHV-1*.

Sequence Analysis

PCR product (478bp), which targeted *BoHV-1 gB* and (173bp), which targeted *BoHV-1 gC* of the isolated samples that had the sharpest band on gel electrophoresis, were purified from the gel using the QIAquick Gel Extraction kit (Qiagen, Hilden, Germany, cat. no. 28704) according to manufacturing instructions. Bi-directional DNA sequencing was performed on gel purified DNA at (Macrogen, South Korea).

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Obtained DNA sequences were deposited to GenBank with accession numbers OP777904, OQ161992 and OQ161993. Basic Local Alignment Search Tool (BLAST®) was established to investigate sequence similarity between sequences deposited in GenBank and our sequences (Altschul *et al.*, 1990) using Clustal W (http://www.ebi.ac.uk/Tools/msa/clustalw2).

The *BoHV-1* strains were compared with other alpha herpesviruses related to *BoHV-1* in terms of percentage similarities and differences in nt and AA identities.

Neighbor-joining phylogenetic tree was constructed with 1000 repeat bootstrap tests and identity percentages were calculated using MEGA X software (MEGA11) (http://www.megasoftware.net/). Nucleotide sequence analysis was done using Bioedit software version 7.1 (Hall, 1999).

Table 1: Nucleotide sequences of the PCR primers for glycoprotein B gene (gB) of *BoHV-1*.

gB	Sequences (5´ to 3´)	Primer location	Product size (bp)	Reference
gB1 F	*TAC GAC TCG TTC GCG CTC TC*	883–902	478	(Fuchs *et al.*, 1999)
gB2 R	*GGT ACG TCT CCA AGC TGC CC*	1341–1360		

(Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.).

Table 2: Nucleotide sequences of the PCR primers for glycoprotein C gene (gC) of *BoHV-1*.

gC	Sequences (5´ to 3´)	Primer location	Product size (bp)	Reference
gC1 F	*CTG CTG TTC GTA GCC CAC AAC G*	763 to 785	173	**(Van Engelenburg *et al.*, 1993)**
gC2 R	*TGT GAC TTG GTG CCC ATG TCG C*	935 to 913		

(Thermo Fisher Scientific, Waltham, Massachusetts, U.S.A.).

RESULTS

Clinical examination of cattle with BRD showed classic and pathognomonic signs of BRD; extremely extended head and neck with bulging of jugular vein, ears drooping, oral breathing, frothing on mouth, puffed out cheeks, congested conjunctival mucous membrane and extremely dilated nostril with tenacious nasal discharge (Figure 1a, b). Inflamed nostrils with erosions and ulcerations were observed (Figure 1c).

Necropsy examination of recently succumbed animals and/or emergency slaughtered animals inside the abattoir and/or outside the abattoir with severe signs of BRD elucidated that interstitial lobar pneumonia with diffuse distribution, heavy, rubbery texture and 'port-wine' staining of both right and left lung (Figure 2a). Transverse section of the pulmonary parenchyma showed a "meaty" appearance and edema, but no exudate is present in airways or on the pleural surface (Figure 2b).

Sixty-three samples of pneumonic lungs (28 native breeds and 35 imported breeds) were subjected to conventional PCR for detection of *BoHV-1*, and 48 (76.19%) of 63 samples were molecularly positive for *BoHV-1*. The specific band of 173 bp after PCR amplification of the gC gene of *BoHV-1* could be detected with 66.7% (32/48) (Figure 3). The specific band of 478 bp after PCR amplification of the gB gene of *BoHV-1* could be detected with 33.3% (16 of 48) (Figure 4).

Partial nucleotide sequencing of both gB and gC genes for *BoHV-1*, isolated in 2022 from the pneumonic lungs of cattle from Sohag Governorate, was carried out. Sequences were submitted to GenBank under accession numbers (OQ161992, OQ161993) and (OP777904) for gB and gC genes, respectively.

Comparative sequence analyses of glycoprotein C gene revealed that field *BoHV-1* obtained in the current study shared 99% to 100% identity on both nucleotide (nt) and amino acid **(A.A)** sequences with *BoHV-1* isolates from the United States Cooper strain (KU198480.2) and commercial vaccines: Nasalgen IP MLV vaccine (MH724210.1), and Bovishield Gold FP 5 MLV vaccine (MH724209.1). However, the gC gene of *BoHV-1* isolate Egypt/Sohag/2022 (OP777904) shared 99.95% identity with *BoHV-1* from Egypt (MW805266.1), 99.93% identity with *BHV-1.2* from the United States (KM258880.1) (Table, 3)

Phylogenetic relationship of reference *BoHV-1* isolates in the phylogenetic tree showed that *BoHV-1* isolate Egypt/Sohag/2022 gC gene partial cds (OP777904) was clustered together with other reference *BoHV-1* isolates in the same clade, while other reference *BoHV-1* isolate BHV-1.2 from Australia (KM258881.1) and *BoHV-1* from Egypt (MW805266.1) were divergent from *BoHV-1* isolate Egypt/Sohag/2022 gC gene partial cds (OP777904) were located in another clade (Figure 5).

