Research Article

Molecular Identification of Bovine Alpha Herpesvirus Subtype 1.1 in Field Isolates in Egypt by Phylogenetic Analysis of Glycoprotein B Gene

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Abstract | Bovine alphaherpesvirus 1 (BoHV-1) is an enveloped double-stranded DNA virus of *Herpesviridae* family. Closely related alpha herpesviruses; BoHV-1 and Bovine herpes virus-5 (BoHV-5) have the same antigenic and genetic relatedness. BoHV-1 is one of the viruses that incriminated in respiratory disease; affects the genital tract and causes significant economic losses to domestic cattle in Egypt. Although BoHV-5 mainly causes encephalitis in calves, the virus is excreted in nasal discharge under stress factors, so herpesvirus latency reactivation cycle is the hallmark of the disease. In the present study, molecular screening of 60 nasal swabs, collected from clinically suspected animals, was established by polymerase chain reaction (PCR) targeting glycoprotein B (gB) gene. Six PCR positive samples were isolated on chorioallantoic membranes (CAMs) of 11 days old Embryonated Chicken Eggs (ECEs) for three blind passages. The CAMs showed thickening and congestion at 1st passage and typical pock lesions appeared at 3rd passage. Indirect immunofluorescent technique (IFT) was used for confirmation of BoHV-1 presence in CAM; the CAM with clear pock lesions showed yellowish-green fluorescence under the fluorescent microscope. The phylogenetic tree constructed from partial sequencing of gB of our isolate was divided into two clades; clade 1 BoHV-1 (BoHV-1.1 and BoHV-1.2) clade 2 (BoHV-5). Our isolate showed 100% homology to BoHV-1.1 reference strains away from BoHV-5 reference strains that were clustered in the other clade. BoHV-5 reference strains were different from our isolate by 25 nucleotide substitutions, which led to five amino acid substitutions (E316D, A378T, S410G, I425T, E429G).

Keywords | Bovine alphaherpesvirus 1, Glycoprotein B, Immunoflourescent technique, Phylogenetic tree, Nucleotide and amino acid substitution

Received | April 21, 2022; **Accepted** | May 08, 2022; **Published** | July 14, 2022
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Citation | Abukhadra BA, Mosad SM, El-Rahman SA (2022). Molecular identification of bovine alpha herpesvirus subtype 1.1 in field isolates in Egypt by phylogenetic analysis of glycoprotein B gene. Adv. Anim. Vet. Sci. 10(8):1739-1746.
DOI | https://dx.doi.org/10.17582/journal.aavs/2022/10.8.1739.1746
ISSN (Online) | 2307-8316

INTRODUCTION

BoHV-1 is a member of the genus *Varicellovirus* within the subfamily *Alphaherpesvirinae* of the family *Herpesviridae* and order *Herpesvirales* (ICTV, 2019). The BoHV-1 genome is linear double-stranded DNA of approximately 135-139Kbp length and it is classified as a class D herpesvirus genome, it has two distinct regions; a unique long (UL) of approximately 102-104 kbp length and a unique short (US) of about 10.5-11 kbp length. Internal repeats (IR) and terminal repeats (TR) of about 24 kbp length flank the unique short region (Muylkens et al., 2007). Although it was reported that UL region is fixed and the US region can be inverted, it was noticed that the UL region could be inverted in some virions as well (Schynts et al., 2003). BoHV-1 genome codes 33 structural proteins (Misra et al., 1981); ten of these structural proteins are, glycoproteins and associated with the envelop,

arranged as six in the UL region; gK (UL53), gC (UL44), gB (UL27), gH (UL22), gM (UL10), gL (UL1) and four in the US, gG (US4), gD (US6), gI (US7) and gE (US8) (Muylkens et al., 2007).

Restriction endonuclease analysis (REA) divides BoHV-1 into two subtypes; BoHV-1.1 (infectious bovine rhinotracheitis) and BoHV-1.2. Although BoHV-1.2 variants (BoHV-1.2a- BoHV-1.2b) are less virulent than BoHV-1.1, it is associated with genital and respiratory infections (d'Offay et al., 2016). Closely related alphaherpesvirus (BoHV-5) that was previously known as BoHV-1.3 is associated with encephalitis (Nandi et al., 2009). Antigenically and genetically, BoHV-1 and BoHV-5 are similar, it has been reported that BoHV-5 can be occasionally isolated from the respiratory tract because of its reactivation from latency in trigeminal ganglia and its secretion in nasal discharge (Marin et al., 2016; Kumar et al., 2020).

