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ISOLATION, IDENTIFICATION AND PROTEIN CHARACTERIZATION OF BOVINE HERPESVIRUS-1 ISOLATES

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ABSTRACT

The main goal of this study was check of antigenic drift from field isolates with regard to the international reference strain Colorado and ascertains whether the viral infection can be controlled by the vaccines. To achieve that, the BoHV-1 isolates were isolated with antigenic and genetic characterization and Sodium Dodecyl Sulphate-Polyacrylamide Gel electrophoresis (SDS-PAGE) the soluble proteins of these isolates with dendrogram analysis the results. From 3 different farms at Delta regions, Egypt, samples were collected From the 1st farm, 3 vaginal swabs represented 17 diseased cattle suffer from pustular vulvovaginitis, from the 2nd farm, tissues (liver, spleen and lung) of 2 aborted feti at 5 and 6 months of gestation and from the 3rd one, 3 nasal swabs of recovery cattle from respiratory manifestations after treated with antibacterial, antipyretic and non steroid anti-inflammatory were collected and processed. The samples were tested by direct fluorescence antibody (FA), nested PCR (nPCR), and isolated on Madin-Darby Bovine Kidney (MDBK) cellular culture and dendrogram analysis of electrophoretic pattern the soluble proteins from field isolates. Five samples (3 vaginal swabs and tissues of 2 aborted feti) were showing fluorescence granules. The DNA of the 5 FA positive samples was amplified within gB gene and yield amplicons sized 294 bp. The viruses from the 5 FA and PCR positive samples were isolated and propagated to antigenic and genetic characterization. To achieve that and at the third passage on MDBK cell culture, clear cytopathic effects (CPEs) were seen. Concerning to the dendrogram analysis, overall homology between the different field isolates and the reference international strain (Colorado) was 74-90% and among the field isolates was 81 to 94%. In conclusion, overall homology between the different Egyptian isolates and the reference international strain (Colorado) and among the Egyptian isolates was high. This can help to reduce the scale of future BoHV-1 infections and reduce the extent of culling or emergency vaccination needed.

Key words: *Isolation, Identification, Protein Characterization Herpesvirus-1*

INTRODUCTION

Studies on antigenic and genomic properties of bovine herpesvirus-1(BoHV-1) are valuable to monitor the viral infection. Infection with BoHV-1 occurs globally and is imposing large direct and indirect productive and reproductive problems causing great financial losses in beef and dairy farms.

BoHV-1 is problematic because of its ability to enter a latency phase within the host. Sciatic nerve and trigeminal nerve are the sites of latency, and meaning that an apparently healthy animal might suddenly resume the virus's spread (Van Engelenburg *et al.*, 1995 a,b; Engels and Ackermann, 1996; Vogel *et al.*, 2004 and Labiuk *et al*., 2009). Also, it has many mechanisms to evade the host's immune systems of both innate immunity and adaptive immunity (Muylkens *et al*., 2007). If the virus infection is not contained and is allowed to spread to the entire herd, there are a growing threat incurred from deaths, abortions, reduced body mass, reduced dairy production, and impairment of international trade (Pfizer Animal Health, 2012).

BoHV-1 is a member of the order *Herpesvirales*, family *Herpesviridae*, subfamily *Alphaherpesvirinae*, genus *Varicellovirus* (Davison, 2010 and International Committee on Taxonomy of Viruses ICTV, 2015).

The viral genome is a single linear double-stranded DNA (dDNA) molecule of approximately 135 to 140 kilo base pairs (kbp) with 72% guanine/cytosine content that coded for 73 open reading frames (ORFs) (Wyler *et al.*, 1989 and Muylkens *et al*., 2007). Of these 73 ORFs, 33 have been determined to be essential for viral replication (Robinson *et al*., 2008).

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Most of the virus's genes have homologous counterpart's common to all alphaherpesviruses, although some are exclusive to the varicelloviruses. Only one of these genesunique long segment 0.5 (UL0.5) is specific to BoHV-1 alone (Muylkens *et al*., 2007).