Comparative sequence analyses of glycoprotein B gene revealed that field *BoVH-1* obtained in the current study (OQ161992 and OQ161993) shared 99% to 100% identity on both nt and A.A sequences in between and 99% to 100% with *BoHV-1* isolates from USA Cooper strain (KU198480.2), from Egypt (MW805275.1). The commercial vaccines Nasalgen IP MLV vaccine (MH724210.1), and Bovishield Gold FP 5 MLV vaccine (MH724209.1) were 99% and 100% identical to the *BoHV-1* isolates Egypt/Sohag/2022 (gB) (OQ161992 and OQ161993) respectively (Table, 4).

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Phylogenetic relationship of reference *BoHV-1* isolates in the phylogenetic tree showed that *BoHV-1* isolates Egypt/Sohag/2022 gB gene partial cds (OQ161992) and (OQ161993) were clustered together with other reference *BoHV-1* in similar subgroup while other reference *BoHV-1* isolates from USA (KM258883.1) and from Egypt (MW805275.1) were not located in the similar subgroup (Figure 6).

Comparative sequence analysis of *BoHV-1* gC gene reveal that *BoHV-1* isolates Egypt/Sohag/2022 gC gene partial cds (OP777904) differ from reference strain (MK552112.1), (KU198480.2), and (AJ004801.1), (MH724208.1) as only in one AA substitution (H/6) (Figure 7) where *BoHV-1.1* from Brazil with accession number (AY052397.1) differ from other *BoHV-1* in three AAs substitutions (V/5, G/19 and T/33) with seven amino deletions. *BoHV-1* from Egypt with accession number (MW805266.1) differ from other *BoHV-1* in one AA substitution (A/52) with one AA deletion (Figure 7).

On the other hand, no differences between gB gene of *BoHV-1* partial cds of isolates (OQ161992 and OQ161993) and other *bovine alpha herpesviruses* in this study (100% nucleotide and AA identities). *BoHV-1* gB gene partial cds (OQ161992) has 99.99% similarity in AAs with only one AA substitution (L/2) (Figure 8).

Table 3: Nucleotides and Amino acids Identities of glycoprotein C gene of *BoHV-1* strain compared with selected sequences

Nucleotides Identities																								
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1	MK552112.1		100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
2	**OQ161992**	99.99		100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
3	**OQ161993**	100	99.99		100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
4	MH751901.1	100	99.99	100		100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
5	MH724210.1	100	99.99	100	100		100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
6	MH724209.1	100	99.99	100	100	100		100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
7	MH724208.1	100	99.99	100	100	100	100		100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
8	MH724207.1	100	99.99	100	100	100	100	100		100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
9	MH724206.1	100	99.99	100	100	100	100	100	100		100	100	100	100	100	100	100	100	100	100	100	100	100	100
10	MH724205.1	100	99.99	100	100	100	100	100	100	100		100	100	100	100	100	100	100	100	100	100	100	100	100
11	MH724204.1	100	99.99	100	100	100	100	100	100	100	100		100	100	100	100	100	100	100	100	100	100	100	100
12	MH724203.1	100	99.99	100	100	100	100	100	100	100	100	100		100	100	100	100	100	100	100	100	100	100	100
13	MH724202.1	100	99.99	100	100	100	100	100	100	100	100	100	100		100	100	100	100	100	100	100	100	100	100
14	MH791338.1	100	99.99	100	100	100	100	100	100	100	100	100	100	100		100	100	100	100	100	100	100	100	100
15	MH598938.1	100	99.99	100	100	100	100	100	100	100	100	100	100	100	100		100	100	100	100	100	100	100	100
16	MF421714.1	100	99.99	100	100	100	100	100	100	100	100	100	100	100	100	100		100	100	100	100	100	100	100
17	MG407792.1	100	99.99	100	100	100	100	100	100	100	100	100	100	100	100	100	100		100	100	100	100	100	100
18	KU198480.2	100	99.99	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100		100	100	100	100	100
19	KF584168.1	100	99.99	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100		100	100	100	100
20	NC_063268.1	100	99.99	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100		100	100	100
21	Z78205.1	100	99.99	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100		100	100
22	KM258883.1	100	99.99	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100		100
23	MW805275.1	100	99.99	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	
Amino Acid Identities																								

Table 4: Nucleotides and Amino acids Identities of glycoprotein B gene of *BoHV-1* strain compared with selected sequences