Although the gold standard method of virus isolation can be done in cell culture either in primary cell culture or continuous cell line as Madin Darby Bovine Kidney (MDBK) (Nandi et al., 2009), there are many successive trials for adaptation of the virus on CAM of ECEs and pock lesion was appeared after serial passages (Samrath et al., 2016)

The field diagnosis is not enough to detect BoHV-1 as the virus is miss diagnosed with many etiological agents or due to mixed infection so laboratory diagnosis is critical (Gu and Kirkland, 2008). PCR represents an excellent, sensitive and rapid molecular diagnostic tool than isolation to detect BoHV-1 (Nandi et al., 2009). The major envelope glycoproteins of BoHV-1 are gB, gC, gD (Niță et al., 2010), glycoprotein B (gB) is a highly conserved gene and acts as a special target for BoHV-1 detection (Ros and Belák, 1999). Despite there were homology of gB genes among different strains of BoHV-1 (Whetstone et al., 1993), gB gene has enough diversity which allows the building of a phylogenetic tree that can be used to divide the virus into subtypes (Surendra, 2015; Kumar et al., 2020).

The aim of this study is molecular identification of BoHV-1 by conventional PCR and establishes baseline information about Egyptian subtypes based on phylogenetic analysis of BoHV-1 isolated on CAM of ECE.

MATERIALS AND METHODS

Samples collection

From seven different localities in different governorates in Egypt, sixty nasal swabs were collected during years 2020 and 2021 from cattle of different ages suffering from severe respiratory signs including red noses, open mouth breathing, lacrimation, and corneal opacity (Figure 1). All samples were prepared according to OIE (2018) for molecular detection and isolation of the virus (Table 1).

Figure 1: Clinically diseased animals suspected to be infected with BoHV-1 showing: (A) Red nose and nasal discharge; (B) open mouth breathing; (C) Corneal opacity.

Sampling was performed according to relevant national regulations recommended by the ethical committee of the faculty of veterinary medicine. Veterinarian supervisors of infected farms and cattle collected all sample

BoHV-1 reference Ag

Reference BoHV-1 (Bovilis IBR marker vaccine, MSD, USA) with a titer of $10^{5.7}$ TCID50/ml. It was used as a positive control in PCR and for the preparation of hyperimmune serum in rabbits.

Conventional PCR and gel electrophoresis for detection of the virus in original samples

DNA extraction

Viral DNA was extracted from the supernatant of nasal swab original samples using QIAamp ® Mini Elute ® virus spin Kit (QIAGEN, GmbH, Hilden, Germany) according to kit instructions. Bovilis IBR modified live vaccine (MSD, USA) used as a positive control sample and PBS as a negative control in PCR

Conventional PCR

Conventional PCR assay was used for molecular screening of BoHV-1 in original samples using a previously reported specific set of primers synthetized by (Metabion, Germany) described in (Table 2) for amplification of 478bp gB gene product (Fuchs et al., 1999). Dream Taq Green PCR Master Mix (Thermo Scientific, USA) was used as flow, 25 µl of 2x Dream Taq Green PCR Mix, 1 µl of each primer (10pmol/ µl), 5 µl of DNA template and up to 50 µl of nuclease-free water. The mixture was subjected to an adjusted thermocycler (Biometra, Germany) under the following conditions; an initial denaturation step of 15 min at 95°C, 40 cycles consisting of 45 sec at 95°C (denaturation), 45 sec at 56°C (annealing), 1 min at 72°C (extension) and 1 cycle for 10 min at 72°C (final extension) (Albayrak and Ozan, 2020).

Table 1: Data of collected samples including (sampling localities, No of samples, type of samples, average age, clinical signs, and history of vaccination.

