

Comparative molecular characterization of bovine herpesvirus-1 strains from Egypt and the United States

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Abstract: Bovine herpesvirus 1 (BoHV-1) is an important contagious viral pathogen negatively affecting the livestock industry because of respiratory illness and /or abortion. Most of the disease protection programmes in Egypt depend on imported vaccines and unfortunately molecular studies on molecular and phylogenetic analysis of viral strains are rare. Such studies are needed to determine which viral strains should be used in vaccine manufacture so as to provide proper protection to susceptible animals in Egypt. The gB, gC, gD and gE glycoproteins of BoHV-1 are the most important because of their functional properties including virus entry into target cells, viral gene expression, DNA replication, and phylogenetic relationships. This is the first report in which glycoproteins of Egyptian BoHV-1.1 virulent strains have been sequenced and compared with U.S. strains, thus increasing our understanding about the genetic relatedness of various strains of BoHV-1 and between bovine alphaherpesviruses (BoHV-1 and BoHV-5). Our results indicate that the Egyptian Abu-Hammad strains are 100% similar to each other and with U.S. Cooper reference strain suggesting that vaccines based on the latter strain are adequate in providing protection to susceptible animals in Egypt.

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1. Introduction

Bovine herpes virus type 1 (BoHV-1) is an economically important pathogen of domestic and wild *Bovidae* and is distributed worldwide causing respiratory tract manifestations and/or abortion (El-Kholy and Abdelrahman, 2006; Tikoo et al., 1995). The BoHV-1 has a positive sense double stranded DNA genome and belongs to *Varicellovirus* genus of *Alphaherpesviridae* subfamily within the family *Herpesviridae*. Coding sequences of the BoHV-1 genome are named gB (gI), gC (gIII), gD (IV), gE, gG, gH (gII), gI, gK, gL and gM, in accordance to their homology with herpes simplex virus 1 (HSV-1), which is a prototype of the subfamily *Alphaherpesviridae*. Six glycoprotein genes gB (UL27), gC (UL44), gH (UL22), gK (UL53), gL (UL1) and gM (UL10) are located in the unique long region (UL) of the genome and the remaining four genes gD (US6), gE (US8), gG (US4) and gI (US7), are clustered in the unique short region (Tikoo et al., 1995).

Outbreaks of respiratory illness in cattle due to BoHV-1.1 infections have occurred occasionally in Egypt since 1976 (Mahmoud et al., 2009; Elshemy

and Hassan, 2010). The virus has also been isolated from other animals such as sheep (Mahmoud and Ahmed, 2009) and camel (Abou-Zaid et al., 2001). The control of the disease in Egypt depends mainly on vaccination with either USA imported vaccine (Cattle Master-4), which contains a unique combination of killed BVDV (bovine viral diarrhoea virus - two strains), modified live BRSV (bovine respiratory syncytial virus), and temperature sensitive BoHV-1 and PI3 (parainfluenza virus type 3) or with a locally prepared vaccine (Pneumo-3) which contains IBR, PI-3 and BVDV (Fayed et al., 2013).

Studies on molecular characterization of Egyptian strains of BoHV-1 are not available. This information would be helpful for comparing old and new virus strains and to determine which strains can be used in the vaccine manufacture. This study was undertaken to characterize and compare Egyptian and American strains of BoHV-1. For this purpose, we relied on the characterization of four viral genes e.g., gB, gC, gD and gE, which are essential for virus penetration (gB), attachment of virions to target cells (gC), induction of a strong and consistent cellular immune response (gD), and facilitation of cell-to-cell

spread of the virus in vivo (gE) (Biewett and Misra, 1991; Chowdhury, 1997; Lyaku et al., 1999; Kevin et al., 1994).

In general, BoHV-1 is divided into 2 subtypes: respiratory isolates (BoHV-1.1) and genital isolates (BoHV-1.2) (Rijsewijk et al., 1999; Spilki et al., 2005). The BoHV-5, which was previously classified as BoHV-1.3, has historically been associated with acute meningoencephalitis in calves (Chowdhury et al., 2000; Traesel et al., 2013) and genital tract infections in cattle (Esteves et al., 2003). Despite the high similarity, differentiation among BoHV-1.1, BoHV-1.2 and BoHV-5 can be achieved by antigenic or molecular analysis of gC (Claus et al., 2005; Silva et al., 2007). In this context, the aim of the present study was to molecularly characterize gB, gC, gD and gE glycoproteins of Egyptian BoHV-1 and to compare them with US strains to determine which strains should be included in vaccine manufacture.