Nucleotides Identities																					
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	MK552112.1		99.99	100	100	100	100	100	100	100	100	100	100	100	100	100	99.98	100	99.98	100	100	100
2	**OP777904**	99.98		99.99	99.99	99.99	99.99	99.99	99.99	99.99	99.99	99.99	99.99	99.99	99.99	99.99	99.97	99.99	99.98	100	99.99	99.99
3	MH751901.1	100	99.98		100	100	100	100	100	100	100	100	100	100	100	100	99.98	100	99.98	100	100	100
4	MH724210.1	100	99.98	100		100	100	100	100	100	100	100	100	100	100	100	99.98	100	99.98	100	100	100
5	MH724209.1	100	99.98	100	100		100	100	100	100	100	100	100	100	100	100	99.98	100	99.98	100	100	100
6	MH724208.1	100	99.98	100	100	100		100	100	100	100	100	100	100	100	100	99.98	100	99.98	100	100	100
7	MH791339.1	100	99.98	100	100	100	100		100	100	100	100	100	100	100	100	99.98	100	99.98	100	100	100
8	KY215944.1	100	99.98	100	100	100	100	100		100	100	100	100	100	100	100	99.98	100	99.98	100	100	100
9	KU198480.2	100	99.98	100	100	100	100	100	100		100	100	100	100	100	100	99.98	100	99.98	100	100	100
10	AJ004801.1	100	99.98	100	100	100	100	100	100	100		100	100	100	100	100	99.98	100	99.98	100	100	100
11	KM258881.1	100	99.96	99.98	99.98	99.98	99.98	99.98	99.98	99.98	99.98		100	100	99.99	100	99.98	100	99.98	100	100	100
12	JN787953.1	100	99.98	100	100	100	100	100	100	100	100	99.98		100	100	100	99.98	100	99.98	100	100	100
13	JN171865.1	100	99.98	100	100	100	100	100	100	100	100	99.98	100		100	100	99.98	100	99.99	100	100	100
14	JN022594.1	99.98	99.96	99.98	99.98	99.98	99.98	99.98	99.98	99.98	99.98	99.96	99.98	99.98		99.99	99.98	100	99.98	99.99	100	100
15	MW805266.1	99.98	99.96	99.98	99.98	99.98	99.98	99.98	99.98	99.98	99.98	100	99.98	99.98	99.95		99.98	100	99.98	100	100	100
16	AY052397.1	99.94	99.92	99.94	99.94	99.94	99.94	99.94	99.94	99.94	99.94	99.94	99.94	99.94	99.98	99.95		99.98	99.98	99.98	99.98	99.98
17	KC756965.1	100	99.98	100	100	100	100	100	100	100	100	99.98	100	100	100	99.95	99.98		99.99	100	100	100
18	KM258880.1	99.95	99.93	99.95	99.95	99.95	99.95	99.95	99.95	99.95	99.95	999.96	99.95	99.95	100	99.95	99.98	100		99.98	99.98	99.98
19	EU272478.1	99.98	99.98	99.98	99.98	99.98	99.98	99.98	99.98	99.98	99.98	99.98	99.98	99.98	99.95	99.98	99.93	99.98	99.95		100	100
20	MG407780.12	100	99.98	100	100	100	100	100	100	100	100	99.98	100	100	99.98	99.98	99.94	100	99.95	99.98		100
21	MF421714.1	100	99.98	100	100	100	100	100	100	100	100	99.98	100	100	99.98	99.98	99.94	100	99.95	99.98	100	
Amino Acid Identities																						

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Figure 1. Classic and pathognomonic signs of BRD

(A) Three years old native breed cow; extremely extended head and neck with bulging of jugular vein, ears drooping, oral breathing, frothing on mouth, puffed out cheeks, congested conjunctival mucous membrane and extremely dilated nostril with tenacious nasal discharge. (B) One week neonatal Friesian calf with enzootic pneumonia; reluctant to move with stiffness gait lowered and extended head and neck, abduction forelimb with oral breathing (white arrows). (C) Erosion and ulceration of nostrils (red arrows) with bilateral mucopurulent nasal discharge (black arrow) in 2 years mixed breed cow.