Sampling localities	No of samples	Type of samples	Average age	Clinical signs	History of vaccination
Ismailia farm 1	4	Nasal swabs	6 months	Severe respiratory signs	animals were vaccinated by live deleted marker vaccine at 2 weeks – 3 months of age
Damietta farm 2	5	Nasal swabs	4-6 months	Severe respiratory signs respiratory signs	Non vaccinated
	5	Nasal swabs	3-4 years	The first evidence of abortion on the farm was noticed about 1 year prior to the sampling	
Nubariya farm3	5	Nasal swab samples were taken after 3 days from emergency vaccination.	4-6 months	Respiratory signs include open-mouth breathing corneal opacity	Emergency vaccination I/m by cattle master 4
	2	Nasal swab samples were taken after 20 days from emergency vaccination	Different ages	Apparently healthy animals	
Banha farm 4	12	Nasal swabs	4-6 months	respiratory signs(lacrimation, cataract	Non vaccinated
Sporadic cases in Banha	2	Nasal swabs	3 months	Fever-respiratory signs- red nose-salivation	Non vaccinated
Nubariya farm 5	6	Nasal swabs samples were taken 3 days after vaccination	Newly-born	Fever-respiratory signs-(multisystemic effect and animals dead after 5 days from birth History of abortion in cows in the same farm and Pustules on vulva and vagina	Emergency vaccination by live deleted marker vaccine
Gamasa farm 6	5	Nasal swabs	4-6 months	Respiratory signs with a red nose	Non vaccinated
Demiana farm 7	14	Nasal swabs	3-4 years	Fever, respiratory signs nasal lacrimation The farm suffering from history of late abortion, decrease in milk production, dead newly born calves	Non vaccinated

Table 2: Details of BoHV-1 gB primers used in the study including (target gene, primer sequence, annealing temperature, product size, and reference).

Target gene	Primer sequence 5'-3'	Annealing temperature	Product size	References
gB (UL27)	Forward TACGACTCGTTCGCGCTCTC Reverse GGTACGTCTCCAAGCTGCCC	56°C	478	(Fuchs *et al.*, 1999)

Gel electrophoresis

The obtained PCR products were visualized by gel electrophoresis in 1% agarose gel (Sigma, USA) stained with ethidium bromide (0.5 µg/mL) and run at 80 mV for 1 h in 1X TAE buffer against Gene Ruler 100pb plus DNA Ladder (Thermoscientific, USA).

Virus isolation

Embryonated chicken eggs (Specific pathogen-free)

For the adaptation of the virus in ECEs, the virus was isolated on CAM of ECEs through inoculation of 200µl supernatant fluid of nasal swabs PCR positive samples in 11days old specific pathogen-free (SPF) embryonated chicken eggs (Nile SPF, Koam Oshiem, Fayoum, Egypt) for 3 serial blind passages. The inoculated eggs were incubated at 37 °C for 6 days and the harvested CAM were examined for pathological lesions (Samrath et al., 2016).

Serological diagnosis of BoHV-1

Preparation of hyperimmune serum in rabbits

Polyclonal antibodies were prepared according to Bashir et al. (2011). With some modifications as follows 2ml of

BoHV-1 reference Ag was mixed with an equal volume of Montanide oil (SEPPIC, France), inoculated I/M four times with one week interval and the serum was collected after one week from the last inoculation.

Indirect immunofluorescent technique (IFT)

Indirect IFT was done for confirmation of BoHV-1 presence in CAM. CAM showed clear pock lesions was Cryosectioned then fixed in acetone 80%. Polyclonal antibodies were diluted at 1:100 in PBS, then added to cryosections and incubated in a humid chamber at 37 ˚C/1hr. The sections were then washed three times with PBS (5 minutes each). Secondary antibodies conjugated with Fluorescein isothiocyanate (FICT) (Anti- rabbit IgG FICT antibody produced in goat, Sigma, USA) diluted 1:50 in PBS were incubated on CAM at 37 ˚C/1hr then washed three times with PBS (5 minutes each) before the examination. The slides were examined for yellowish-green fluorescence under the fluorescent microscope (El-Kholy, 2005).

Molecular identification of isolated sample using PCR and gel electrophoresis

The CAMs of ECEs that showed clear pock lesion were homogenized with PBS and antibiotic solution using sterile mortar and pestle to make 10% suspension then centrifuged and the supernatant was taken for DNA extraction, PCR and gel electrophoresis as described before in (2.3.1, 2 and 3).

Sequence analysis

PCR product (478bp), which targeted BoHV-1gB of an isolated sample that had the sharpest band on gel electrophoresis, was purified from the gel using the QIAquick Gel Extraction kit (Qiagen Inc., Valencia, CA, and USA). Bi-directional DNA sequencing was performed on gel purified DNA at (Macrogen, South Korea). Obtained DNA sequence was deposited to GenBank with accession number OM803183. Basic Local Alignment Search Tool (BLAST®) was established to investigate sequence similarity between sequences deposited in GenBank and our sequence (Altschul et al., 1990) using Clustal W (http://www.ebi.ac.uk/Tools/msa/clustalw2). Neighbor-joining phylogenetic tree was constructed with 1000 repeat bootstrap tests and identity percentages were calculated using MEGA X software (http://www.megasoftware.net/). Nucleotide and deduced amino acid sequence analysis was done using Bioedit software version 7.1 (Hall, 1999).