BoHV-1 specifies at least 11 glycoproteins, designated gB, gC, gD, gE, gG, gH, gI, gK, gL, gM, and gN (Whitbeck *et al*., 1988; Tikoo *et al*., 1990; Leung-Tack *et al*., 1994; Vilcek *et al*., 1995; Schwyzer and Ackermann, 1996). gB is the most conserved glycoprotein in the family *Herpesviridae*, suggesting that this glycoprotein plays a critical role (s) in propagation of the virus.

Genital infections may result in development of pustular vulvovaginitis in females or balanoposthitis in males, occur transiently and resolve spontaneously in 1 to 2 weeks. Clinically, the diseases are characterized by formation of variable number of small nodules, vesicles, focal erosions, or ulcers visible on inflamed mucosalmembranes, (Straub, 1990).

Gross lesions are not observed in aborted fetuses, but microscopic necrotic foci are present in most tissues and the liver is consistently affected (Bosch *et al*., 1997).

There is growing awareness of the desirability to eradicate BoHV-1, although knowledge on the genomic and antigenic characterization of Egyptian field strains and the homology regard to the international reference strains are lacked.

The main goal of this study was check for antigenic drift of field isolates with regard to the international reference strain Colorado and ascertains whether the viral infection can be controlled by the vaccines. To achieve that, the BoHV-1 isolates were isolated with antigenic and genetic characterization and dendrogram analyses of the soluble protein electrophoretic pattern of these isolates.

MATERIALS AND METHODS

A) Samples collection and preparation:

From 3 different farms at Delta regions, Egypt, samples were collected: From the 1st farm, 3 vaginal swabs represented 17 diseased cattle suffer from pustular vulvovaginitis, that small vesicles, focal erosions, and ulcers on inflamed mucosal membranes (figure 1) and 3 vaginal swabs from apparently healthy cattle. From the 2nd farm, tissues (liver, spleen and lung) of 2 aborted feti at 5 and 6 months of gestation were homogenized in phosphate buffered saline (PBS) and suspended in 200 µl PBS with antibiotic/antimycotic. And from the 3rd one, 3 nasal swabs of recovery cattle from respiratory manifestation after treated with antibacterial, antipyretic and nonsteroid anti-inflammatory. The swabs were collected and processed in PBS and the cell debris were washed twice and suspended in 200 µl PBS with antibiotic/antimycotic.

B) Direct detection of viral antigens:

B-1) Immunofluorescence detection the viral antigens:

On glass cover slips, cellular suspension (15 µl) of each sample was dried at room temperature (RT) and fixed by chilled acetone for 15 minutes. Florescence isothiocyanate (FITC) conjugated anti-BoHV-1 purchased from VMRD, USA, (catalog No.CJ-F-IBR-10ML) were used for direct detection of viral antigen on cellular fixed coverslips (Xingnian and Kirkland, 2008).

B-2) Genomic detection:

B-2.1) DNA extraction:

Total DNA were extracted from 100 µl of cellular suspension of each sample by Gene Jet genomic DNA purification kit (Thermo scientific, K0721) as described by the manufacturer.

B-2.2) The primer sets and viral genomic amplification:

Based on complete BoHV-1genome sequence Database (Gene Bank accession No. BHV1CGEN), and as illustrated in Ros and Belak (1999), the primer sets were selected and synthesized in biobasic, Canada. The primer set 1 (CR30 and CR31) was used for the first round of amplification at a condition of 95^0C/7 min as one step for first denaturation followed by 35 cycles. Each cycle is 3 steps; 95^0C /1 min, 60^0C /1 min and 72^0C/1 min and a finally one extension step at 72^0C/7 min. The primer set 2 (CR32 and CR33) was used for second (internal) round amplification at the same condition of the first round except, the annealing temperature is 62^0C. The primer sets as CR30 (5'-CCA GTC CCA GGC RAC CGT CAC-3'), CR31 (5'-TCG AAR GCC GAG TAC CTG CG-3'), CR32 (5'-TGG TGG CCT TYG ACC GCG AC-3') and CR33 (5-'GCT CCG GCG AGT AGC TGG TGT G-3') were used at a final concentration 15 picomol/reaction for each one.