2. Materials and Methods

2.1. Viruses, cells, and DNA extraction

The six US strains of BoHV-1 used in this study were isolated at the University of Minnesota, Veterinary Diagnostic Laboratory (MVDL), from 2004 to 2012 from cows suffering from respiratory illness. The two Egyptian strains of BoHV-1 were isolated at the Veterinary Serum and Vaccine Institute-Abbasia, Cairo, Egypt, from cattle with cough, nasal discharge and conjunctivitis (Table 1). The viruses from Egypt were transported to Minnesota on classic Whatman FTA cards which results in the lysis of all proteins but maintains viral DNA (Liang et al., 2014). The six U.S. strains of BoHV-1 were propagated on Madin-Darby bovine kidney (MDBK) cell monolayers maintained in MEM with Earle's salts supplemented with 2% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. Inoculated cells were harvested when the cytopathic effects (CPE) involved about 80% of the monolayer and then centrifuged at 1,200 xg for 10 min to remove cellular debris. Viral DNA from supernatants of infected cells and from FTA cards was extracted using the DNeasy blood and tissue kit (Qiagen, Valencia, CA) following manufacturer's instructions.

2.2. Polymerase chain reaction (PCR)

Primers were designed to amplify 668, 575, 412, and 425 bp of gB, gC, gD and gE genes, respectively (Table 2). Extracted DNA was subjected to PCR using HotStarTaq master mix kit (Qiagen, Valencia, CA). In case of gC we added 1µl of glycerol 5% in the reaction mixture. Amplification reactions (25µl reaction mixture) were performed in a thermocycler (Mastercycler Eppendorf) under the following conditions: an initial denaturation step of 15 min at 95°C, followed by 35 cycles of 1 min at 94°C (denaturation); 1 min at 58°C for gC, 52°C for gD and 51°C for both gB and gE (annealing); 1 min at 72°C (extension) and one final extension step of 10 min at 72°C. The PCR products were analyzed by 1.2% agarose gel electrophoresis in Tris- acetate-EDTA buffer followed by staining with ethidium bromide. A single band of expected product size confirmed the presence of target glycoprotein.

2.3. Sequencing of purified PCR products

The PCR products were purified using QIAquick PCR purification kit (Qiagen) as per manufacturer's instructions. The purified PCR products were submitted to University of Minnesota Genomics Center (UMGC) for sequencing with the same forward and reverse primers as used in PCR reaction with the dideoxy chain termination method and primer walking strategy. The obtained sequences were curated and aligned using "Sequencher 5.1" software (<https://genecodes.com>) followed by BLAST analysis in GenBank data base for comparing with other BoHV sequences. The compatible nucleotide (nt) sequences were aligned by using the Clustal W option in MEGA 6.0 (Molecular Evolutionary Genetic Analysis) to obtain a consensus sequence. The phylogenetic correlation comparison and tree construction were also done by using MEGA 6.0. A phylogenetic tree of aligned sequences was constructed by selecting the best fit Maximum Likelihood model (Tamura 3-parameter) in Mega 6.0 based on lowest BIC score (Bayesian Information Criterion) (Tamura et al., 2013). The GenBank accession numbers for these genes are shown in Table 3.

Table 1: Bovine herpesviruses used.

S. No.	Year of isolation	State /Country	Sample type	Virus name
1	2004	Minnesota/USA	Lung homogenate	BHV-1/cattle/ MN1/USA/ 2004
2	2008	Minnesota/USA	Stomach contents	BHV-1/cattle /MN2/USA/ 2008
3	2009	Minnesota/USA	Lung homogenate	BHV-1/cattle /MN3/USA/2009
4	2010	Minnesota/USA	Eye swab	BHV-1/cattle /MN4/USA/ 2010
5	2010	Minnesota/USA	Nasal swab	BHV-1/cattle /MN5/USA /2010
6	2012	Minnesota/USA	Nasal swab	BHV-1/cattle /MN6/USA/ 2012
7	2013	Sharkia/Egypt	Nasal swab	BHV-1/cattle/ Abu-Hammad/ Egypt/1/ 2013
8	2013	Sharkia/Egypt	Nasal swab	BHV-1/cattle/ Abu- Hammad/ Egypt/2/ 2013

Table 2: Forward and reverse primers with size and annealing temperatures.