RESULTS AND DISCUSSION

Molecular identification of BoHV-1

Sixty nasal swabs from clinically suspected animals were subjected to conventional PCR for amplification of gB using a specific set of primers. Electrophoresis of amplified products revealed six positive samples (10%) with correct expected band size (478bp). The control positive (BoHV-1 reference Ag) had the same amplicon size (478bp) as the samples without a significant difference between them.

Isolation of virus on CAM of ECEs

The supernatant fluids of six PCR positive samples were inoculated on CAMs of 11 days old SPF-ECES for three successive passages. The lesion on CAM progressed after serial passages. Firstly appeared as thickening, edema and congestion of CAM then small pock lesions appeared in the second passage which increased in size and number in the third passage (Figure 2).

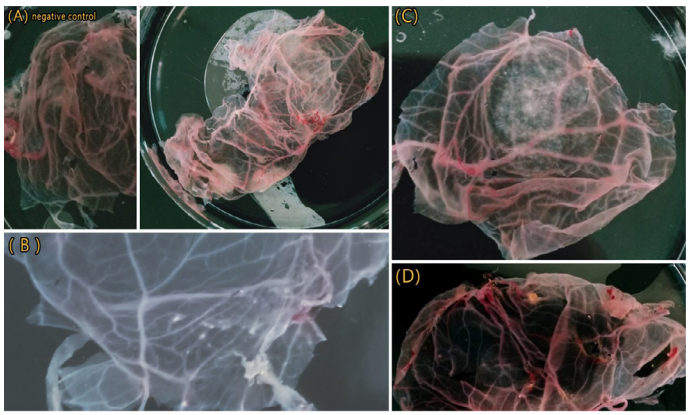

Figure 2: Results of BoHV-1 isolation on CAM showing. (A) Control negative and CAM of 1st passage showing thickening and congestion. (B) CAM of 2nd passage showing Small number of pocks. (C) CAM of 3rd passage showed typical pock lesion. (D) CAM of 3rd passage showed small and large pock lesion.

Serological diagnosis of BoHV-1 using IFT

Chorioallantoic membranes that showed clear pock lesions at 3rd passage was examined by IFT. Yellowish-green fluorescence was detected in examined CAM under the fluorescent microscope (Figure 3).

Phylogenetic analysis of gB gene of isolated BoHV-1

The neighbor-joining tree was constructed based on partial sequencing of gB (UL27) gene of BoHV-1 isolate that had the sharpest band on gel electrophoresis (Figure 4). The phylogenetic tree revealed the genetic relatedness among different strains of BoHV-1 and was divided into two clades; clade 1 BoHV-1 (BoHV-1.1 and BoHV-1.2) and clade 2 (BoHV-5). Our isolate showed 100% homology with BoHV1.1 reference strains from the USA with accession no (AJ004801, KU198480 and MG407777), (99.7%) with BoHV-1.2 reference strain with accession no (KM258881). BoHV-5 reference strains were clustered in a separate clade, as they were genetically different from our