C) Virus isolation and propagation:

The FA and PCR positive samples (n=5) were isolated and propagated in MDBK with daily examination for the cytopathic effects (CPEs) development along 5-7 days for 3 passages as cited by George *et al*. (1996).

D) Concentration and Purification the Viral isolates:

For each isolate, the viral suspension was collected, concentrated and purified using polyethylene glycol 6000 (PEG-6000) as described briefly by Kelling *et al.* (1990).

E) Quantitation of antigen concentration:
Total antigen concentration was measured for each isolates using total protein liquidcolour reagent; Stanbio laboratory, Boerne, Tx, USA, as in the manufacturer's leaflet. The antigen concentration (µg/µl) was calculated as:

OD of the sample/OD of the standard×concentration of Standard (10 g/dl).

F-1) Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE):
The soluble proteins of the five purified isolates and the international reference Colorado strain were denaturated and separated in 12% sodium dodecyl sulphate-30% polyacrylamide separating gel electrophoresis (SDS-PAGE) according to Laemmli (1970). Using mini-protein II electrophoresis cell (Bio-Rad) at 50 volts for 4 hrs. The electrophoretic patterns of the structural viral proteins were matched with the full-range molecular weight protein marker ranging from 6.5 to 200 KDa (SERVA Electrophoresis, Cat. No. 39215).

The different fractions were quantified using Bio-Rad GS 700 imaging densitometer molecular analysis software.

F-2) Dendrogram analyses:
Dendrogram was constructed to reveal the relatedness percent between the different isolates using gel proanalyzer version 4.5 cypermedica, USA.

RESULTS

Clinically diseased cows with pustular vulvovaginitis:
With various degrees in vaginal infected 17 cattle, small pustules became visible in the vulva and caudal vaginal region. They enlarge and spread over the whole epithelium in a plaque-like causing edema and hyperemia with serous to mucopurulent discharge.

Immunofluorescence detection of the viral antigens:
In the present study, 5 samples (3 vaginal swabs from clinically diseased cattle and tissues of 2 aborted feti) were showing fluorescence granules (figure 3) using FITC conjugated anti-BoHV-1.

Genomic detection:
The DNA of the 5 positive FA samples was amplified within gB gene and yield amplicons sized 294 bp as well as the reference Colorado strain. The DNA of the 3 nasal swabs wasn't amplified as well as the negative control one (figure 4).

Virus isolation and propagation:
The viruses from the 5 FA and PCR positive samples were isolated and propagated on MDBK cell culture. At the third passage, clear CPEs were seen (figure 6) as the cells became rounded, dispersed with grapes-like appearance in the fluid phase and intranuclear inclusion bodies as compared to the cellular control (figure 5).

Concentration, Purification and Quantitation the Viral antigens:
For each isolate, the viral suspension was collected, concentrated, purified and antigen concentration was measured. According to the formula illustrated by the manufacturer leaflet, the antigen concentration for each isolate was calculated as µg/µl. They were 4.6 µg/µl for the reference strain, 3.9, 4.19, 4.4, 4 & 3.6 µg/µl for the 5 field isolates.

SDS-PAGE Polyacrylamide Gel Electrophoresis and dendrogram analysis:
Concerning to the results as clarified and illustrated in the figures 7 and 8 and in the table, the overall homology between the reference strain and the field isolates was 74 to 90% and among the field isolates manner was 81 to 94%.

Figure 1 and 2: Small pustules become visible in the vulva and caudal vaginal region. They enlarge and spread over the whole epithelium in a plaque-like manner causing edema and hyperemia.