Primer name	Primer sequence	Annealing temperature	Amplicon size	Sequence Region
BHV-1 GB	F 5'-GTA CAC GTT CAA GGC CTA CA-'3 R 5'- TCG TCT CGC AGC ATT TC- '3	51 °C	668bp	56067-56086 56719-56735
BHV-1 GC	F 5'-CGG CCA CGA CGC TGA CGA- '3 R 5'-CGC CGC CGA GTA CTA CCC- '3	58 °C	575 bp	16786-16803 17360-17343
BHV-1 GD	F 5'-GGA AGC ACT TTG GGT ACT GC-'3 R 5'-TAG CCC TTC GAC TCC TCA AA- '3	52 °C	412bp	392-411 784-803
BHV-1 GE	F 5'-GCG ATT CCT TTC TGC TAT CG- '3 R 5'-GCT GTA GTC CCA AGC TTC CA- '3	51 °C	425bp	721-740 1126-1145

Table 3: GeneBank accession numbers for glycoprotein of BHV-1 isolates.

Virus name	GenBank accession numbers for indicated glycoproteins			
	gB	gC	gD	gE
BHV-1/cattle/ MN1/USA/ 2004	KJ652513	KJ652521	KJ652529	KJ652505
BHV-1/cattle /MN2/USA/ 2008	KJ652514	KJ652522	KJ652530	KJ652506
BHV-1/cattle /MN3/USA/2009	KJ652515	KJ652523	KJ652531	KJ652507
BHV-1/cattle /MN4/USA/ 2010	KJ652516	KJ652524	KJ652532	KJ652508
BHV-1/cattle /MN5/USA /2010	KJ652517	KJ652525	KJ652533	KJ652509
BHV-1/cattle /MN6/USA/ 2012	KJ652518	KJ652526	KJ652534	KJ652510
BHV-1/cattle/ Abu-Hammad/ Egypt/1/ 2013	KJ652519	KJ652527	KJ652535	KJ652511
BHV-1/cattle/ Abu- Hammad/ Egypt/2/2013	KJ652520	KJ652528	KJ652536	KJ652512

3. Results

The identity of viral strains was confirmed by PCR and sequencing of glycoproteins gB, gC, gD and gE (Table 3). Even though the eight viruses were isolated from different sample types and from different years (Table 1), there was no change in glycoprotein sequences. Molecular and phylogenetic analysis of nt sequences of the four genes (gB, gC, gD and gE) of Egyptian Abu- Hammad strains and Minnesota strains revealed 100% similarity among them. The results of phylogenetic analysis of different glycoproteins are given below.

3.1. Phylogenetic analysis of glycoprotein B: The Egyptian and US strains were 100% similar to the US Cooper (JX898220) strain and to BoHV-1.1 strains from Brazil (DQ006855, DQ006851 and AY745877.1) and India (KF734608 and KF734615.1). The pairwise distance analysis showed that there was 100% similarity between BoHV-1.1 and BoHV-1.2 Indian strains (JX127203 and JF927975) while 96% similarity was detected between BoHV-1 and BoHV-5 from Brazil (AY261359 and AY745876) and Switzerland (AF078726). The latter three strains were found in another cluster but were still related to the ancestor (Figure 1).

3.2. Phylogenetic analysis of glycoprotein C: The nucleotide identity was 100% with US Cooper (JN173204 and DQ173733), Netherlands LAM

(JN173205) and Croatia (GQ169130) strains. The similarity was 99% with Brazilian (JN173209 and JN173219) and Indian (JX127204 and KC682099) strains of BoHV-1.2 while similarity with U.S. (U35883) and Brazilian strains (DQ173725) of BoHV-5 was 90% (Figure 2).

