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# Characterization of Recombinant Baculovirus Expressing envelope glycoprotein E of the local BoHV-1

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## SUMMURY

A recombinant baculovirus expressing envelope glycoprotein E (gE) of the local Bovine Herpesvirus type-1 (BoHV-1) "Abu-Hammad strain" was characterized. PCR analysis was conducted on the genomic DNA of recombinant baculovirus to verify the presence of the inserted gE gene. *Spodoptera frugiperda* (Sf9) cells infected with the recombinant baculovirus expressed a high level of immunoreactive recombinant gE protein that was demonstrated by indirect immunoflurescence and Western blot analyses. The recombinant gE protein was used as a coating antigen in an indirect ELISA for detection of BoHV-1 anti-gE antibody to differentiate cattle vaccinated with the local of BoHV-1 "Abu-Hammad strain" from those vaccinated with $gE^-$ negative marker vaccine. A limited number of bovine serum samples which previously tested by the indirect ELISA, virus neutralization Test (VNT) and commercial blocking gE ELISA (IDEXX) were used to be tested by indirect ELISA. The obtainted results proved the reactivity of the recombinant gE protein and its utility as a diagnostic antigen in a gE based indirect ELISA for diagnosis of BoHV-1 as well as distinguishing infected animals within a gE marker vaccinated herd.

**Key Word:** (BoHV-1-glycoprotien E-baculovirus – Sf9 – ELISA).

## INTRODUCTION

Bovine Herpesvirus type 1 (BoHV-1), a member of the Alphaherpesvirinae subfamily, is a major pathogen of cattle, causing infectious bovine rhinotracheitis (IBR), infectious pustular vulvovaginitis (IPV) and infectious balanoposthitis (IBP) (Gibbs and Rweyemamu, 1977; Schwyzer and Ackermann, 1996). Bovine herpesvirus-1 infection constitutes one of the most important diseases of cattle and causes significant economical losses to livestock worldwide.

BoHV-1 genome consists of approximately 136 – 140 kilo base pairs comprising a long unique region (UL) of 104 kbp and short region which contains a unique segment (US) of 10 kbp flanked by internal and terminal inverted repeats of 12 kb each (Wyler *et al.*, 1989 and Schwyzer & Ackerman 1996).The gE envelope glycoprotein encoded by US8 gene is a minor antigen in contrast to the other immunodominant glycoproteins such as gB, gC and gD (Rijsewijk *et al.*, 1992; Rebordosa *et al.*, 1994). BoHV- 1 gE is a 575 amino-acid glycoprotein with a calculated molecular weight (MW)of 61 kDa and an apparent MW of 92 kDa. The gE glycoprotein is involved in cell-to-cell and neuronal spread (Rijsewijk *et al.*, 1994; Rebordosa *et al.*, 1996).

Serum antibodies against gE could be detected as early as 11 days after experimental infection and were found to persist at a high and stable level for at least 2–3 years after experimental intranasal or contact infection with either a BoHV-1 subtype 1 or BoHV-1 subtype 2 strain (Van Oirschot et al., 1997). Using the recombinant gE protein as a more specific antigen instead of whole virus in the gE ELISA for the detection of antibodies directed against the gE of BoHV-1 in serum, might reduce or eliminate non specific reactions (Van Oirschot *et al.*, 1997).

A BoHV1 glycoprotein E (gE) enzyme-linked immunosorbent assay (ELISA) was used in combination with Marker vaccine to differentiate infected cattle with wild-type of BoHV-1 from vaccinated cattle with gE negative vaccine. antibodies against wild-type BoHV-1 was detected, whereas antibodies against the marker vaccine from which gE is deleted are not detected (Kaashoek *et al.*, 1995).

The aim of the study was to prepare gE-ELISA based on recombinant baculovirus expressing the gE protein of BoHV-1 for measurement of antibodies directed against gE of BoHV1 and to be used as a companion diagnostic test for gE-negative BoHV1 vaccines.

## MATERIALS AND METHODS

### 1. Cells and Viruses

The Spodoptera frugiperda (Sf9) insect cells (Invertogen) were maintained at 27°C in complete Grace's insect medium [Biochrom] including 10% fetal bovine serum [PAA] in the presence of 1X L- Glutamin [Euro Lone, 100X], 1X *Lactalbumin* Enzymatic Hydrolysate [Sigma, 50X], 1X Antibiotic-antimycotic mix [PAA], for each 100 ml medium. The recombinant baculovirus expressing the gE protein of BoHV-1was constructed and developed through a research project sponsored by the National Academy for scientific Research and Technology (PI Dr. Alaa El-Kholy) and used to be characterized. The recombinant baculovirus

was grown in monolayer of Sf9 as described by summers and Smith (1987).