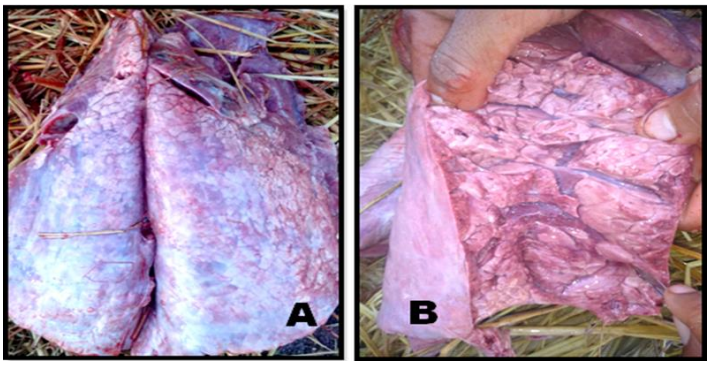

Figure 2. Acute interstitial lobar pneumonia of neonatal Friesian calf recently succumbed with signs of lobar pneumonia

(A) Diffuse distribution with heavy, rubbery texture and 'port-wine' staining of both right and left lung. (B) Transverse section; the pulmonary parenchyma has a "meaty" appearance and edema, but no exudate is present in airways or on the pleural surface.

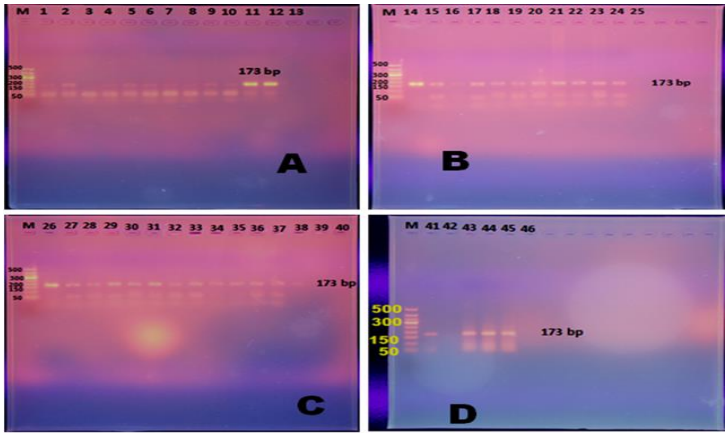

Figure 3. Agarose gel electrophoresis of PCR amplification products of glycoprotein C gene of *BoHV1* with amplified products at 173 bp. Lane M: DNA Ladder (Marker) 50 bp.

(A): (Lanes 11 and 12 control positive. Lane 13 control negative. Lane 3: negative tested sample. Lanes 1, 2, and Lanes 4—10: positive tested samples with amplified products at 173 bp). B: (Lane 14 control positive. Lane 25 control negative. Lanes 15-24: positive tested samples). C: (Lane 26 control positive. Lane 40 control negative. Lane 39 negative tested sample. Lanes 27-38: positive tested samples). D: (Lane 41 control positive. Lane 42 control negative. Lane 46 negative tested sample. Lanes 43, 44, and 45: positive tested samples).

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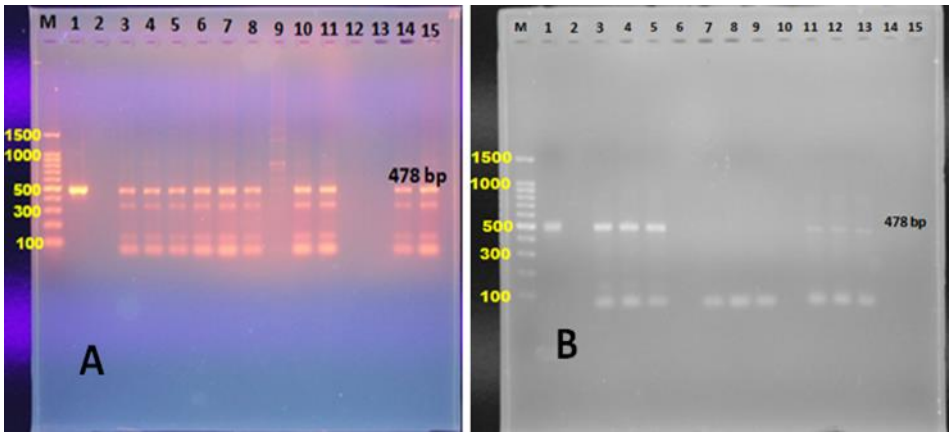

Figure 4. Agarose gel electrophoresis of PCR amplification products of glycoprotein B gene of *BoHV-1* with amplified products at 478 bp. Lane M: DNA Ladder (Marker) 100 bp. Lane 1 control positive. Lane 2 control negative.

A: (Lanes 9, 12, and 13 negative tested sample. Lanes 3-8 and 10, 11, 14, and 15: positive tested samples). B: (Lanes 6-10, 14, and 15 negative tested samples. Lanes 3, 4, 5, 11, 12, and 13: positive tested samples).