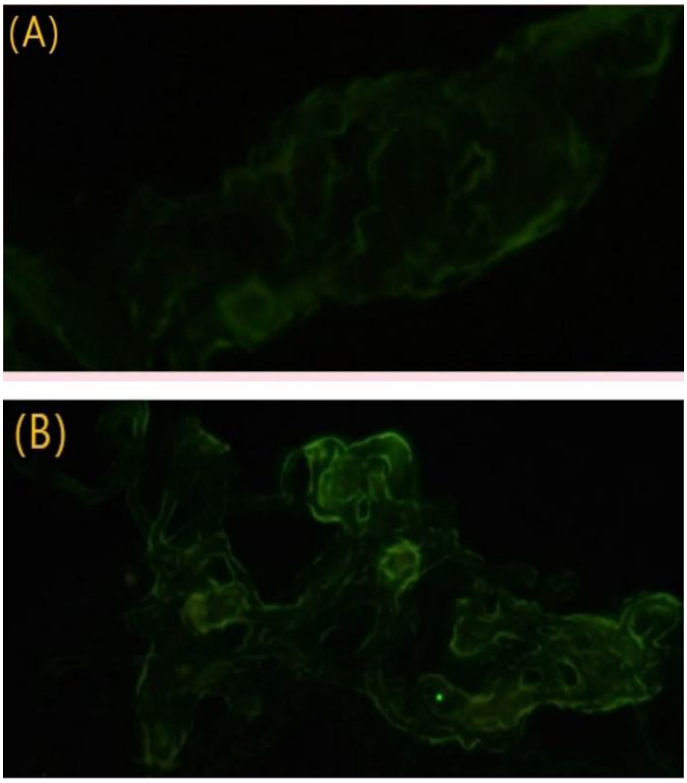

Figure 3: Result of IFT from CAMs. (A) Control negative; (B) CAM from the third passage shows yellowish-green fluorescence.

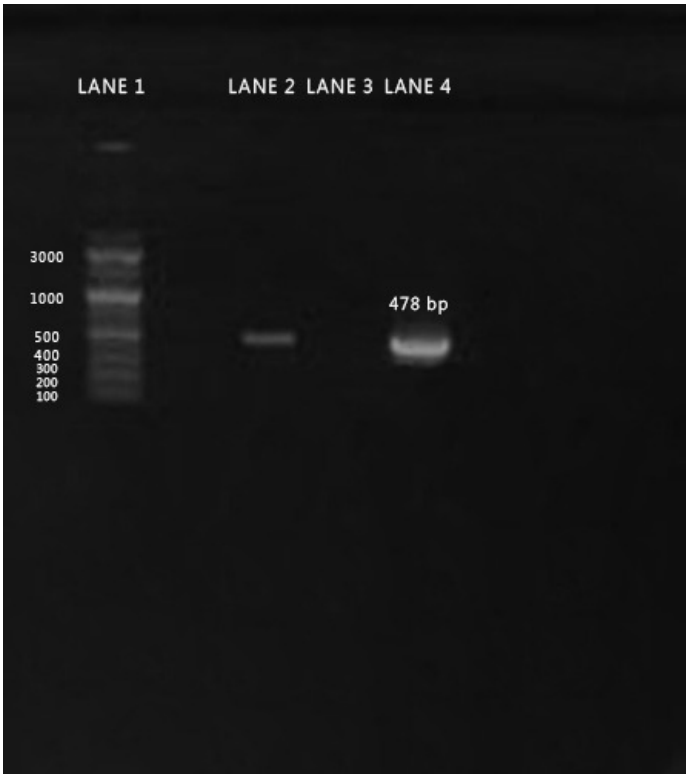

Figure 4: Agarose gel electrophoresis of PCR amplicon for gB gene of BoHV-1isolate separated on 1% agarose gel and stained with ethidium bromide (Positive PCR amplicons are at the size of approximately 478 bp). Lane 1 denotes for 100 bp DNA Ladder, Lane 2: Positive sample, Lane 3 negative control. Lane 4: positive control.