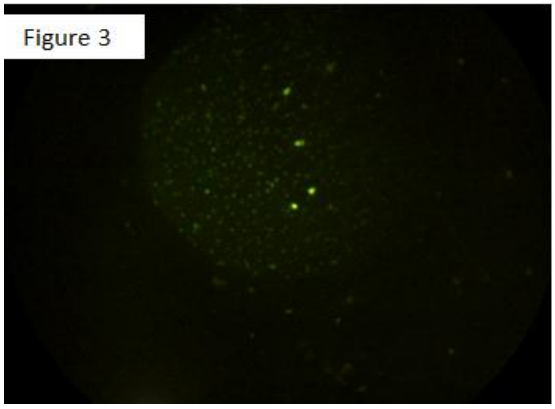


Figure 3: fluorescence granules were observed in the positive samples, X 100

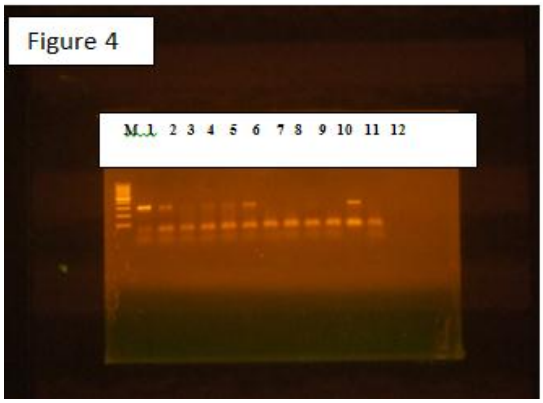


Figure 4: Agarose gel (1%) analysis of nPCR amplified products with type specific primers. Lane M is 100 bp DNA ladder (Aβ gene, UK), lane 1, 11 are Colorado strain as positive controls. I (294 bp), and lane 2-7 are positive samples, lane 8-10 are negative samples and lane 12 is the negative control.

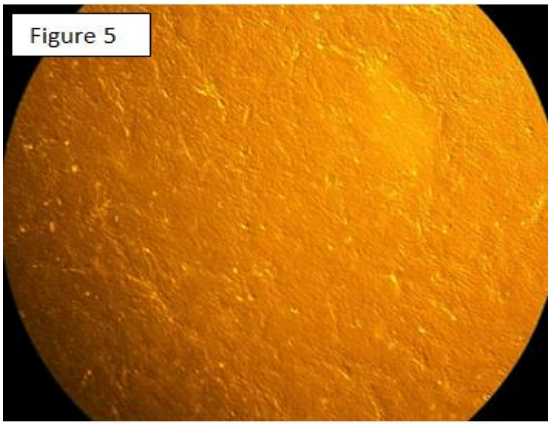


Figure 5: MDBK cellular control (X40)

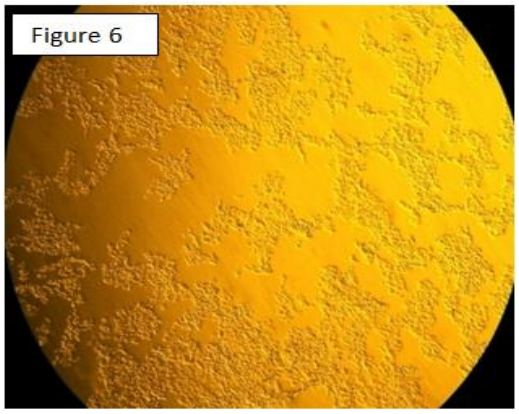


Figure 6: MDBK cellular inoculated with FA and PCR positive vaginal swab, the cells became rounded, dispersed with grapes-like appearance in the fluid phase and intranuclear inclusion bodies (X 100).