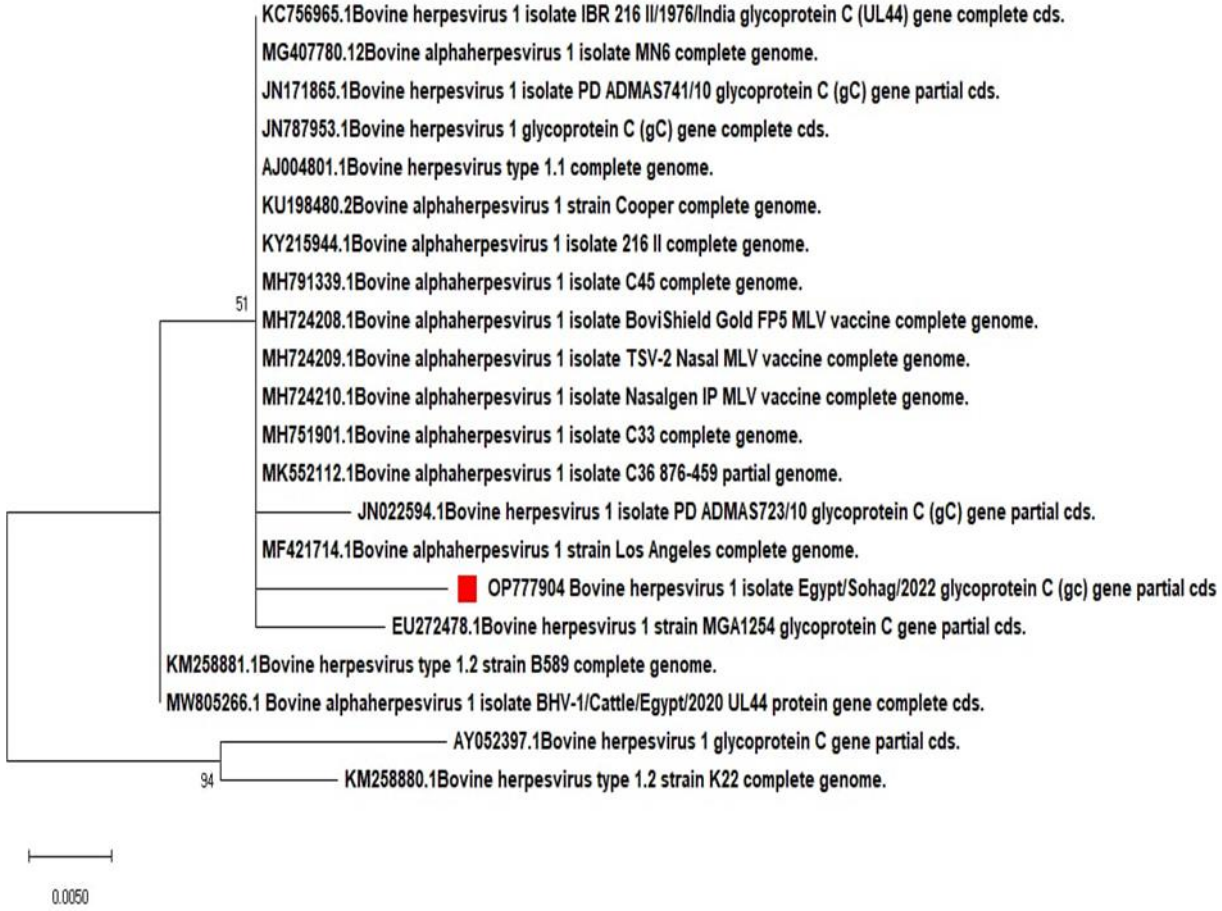

Figure 5. Phylogenetic analysis of Bovine alphaherpesvirus 1 glycoprotein C (gC) gene sequences.

Tree was generated using Mega x program by the neighbor-joining analysis. Bootstrap confidence values were calculated on 1000 replicates according to the maximum-likelihood approach. Sequences obtained in this study are labeled with .