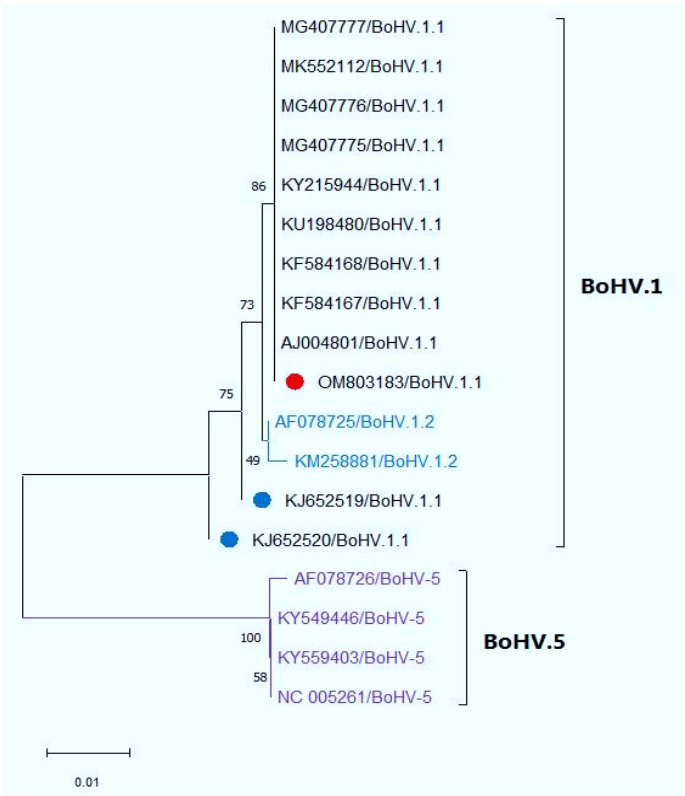

Figure 5: Neighbor-Joining tree was constructed based on partial sequencing of the gB gene showing the genetic relatedness of our Egyptian isolate (OM803183) to other reference strains from GenBank. Our isolate showed 100% homology to BoHV1.1 reference strains Including Egyptian BoHV-1.1 reference strains (blue circle) with query coverage 63% but away from BoHV-5, which are located in a separate clade.

isolate with (94.1-94.7%) identity (Figure 5). Nucleotide and deduced amino acid sequence analysis between our isolate and reference strains from Genbank were done to determine the difference between BoHV-1 subtypes. Nucleotide alignment of 431bp (corresponding to position 885-1315 of AJ004801) of our isolate showed complete similarity to BoHV-1.1 reference strains from Genbank. BoHV-1.1 and BoHV-1.2 reference strains were identical with two nucleotide substitutions in one strain of BoHV-1.2 (KM258881) without amino acid substitution. BoHV-5 reference strains were identical to each other and have 25 nucleotide substitutions (transition and transversion) in comparison to BoHV-1 (BoHV-1.1 and BoHV-1.2) (Figure 6), these 25 nucleotide substitutions led to 5 amino acid substitutions (Figure 7, Table 3).

BoHV-1 is usually misfield diagnosed in our Egyptian farms because the virus has a multi-systemic effect on cattle, it affects the respiratory tract, genital tract, in addition to fetal multi- systemic infection and occasionally encephalitis in calves so the concern should be directed nowadays to laboratory BoHV-1 detection (Gu and Kirkland, 2008; Nandi et al., 2009). Controlling of BoHV-1 in Egypt is challenged by the latency of the virus although massive routine vaccination programs are used.

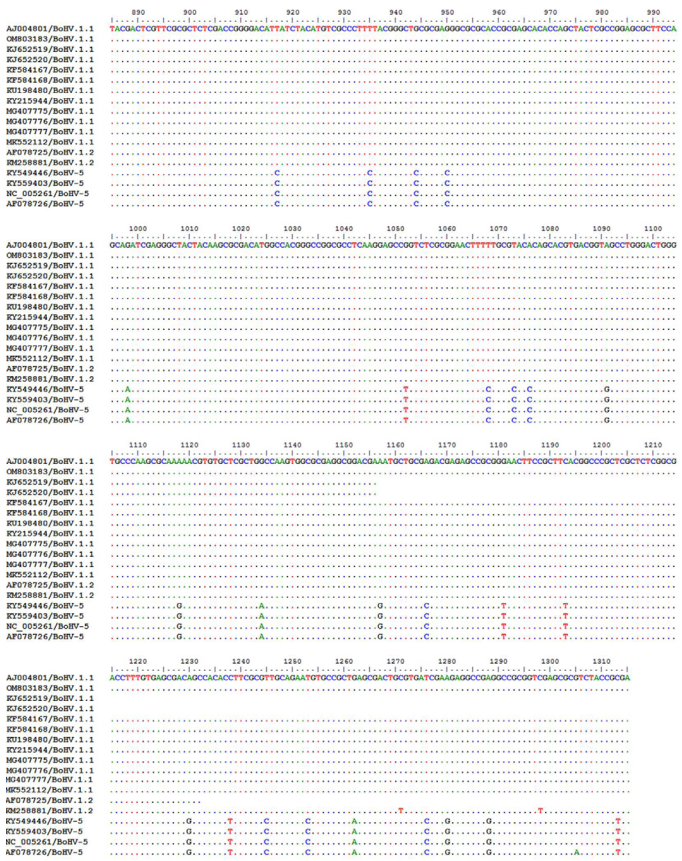

Figure 6: Nucleotide sequence alignment of our isolate in comparison to other strains from Genbank with AJ004801 as reference strain using Bioedit software version 7.1

Table 3: Describe deduced amino acid sequence alignment of gB gene of BoHV-1 isolate with accession no (OM803183) and references strains from Genebank.