3.3. Phylogenetic analysis of glycoprotein D: The nt identity based on sequence alignment and phylogenetic analysis of Egyptian isolates with previously published Egyptian vaccinal strain (AY690484) was 99%. We found six nt insertions (119291 G, 119324 G, 119372 T, 119403 C, 119412 G, 119430 A) and three nt substitutions (A 119382 G, G 119383 A, A 119384 T) that were unique to the Egyptian vaccine strain. Nucleotide similarity with US Cooper (JX898220 and Z98199) and Chinese (JN787954) strains was 100%. The nt similarity was 100% with Indian (JX127205) BoHV-1.2 strain and 88% with US (U14656) BoHV-5 strain (Figure 3).

3.4. Phylogenetic analysis of glycoprotein E: On comparison with previously published Abu-Hammad vaccinal strain sequences from Egypt, all isolates had 100% similarity with Egypt/2008 (EU850282). The phylogenetic analysis and pairwise distance analysis showed 100% similarity with US Cooper (JX898220 and Z98199), Sweden (AF133121), and China (GU591891) strains. The isolate similarity was 100% with BoHV-1.2 (Z23068) and 88% with US strains of BoHV-5 (AY261359 and AF208294) (Figure 4).

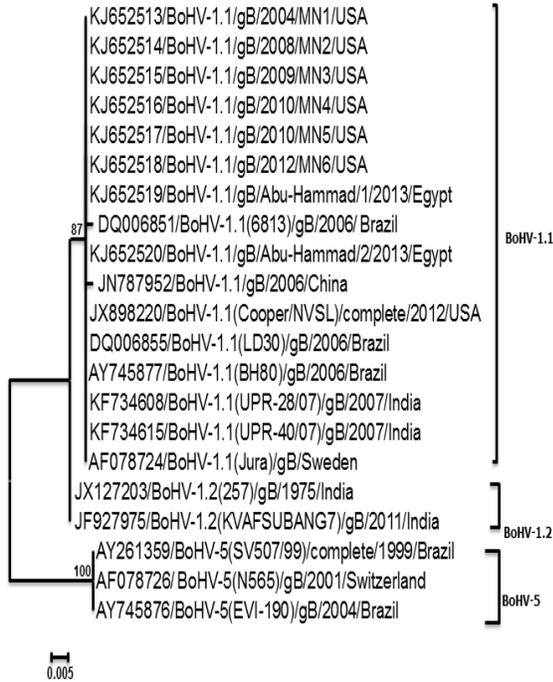


Figure 1. Phylogenetic tree constructed by neighbor-joining method on the basis of partial sequences of the gB gene. The tree shows relationship of Egyptian BHV-1.1 isolates with other strains.

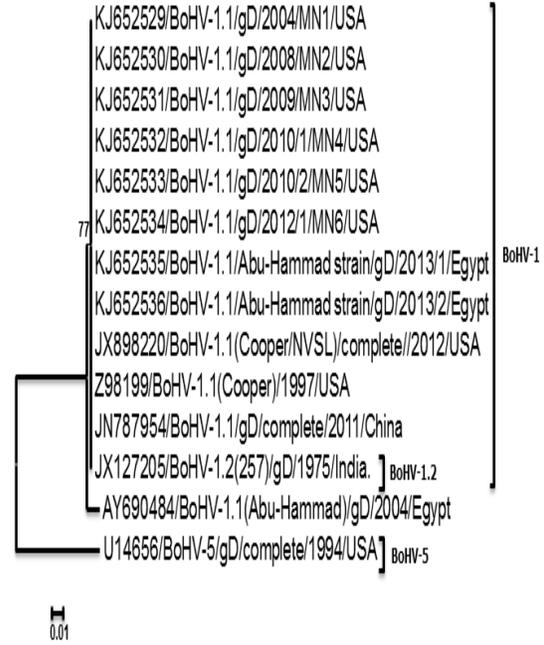


Figure 3. Phylogenetic tree of partial sequences of the gD gene. Tree shows relationship of Egyptian BHV-1.1 isolates with other strains.