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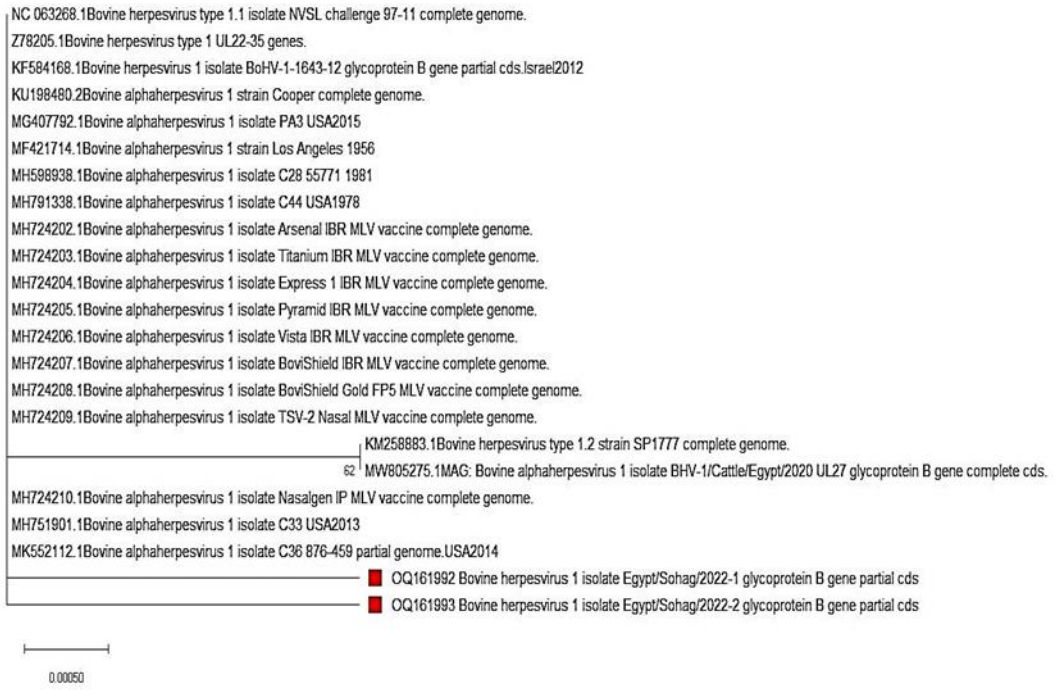

Figure 6: Phylogenetic analysis of Bovine alphaherpesvirus 1 glycoprotein B (gB) gene sequences.

Tree was generated using Mega x program by the neighbor-joining analysis. Bootstrap confidence values were calculated on 1000 replicates according to the maximum-likelihood approach. Sequences obtained in this study are labeled with ■.

MK552112.1Bovine_alphaherpesviru
DSFALSTGDIIYMSPFYGLREGAHREHTSYSPERFQQIEGYYKRDMATGRRLKEPVSRNFLRT
QHVTVAWDWVPKRKNVC
OQ161992 Bovine herpesvirus 1 Eg .L.....
OQ161993 Bovine herpesvirus 1 Eg .....
MH751901.1Bovine_alphaherpesviru .....
MH724210.1Bovine_alphaherpesviru .....
MH724209.1Bovine_alphaherpesviru .....
MH724208.1Bovine_alphaherpesviru .....
MH724207.1Bovine_alphaherpesviru .....
MH724206.1Bovine_alphaherpesviru .....
MH724205.1Bovine_alphaherpesviru .....
MH724204.1Bovine_alphaherpesviru .....
MH724203.1Bovine_alphaherpesviru .....
MH724202.1Bovine_alphaherpesviru .....
MH791338.1Bovine_alphaherpesviru .....
MH598938.1Bovine_alphaherpesviru .....
MF421714.1Bovine_alphaherpesviru .....
MG407792.1Bovine_alphaherpesviru .....
KU198480.2Bovine_alphaherpesviru .....
KF584168.1Bovine_herpesvirus_1_i .....
NC_063268.1Bovine_herpesvirus_ty .....
Z78205.1Bovine_herpesvirus_type_ .....
KM258883.1Bovine_herpesvirus_typ .....
MW805275.1MAG:_Bovine_alphaherpe .....
MK552112.1Bovine_alphaherpesviru
SLAKWREADEMLRDESRGNFRFTARSLSATFVSDSHTFALQNVPLSDCVIEEAEAAVERVYRE
RYNGTHVLSGSLETY
OQ161992 Bovine herpesvirus 1 Eg .....
OQ161993 Bovine herpesvirus 1 Eg .....

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MH751901.1Bovine_alphaherpesviru .....
MH724210.1Bovine_alphaherpesviru .....
MH724209.1Bovine_alphaherpesviru .....
MH724208.1Bovine_alphaherpesviru .....
MH724207.1Bovine_alphaherpesviru .....
MH724206.1Bovine_alphaherpesviru .....
MH724205.1Bovine_alphaherpesviru .....
MH724204.1Bovine_alphaherpesviru .....
MH724203.1Bovine_alphaherpesviru .....
MH724202.1Bovine_alphaherpesviru .....
MH791338.1Bovine_alphaherpesviru .....
MH598938.1Bovine_alphaherpesviru .....
MF421714.1Bovine_alphaherpesviru .....
MG407792.1Bovine_alphaherpesviru .....
KU198480.2Bovine_alphaherpesviru .....
KF584168.1Bovine_herpesvirus_1_i .....
NC 063268.1Bovine herpesvirus ty .....
Z78205.1Bovine_herpesvirus_type_ .....
KM258883.1Bovine_herpesvirus_typ .....
MW805275.1MAG:_Bovine_alphaherpe .....