Accession no	BoHV-1 subtype	Sequence code of AJ004801				
		316	378	410	425	429
AJ004801	BoHV-1.1	E	A	S	I	E
OM803183	BoHV-1.1	E	A	S	I	E
KJ652519	BoHV-1.1	E	A	S	I	E
KJ652520	BoHV-1.1	E	A	S	I	E
KF584167	BoHV-1.1	E	A	S	I	E
KF584168	BoHV-1.1	E	A	S	I	E
KU198480	BoHV-1.1	E	A	S	I	E
KY215944	BoHV-1.1	E	A	S	I	E
MG407775	BoHV-1.1	E	A	S	I	E
MG407776	BoHV-1.1	E	A	S	I	E
MG407777	BoHV-1.1	E	A	S	I	E
MK552112	BoHV-1.1	E	A	S	I	E
AF078725	BoHV-1.2	E	A	S	I	E
KM258881	BoHV-1.2	E	A	S	I	E
KY549446	BoHV-5	D	T	G	T	G
KY559403	BoHV-5	D	T	G	T	G
NC_005261	BoHV-5	D	T	G	T	G
AF078726	BoHV-5	D	T	G	T	G

A: Alanine; D: Aspartic acid; E: Glutamic acid; G: Glycine; I: Isoleucine; T: Threonine; V: Valine

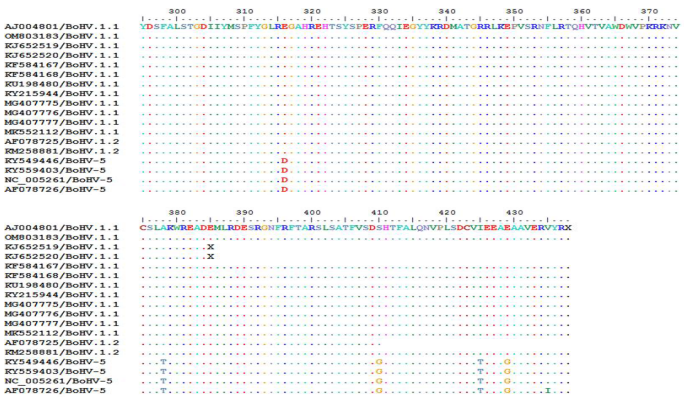

Figure 7: Deduced amino acid sequence alignment of our isolate in comparison to other strains from Genbank with AJ004801 as reference strain using Bioedit software version 7.1

BoHV-1 could be divided into two subtypes; BoHV-1.1 and BoHV-1.2 that divided by REA into (BoHV-1.2a and BoHV-1.2b) (Metzler et al., 1985). BoHV-1.1 is mainly associated with respiratory signs and abortion (Jones and Chowdhury, 2007). Although BoHV-1.2 is mainly associated with genital affection, BoHV-1.2b was previously reported to cause respiratory infection (d'Offay et al., 2016). BoHV-5 is mainly associated with encephalitis however, it was reported that BoHV-5 can be replicated in the respiratory system and a large number of viruses were shed in nasal discharge during the acute phase of infection. It's difficult to differentiate between BoHV-1 and BoHV-5 serologically because they are antigenically and genetically closely related to each other (Del Médico Zajac et al., 2010). In the present study, sixty nasal swabs were collected from seven different localities in different governorates in Egypt from diseased cattle suspected to be infected with BoHV-1. All samples were firstly subjected to PCR for amplification of gB gene (478bp) (Fuchs et al, 1999). Glycoprotein B was selected because its a highly conserved gene and a special target for designing primers used for BoHV-1 detection (Ros and Belák, 2002). Of sixty tested samples, six samples gave a positive result in PCR these results indicated that the prevalence of BoHV-1 obtained in this study was 10% in cattle these results were in agreement with Zeedan et al. (2018), who found that 10 % of nasal swab samples collected from Beni-suef from cattle gave positive result in PCR targeting gB while El-Kholy (2005) found that the prevalence of the virus in cattle was 16% using nested PCR targeting gB. Six PCR positive samples were isolated on CAM for 3 serial blind passages. Firstly the samples showed congestion and thickening of CAM at 1st passage, Then typical pock lesions that were appeared in 2nd passage increased in number and size after serial passages these results were in concurrence with Samrath et al. (2016) and Zeedan et al. (2018), who isolated the BoHV-1 on CAM of ECEs producing pock lesions.