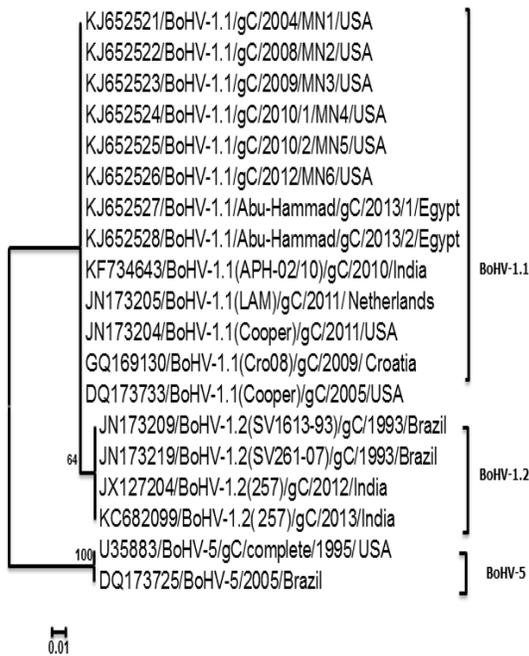


Figure 2. Phylogenetic tree constructed by neighbor-joining method on the basis of partial sequences of the gC gene. The tree shows relationship of Egyptian BHV-1.1 isolates with other strains.

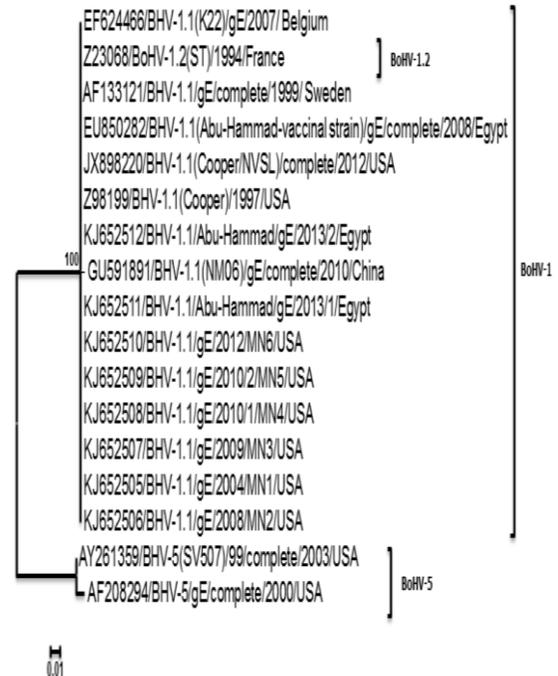


Figure 4. Phylogenetic tree of partial sequences of the gE gene. The tree shows relationship of Egyptian BHV-1.1 isolates with other strains.

4. Discussion

Since 1976, when BoHV-1 was first recorded in Egypt, it has become endemic in cattle herds with periodic outbreaks (Elshemy and Hassan, 2010). The Egyptian strains in our study were isolated from two BoHV-1 non-vaccinated cows suffering from respiratory illness. The animals belonged to two different farmers. Most of the cattle in Egypt are owned by individuals and hence knowledge on vaccination strategy and the importance of vaccination may be lacking among them. On the other hand, most commercial farms depend on imported vaccines containing US strains and for this reason a comparison with US strains is needed.

Despite the fact that IBR is a disease with long history in Egypt, this is the first report on characterization of virulent BoHV-1.1 strains based on sequencing and phylogenetic analysis of glycoproteins gB, gC, gD and gE. The amplicons of gB, gD and gE were obtained by following manufacturer's instructions on HotStarTaq master mix kit (Qiagen, Valencia, CA) without addition of extra reagents. However, PCR amplicons from gC sequences were obtained only with the addition of 5% glycerol to the reaction mixture. This is due to the high G+C content (71-72%) of BoHV-1 sequences (Majumder et al., 2013).

Based on sequence comparison, there were no differences among the eight isolates although they were obtained from two different countries and from different samples and over several years (Table 1). This is in agreement with a South American study in which Esteves et al. (2008) analyzed gC of BoHV-1.1 strains from cattle suffering from rhinotracheitis and abortion. A study of BoHV-1.1 isolation from nasal swabs, semen, vaginal swabs, preputial swabs and brain also revealed high degree of identity in all virus samples (Traesel et al., 2013).