Figure 7: Deduced amino acid sequence of *Bovine alphherpesvirus 1* glycoprotein B (gB) gene. Dots indicate identical amino acid.

MK552112.1Bovine_alphaherpesviru
LLFVAQNGSIAYRSAELGDNYIFPSPADPRNLPLTVRSLTAATEGVYTWRRDMGTKS
OP777904_Bovine_herpesvirus_1_ .....H.....
MH751901.1Bovine_alphaherpesviru .....
MH724210.1Bovine_alphaherpesviru .....
MH724209.1Bovine_alphaherpesviru .....
MH724208.1Bovine_alphaherpesviru .....
MH791339.1Bovine_alphaherpesviru .....
KY215944.1Bovine_alphaherpesviru .....
KU198480.2Bovine_alphaherpesviru .....
AJ004801.1Bovine_herpesvirus_typ .....
KM258881.1Bovine_herpesvirus_typ .....A..
JN787953.1Bovine_herpesvirus_1_g .....
JN171865.1Bovine_herpesvirus_1_i .....
JN022594.1Bovine_herpesvirus_1_i .....M.....
MW805266.1_Bovine_alphaherpesvir .....A.-
AY052397.1Bovine_herpesvirus_1_g ....V.....G.....T.....-------
KC756965.1Bovine_herpesvirus_1_i .....
KM258880.1Bovine_herpesvirus_typ ....V.....G.....A..
EU272478.1Bovine_herpesvirus_1_s --------.....C...-------
MG407780.12Bovine_alphaherpesvir .....
MF421714.1Bovine_alphaherpesviru .....
Dots indicate identical amino acid

Figure 8. Deduced amino acid sequence of *Bovine alphherpesvirus 1* glycoprotein C (gC) gene.

DISCUSSION

BRD is a devastating syndrome for cattle and has a major impact on the health and economic performance (Griffin, 1997). Cattle with severe signs of BRD showed characteristic classic signs and posture, standing motionless with elbows abducted, neck extended, head lowered and extended with the mouth open and the tongue protruding with frothing at the mouth. This posture maximizes the airway diameter and minimizes the resistance to air flow (Kelly, 1974; & Andrews *et al.*, 2004).

During the last few years, many outbreaks caused by some viruses of BRDC have frequently occurred on cattle farms in Egypt (Zeedan *et al.*, 2018; & Nagy *et al.*, 2022). *BoHV-1* plays extraordinary roles in the initiation of BRD by suppression of lung clearance mechanism of the infected cases, allowing the secondary infections (e.g., *Mannheimia haemolytica*, *Pasteurella multocida*, and *Histophilus somni*) inducing severe pneumonia followed by death (Lovato *et al.*, 2003). Viral genes of *BoHV-1* alone can inhibit specific immune responses in the absence of other viral genes through infection of T lymphocytes of cattle, causing directly and

***Corresponding Author:** Ahmed M.E. Yousef

indirectly apoptosis that subsequently lead to suppression of cell-mediated immunity, enhancing establishment of latency and increasing the probability for secondary bacterial infection (Carter *et al.,* 1989; & Bastawecy & Abouzeid, 2016).

In the present study, *BoHV-1* was identified in imported and native breed with respiratory disorders by PCR targeting the gB and gC gene of the virus. The gB and gC are one of the major envelope proteins of herpesviruses, which are responsible for virus penetration and entry (Sobhy *et al.,* 2014; & Levings *et al.,* 2015). Molecular diagnosis and sequencing of the gB and gC gene is one of the methods frequently used by many researchers to understand the epidemiology and distribution of *BoHV-1* worldwide (Patil *et al.,* 2016).

The first study of molecular characterization of *BoHV-1* in Egypt revealed that the Egyptian *BoHV-1* strain had a 100% similarity to *BoHV-1.1* reference strains based on partial sequencing of gB, gC, gE, and gD (Sobhy *et al.*, 2014).

Comparative sequence analyses of glycoprotein C gene revealed that field *boHV-1* obtained in the current study shared 99% to 100% identity on both (nt) and **(A.A)** sequences with *BoHV-1* isolates from the United States Cooper strain (KU198480.2) and commercial vaccines: Nasalgen IP MLV vaccine (MH724210.1) and Bovishield Gold FP 5 MLV vaccine (MH724209.1). However, the gC gene of *BoHV-1* isolate Egypt/Sohag/2022 (OP777904) shared 99.95% identity with *BoHV-1* from Egypt (MW805266.1), 99.93% identity with *BHV-1.2* from the United States (KM258880.1).