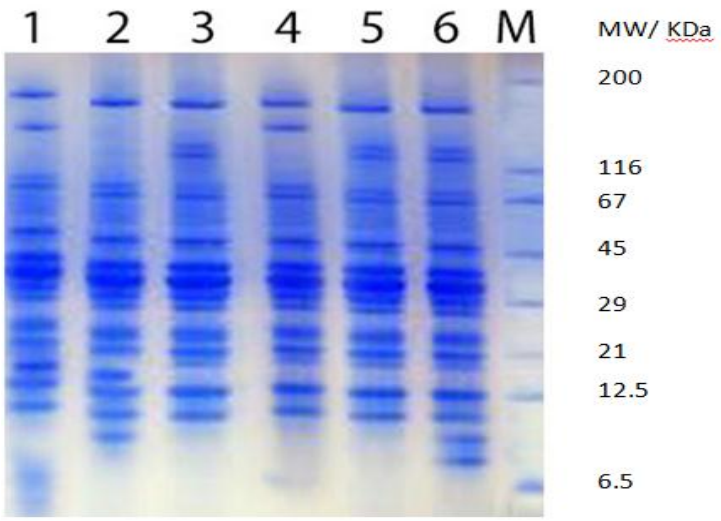
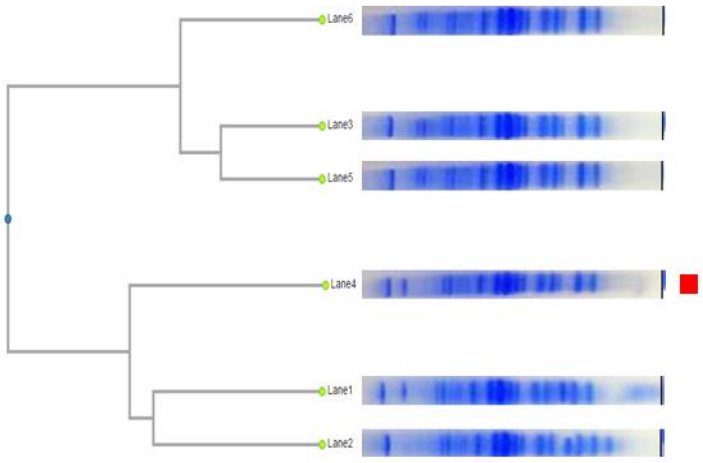


Figure 7: Protein profile of the BoHV-1 viral isolates. Lane 1 represented the reference strain, lanes 2-6 represented the field isolates and lane M is the M wt marker.



- Referance strain

Figure 8: Dendrogram analyses of BoHV-1 reference strain and the 5 field isolates.

Table of protein similarities between the international reference strain (Colorado) and the 5 field isolates.

	Lane1	Lane2	Lane3	Lane4	Lane5	Lane6
Lane1	1	0.9	0.79	0.9	0.79	0.74
Lane2	0.9	1	0.81	0.87	0.81	0.82
Lane3	0.79	0.81	1	0.88	0.94	0.89
Lane4	0.9	0.87	0.88	1	0.88	0.82
Lane5	0.79	0.81	0.94	0.88	1	0.94
Lane6	0.74	0.82	0.89	0.82	0.94	1

DISCUSSION

Global threats from infectious diseases are not lessening, and nations that seek to remain disease-free must have high quality surveillance systems operating at international, national and local levels. This can be improved by rapid diagnosis using the latest scientific techniques that help to reduce the scale of infectious diseases (The Royal Society, 2002 and OIE, 2004).

Infection of the mucosal surfaces ingenital tract has welfare impact that leads to pain and distress in the animals. The progress of infection in these sites leads to secondary clinical signs. The sequelae of secondary infection cause the consistency of the discharges to change (serous to mucopurulent) and to increase local pathology such as necrotic lesions, pustules and ulcers in the genital tracts (Engels and Ackermann, 1996 and EFSA, 2006), as observed clinically in this study.

Laboratory diagnosis still rests partly upon showing the presence of the infecting microorganism after culturing. The virus might be detectable only after multiple cycles of replication, which could take several days or weeks. In spite of the development of new scientific methods to speed up detection and to increase sensitivity and specificity, the culture and characterization of disease causing agents remain important and are performed in parallel with other rapid diagnostic measures.