Immunofluorescent detection of the virus was done on CAMs with clear pock lesions and positive yellowish-green fluorescence was detected, this result was in accordance with El-Kholy (2005), who confirmed the BoHV-1 presence by IFT in MDBK cells.

Many studies were developed to differentiate between BoHV-1 and BoHV-5 using restriction endonuclease analysis, conventional PCR targeting gB and gC (Metzler et al., 1985; Ros and Belak, 2002; Esteves et al., 2008) and real-time PCR (Marin et al., 2016). The first report of molecular characterization of BoHV-1 in Egypt based on partial sequencing of gB, gC, gE and gD revealed that the Egyptian BoHV-1 strain showed 100% homology to BoHV-1.1 reference strains (Sobhy et al., 2014). Scanty phylogenetic analysis studies that were previously performed in Egypt revealed that the Egyptian strain belong to subtype BoHV-1.1 while BoHV-1.2 and BoHV-5 are not reported in Egypt and need further investigation. Therefore, we constructed our study to investigate the circulating BoHV-1 subtype based on phylogenetic analysis of partially sequenced gB gene of BoHV-1 isolate.

PCR product (478bp) of BoHV-1 isolate that had sharpest band on gel electrophoresis was sequenced using the Sanger method and sequence analysis revealed 431bp due to vector trimming and generating consensus sequence of forward and reverse primers. The sequence (431bp) of our isolate with accession no. (OM803183) corresponding to the position from (885-1315) of cooper strain (AJ004801) was aligned with BoHV-1.1, BoHV-1.2 and BoHV-5 reference strains. The neighbor-joining tree was constructed based on a partial sequence of gB gene of our isolate. The phylogenetic tree was divided into two clades; clade 1 BoHV-1 (BoHV-1.1 and BoHV-1.2) and clade 2 BoHV-5. Our isolate showed 100% homology to BoHV-1.1 reference strains (AJ004801, KU198480), 99.7% to BoHV-1.2 reference strain with accession no (KM258881) and (94.1-94.7%) to BoHV-5 reference strain. So our isolate was genetically different from BoHV-5 reference strains that locate at separate clade these results were in agreement with Ros and Belák (2002). Ros and Belák reported that the BoHV-1.1 was genetical different from BoHV-5 with 95.5% identity based on phylogenetic analysis of partial gB gene sequence analysis. Also our results were in accordance with Delhon et al. (2003) who reported 93% identity between BoHV-1.1 and BoHV-5 using phylogenetic analysis of gB gene. Nucleotide alignment and deduced amino acid analysis of our isolate to reference strains from Genbank were used to distinguish between BoHV-1 subtypes. The nucleotide sequence of our 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 rsults were in concurrence with Del Médico Zajac et al. (2010) who reported that deduced amino acid sequence alignment between BoHV-1 and BoHV-5 reference strains showed average more than 90% identity among glycoprotein B of different strains. These 25 nucleotide substitution led to 5 amino acid substitutions (E316D, A378T, S410G, I425T, E429G) which were considered the point of difference in our study between BoHV-1 and BoHV-5.

CONCLUSIONS AND RECOMMENDATION

Phylogenetic analysis of partially sequenced gB gene of our isolate gives worthy information about circulating Egyptian BoHV-1 subtype (BoHV-1.1) which is different from BoHV-5 by 25 nucleotide subistiutions, 5 amino acid substitution (E316D, A378T, S410G, I425T, E429G). finally we found that unlike many studies restricted to gC, gB has enough diversity to distinguish between closely related alphaherpesviruses (BoHV-1 and BoHV-5). Understanding of circulating Egyptian BoHV-1 subtype will help us in the prevention and control of the virus by using commercially available vaccines containing Egyptian BoHV-1 subtype. Further investigation should be applied to give more information about Egyptian BoHV-1 subtypes with evidence of BoHV1.2 and BoHV-5 that were not reported in Egypt.

NOVELTY STATEMENT

BoHV-1.1 is the circulating subtype in field isolate in Egypt based on molecular characterization of BoHV-1gB gene. phylogenetic analysis of BoHV-1gB sequence product is a promising tool to differentiate between closely related alpha-herpes viruses (BoHV-1 and BoHV-5)

AUTHOR'S CONTRIBUTION

All authors except the first one had supervision contribution. The first to third author had the major contribution in paper writing, editing, and reviewing. The second author participate in practical part. finally the corresponding author is the third author.

Conflict of interest

The authors have declared no conflict of interest.

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