In the present study, the phylogenetic analysis based on nt sequences yielded clusters corresponding to viral types and subtypes. Supported by high bootstrap values, three groups were evident: BoHV-1.1, BoHV-1.2 and BoHV-5 (Figure 1-4). This grouping agrees with results of previous phylogenetic studies using the gC COOH-t (Esteves et al., 2008), the gB and the gD coding regions (Ros and Belák, 1999). The gB gene among our isolates was highly conserved and can thus be used as a target for designing primers for detection of BoHV-1 (Majumder et al., 2013). Glycoprotein gC sequences of Egyptian and US isolates matched by 100% with reference Cooper strains (JN173204 and DQ173733) and 90% with BoHV-5 strain (U35883) (Figure 2). This supports previous studies indicating the usefulness of gC glycoprotein sequences in differentiating BoHV-1 and BoHV-5 (Chowdhury et

al., 2000; Claus et al., 2005; Traesel et al., 2013. An 88% match of gD and gE with those of BoHV-5 suggests that these proteins can also be useful in differentiation of BoHV-1 and BoHV-5.

BoHV-1 glycoprotein gD is situated between map units 0.892 and 0.902 and encodes a predicted protein of 417 amino acids (Tikoo et al., 1990). This gene was sequenced and compared with nt sequences of other BoHV-1 and BoHV-5 strains. The results revealed that the maximum nucleotide similarity (100%) was with BoHV-1.1 sequences from USA (JX898220 and Z98199) which is reference Cooper strain (Figure 3). In a previous study by El-Kholy and Abdelrahman, (2006) 98% similarity was detected with reference US Cooper strain when compared with Abu-Hammad/Egypt vaccinal strain gD sequence. The unique six insertions and three substitutions that we detected in Egyptian vaccinal strain (AY690484) could have occurred during manipulation of the virus for making vaccine. Further studies are needed to confirm if gD sequences can be used as a marker to differentiate between virulent and vaccinal strains in Egypt. Sequencing of purified gE PCR amplicon in both directions (Figure 4) agrees with sequence data of the BoHV-1.1 Abu-Hammad /Egypt vaccinal strain gE gene submitted to the GenBank by El-Kholy et al., (2013).

Based on gB, gC, gD and gE, 100% sequence similarity was found among our analyzed sequences and genome sequence of the BoHV-1.1 reference US Cooper strain and grouped together (Figure 1- 4). The pairwise distance analysis revealed that gB, gD and gE are conserved and cannot differentiate between BoHV-1.1 and BoHV1.2 as the similarity was 100% among them. On the other hand, gC is the least conserved gene (Delhon et al., 2003) and is useful in differentiation of BoHV-1.1 and 1.2. The phylogenetic analysis showed a consistent grouping of BoHV-1 and BoHV-5 in clearly separated but related groups with similar tree topology already has been described by Ros and Belak, (1999) using gB and gD genes and by Esteves et al. (2008) using gC gene. Our results also support the studies of Delhon et al. (2003) and Meyer et al. (1999) that genomic sequence of BoHV-5 is very similar to BoHV-1.

An important aspect of BoHV-1 infection is latency in which stress or corticosteroid treatment can activate the virus resulting in virus shedding (Lovato et al., 2003). The shedding of virus in semen can result in transmission of infection to cattle leading to abortion. This can be avoided through proper vaccination. Our findings indicate that the use of US Cooper strain in imported vaccine is appropriate and that it should provide protection against disease. This is supported by Fayed et al. (2013) who found that the local Pneumo 3 vaccine and the imported Cattle

Master 4 vaccine were highly effective in producing immune response against BoHV-1.

In conclusion, the findings of the current study showed that genomic structure of BoHV-1 is very stable. This is the first report for characterization of the four glycoprotein genes of Egyptian Abu-Hammad virulent strain glycoproteins and forms a solid foundation for future studies on BoHV-1 Egyptian strains and related viruses. The comparative genetic analysis reported in this study is useful not only to trace conservation of the Egyptian BoHV-1 among related alphaherpesviruses but also to establish genetic tools for nationwide epidemiological studies. The study also suggests that both local and imported vaccines should provide adequate protection to susceptible animals in Egypt.

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