There are varieties of techniques to direct visualize the captured protein antigens with the use of specific antibodies as fluorescence isothiocyanate (FITC) and horse radish peroxidase (HRP) conjugated antibodies (Collins *et al.*, 1988).

Direct detection of viral nucleic acid makes up the genetic material can be detected and characterized. The technique is able to detect less than 10 molecules of the BOHV-1 genome using nested PCR (nPCR) protocols. When compared with the routinely used virus isolation, the PCR can be several to 100-fold more sensitive (Van Engelenburg *et al.*, 1993).

New isolates are examined to check the antigenic drift and ascertain whether the infecting virus can be controlled by the vaccines available (Edwards *et al.*, 1983). The need to update vaccine strains is therefore recognized. Compared with the international reference strain, viruses can be allocated to topotypes, which represent independent genetic lineages occurring within different geographical regions. This helps to determine the possible geographical distribution of the particular strains of virus.

Concerning to the dendrogram analysis, overall homology between the different field isolates and the reference international strain (Colorado) was 74-90% and among the field isolates was 81 to 94%. This supports the hypothesis that small discrepancies present among the field isolates and the international reference one. The BoHV-1 DNA genome is more stable that encoded high percent (72%) of Guanine-Cytosine (GC) content (Wyler *et al.*, 1989 and Muylkens *et al*., 2007).

In conclusion, overall homology between the different Egyptian isolates and the reference international strain (Colorado) and among the Egyptian isolates was high. This can help to reduce the scale of future BoHV-1 infections and reduce the extent of culling or emergency vaccination needed.

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عزل وتصنيف مع التوصيف البروتيني للمعزولات من فيروس الهربس البقرى-١

ياسر جميل محمود عبد الحفيظ ، رانيا صلاح عبد الفتاح المحمدى ، ايناس محمد جمال الدين

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الهدف الرئيسي من هذه الدراسة التحقق من مدى الانجراف المستضد للمعزولات الحقلية لفيروس الهربس البقرى -١ ومقارنتها بالسلالة المرجعية العالمية كولورادو وللتاكد ما اذا كان يمكن السيطرة على العدوى بالتحصين ضد الفيروس. لذا تم الكشف وعزل فيروس الهربس البقرى -١ من عينات حقلية (ثلاث مسحات مهبلية تمثل ١٧ بقرة تعانى من الالتهاب المهبلى البثرى ، عدد ٢ عينة انسجة كبد ورئة وطحال لجنين مجهض عمر ٥ و ٦ اشهر من الحمل ، ٣ مسحات انفية من ابقار تعانى من اعراض تنفسية وتم معالجتها باستخدام مضادات حيوية وخافض للحرارة ومضادات للالتهابات غير استيرويديه). بالكشف عن الفيروس فى العينات الحقلية باستخدام اختبار الصبغة الفلورسينتي المباشر واختبار البلمرة المتسلسل العشى فى المنطقة الجينية (gB) وجد ان هناك ٥ عينات ايجابية. تم عزل العينات على الخلايا النسيجية MDBK بالتمرير ثلاث مرات مع متابعة التاثير السيتوباثولوجى. وبدراسة التوصيف الانتجينى للمعزولات باستخدام الفصل الكهربائى الراسى (SDS-PAGE) وتحليل النتائج باستخدام برنامج الدينروجرام كانت نسبة التماثل الكلى بين المعزولات المختلفة والسلالة المرجعية العالمية ٧٤-٩٠% وبين المعزولات وبعضها ٨١-٩٤%. خلصت الدراسة الى ارتفاع نسبة التماثل بين المعزولات الحقلية والسلالة العالمية كلورادوا مع انخفاض نسبة الطفرات على المستوى الجينى والانتجينى. مما يعنى ان استخدام التحصين ضد هذا الفيروس يلعب دورا اساسيا فى تقليل نسبة الاصابة ومعدلات النفوق بين الماشية.