Phylogenetic relationship of reference *BoHV-1* isolates in the phylogenetic tree showed that *BoHV-1* isolate Egypt/Sohag/2022 glycoprotein C (gC) gene partial cds (OP777904) was clustered together with other reference *BoHV-1* isolates in the same clade, while other reference *BoHV-1* isolate BHV-1.2 from Australia (KM258881.1) and *BoHV-1* from Egypt (MW805266.1) were divergent from *BoHV-1* isolate Egypt/Sohag/2022 glycoprotein C (gC) gene partial cds (OP777904) were located in another clade. In another study, Zhou *et al.* (2020) reported that 13 PCR products were sequenced, analyzed, and submitted to the GenBank database based on gC of *BoHV-1*. The percentage of similarity of 13 isolates varied from 97.3-99.3% to BoHV-1.1, 98.0 to100% to BoHV-1.2, and 91.1-92.6% to BoHV-5 (Table 2). The isolates had 3-12 nucleotide mutations compared to COOPER strain.

Comparative sequence analyses of glycoprotein B gene reveal that field *Bovine alphherpesvirus 1* obtained in the current study (OQ161992 and OQ161993) shared 99% to 100% identity on both nucleotide and amino acid sequences in between and 99 to 100% with *BoHV-1* isolates from USA Cooper strain (KU198480.2), and from Egypt (MW805275.1). The commercial vaccines Nasalgen IP MLV vaccine (MH724210.1), and Bovishield Gold FP 5 MLV vaccine (MH724209.1) were 99% and 100% identical to the *BoHV-1* isolates Egypt/Sohag/2022 (gB) (OQ161992 and OQ161993) respectively.

Phylogenetic relationship of reference *BoHV-1* isolates in the phylogenetic tree showed that *BoHV-1* isolates Egypt/Sohag/2022 glycoprotein B (gB) gene partial cds (OQ161992) and (OQ161993) were clustered together with other reference *BoHV-1* in similar subgroup while other reference *BoHV-1* isolates from USA (KM258883.1) and from Egypt (MW805275.1) were not located in the similar subgroup.

Therefore, the obtained isolate was closely related to Cooper reference strain genotype 1.1 and genetically different from *BoHV-5* Cattle/Egypt/2020 (MW805274.1) and *BoHV-1* Cattle/Egypt/2020 (MW805275.1) that locate at separate clade these results were in agreement with Nagy *et al.* (2022). Nagy *et al.* (2022) reported 99.57% identity between *BoHV-1* and the Cooper reference strain. The First detection of *BoHV-5* was achieved by Nagy *et al.* (2022). Also our results were in accordance with Abukhadra *et al.* (2022) who reported 94.194.7% identity between *BoHV- 1.1* and *BoHV-5* using phylogenetic analysis of the gB gene.

The phylogenetic analysis revealed that our isolates (OQ161992 and OQ1993) in the similar cluster with subtype 1.1 of *BoHV.1*, corresponding to the gB gene nucleotide sequences of *BoHV-1* obtained from the field strain that includes the reference Cooper strain and vaccine strains these results were in agreement with Albayrak *et al.* (2020). Albayrak *et al.* (2020) reported 100% nucleotide (nt) homology between *BoHV-1* and the reference Cooper strain. Subtype 1.1 usually comprises strains responsible for respiratory disease. This result is unsurprising, since the current isolate had been detected in cattle with severe respiratory signs (Albayrak *et al.,* 2020). Abukhadra *et al.* (2022) reported that the nucleotide sequence of their isolate was completely identical to the nucleotide sequence of *BoHV-1.1* reference strains and was genetically different from *BoHV-5* reference strains that were located at separate clade by 25 nucleotide substitutions at different locations. These 25 nucleotide substitution led to 5 amino acid substitutions. In another study, Nišavić *et al.* (2018) mentioned that the phylogenetic analysis of the gB gene nt sequences of all four *BoHV-1* strains isolated from Serbia showed that they were 100% similar to each other and branched with *BoHV-1* strains isolated in Egypt and USA.

CONCLUSION

Due to the high percentage of similarity between our isolates and commercially available vaccines containing the Cooper reference strain, which have reached 99.98% to 100% similarity in the gC gene

***Corresponding Author:** Ahmed M.E. Yousef

and 99.99% to 100% similarity in the gB gene, it is advisable to use respiratory viral vaccines such as Naslagen and/or Bovishield Gold FP-5 modified live vaccines as preventive measures against BoHV-1. Imported breed cattle are now not only used for meat consumption but are also reared in Egyptian farms, making them a major source of infection for *BoHV-1*, particularly in the south of Egypt, where respiratory viral vaccines are either scarce or ineffective, resulting in worsening clinical signs and increased economic losses. Good control to BRD depends mainly on vaccination program of cattle against respiratory pathogens in parallel with good management and hygienic measures.

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***Corresponding Author:** Ahmed M.E. Yousef