**2. Bovine sera**

To assess specificity and sensitivity of the prepared gE based ELISA, anti- gE serum [IDEXX], control negative serum [IDEXX], fetal calf serum [PAA and Biowest] as negative controls, and serum samples obtained from immunized calves with Killed BoHV-1 vaccine and gE negative vaccine (obtained from Veterinary Serum and Vaccine Research Institute, Abbassia, Cairo) were used.

**3. Confirmation analysis of recombinant protein:**

PCR analysis was used to verify the purity of recombinant baculovirus expressing the gE protein of BoHV-1. PCR analysis was performed using set of primers flanking the polyhedrin region that are compatible with all polyhedrin promoter based baculovirus transfer vectors, and applied on DNA of The recombinant baculovirus expressing the gE protein which extracted by using **JETQUICK** Blood &cell culture **DNA** spin Kit. The primers for PCR were as follows: baculovirus forward primer, 5′-TTTACTGTTTTCGTAACAGTTTTG-3′ (binds from -44 nt 4049 to -21 nt 4072 in front the start of the polyhedrin gene) and baculovirus reverse primer, 5′ CAACAACGCACAGAATCTAGC- 3′ (binds at +794 nt 4886 to +774 nt 4866 3′ to the polyhedron gene). The PCR reaction was performed as described by Taq PCR Core kit [QIAGEN] in a total volume of 50 µl containing: 5µl Extracted viral DNA, 25µl Nuclease free water, 5µl Coral buffer, 5µl Q buffer, 1.5µl from each primers, 1µl dNTPS and 1µl Taq polymerase enzyme ( 2.5 U/ 1 µl). The PCR cycling condition consisted of one cycle of 94°C for 2 min for initial denaturation, 30 amplification cycles were conducted, each cycle consists of 94°C for 1 min (denaturation), 55°C for 2 min (annealing) and 72°C for 3 min (extension) followed by a final extension cycle of 7 min at 72°C. The PCR products were analyzed on 1% agarose gel containing 0.5 µg/ml ethidium bromide.

Other specific set of primers to gE encoding sequences were applied on DNA extract to amplify their full length yielding a PCR amplicons. The PCR reaction was conducted similar to abovementioned using Taq PCR Core kit [QIAGEN] and The PCR cycling condition as previously with 58°C for annealing, 3 min for extension, and total 40 cycle

**4. Characterization of expressed baculovirus recombinant gE protein Fluorescent antibody technique (FAT):**

Indirect FAT was performed as previously described by Ausubel et al. (1994) with some modifications. Briefly, Sf9 cells were seeded in 8 - chamber multitested slide and infected with multiplicity of infection

(MOI) 10 of recombinant baculovirus expressing the gE protein of BoHV-1. Two days post-infection, the cells were washed with PBS and fixed with 80% cold acetone for 20 min at 4°C. The cells were then incubated with a anti-BoHV-1 (VMRD) for 1 hr at 37°C, and washed three times with PBS. anti-bovine FITC conjugate (diluted 1/40 in PBS) was added and incubated at 37 °C for 1hour. Followed by three times of washing and examined under fluorescent microscope for detection of the fluorescent reaction.

**Preparation of antigen**

The preparation and harvest of expressed protein were conducted as described by Ausubel *et al.* (1994) with some modifications. Briefly, Sf9 cells ($3X10^7$ cells/5ml Grace's insect medium containing 2% FBS) were seeded in 25 $cm^2$ flasks then inoculated by 5 MOI of recombinant baculovirus expressing gE protein and incubated at 27°C. The optimum time for protein expression was determined by harvesting the recombinant virus-infected Sf9 at 24, 72 and 96 hours postinfection when CPE was appeared. The harvest time which gives the highest protein yield was predetermined by a time course of protein expression. Positive control antigens were prepared by infecting Madin Darby Bovine Kidney (MDBK) cells with 2 MOI of BoHV-1 (Abu Hammed strain) and harvesting cells when CPE was complete. Infected SF9 and MDBK cells were clarified by centrifugation at 3000 rpm for 10 min at 4°C. Supernatant was collected and the pellet was suspended in lysis buffer (5ml PBS pH 7.2, 50µl triton X-100 and protease inhibitor mix [ROTH] following supplier's recommended concentrations. The suspension was exposed to freezing and thawing on ice 3 time then centrifuged at 3000 rpm for 10 min at 4°C. The supernatant containing antigen was diluted in the proper buffer for ELISA or western blotting as described below.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blot Assays**

SDS-PAGE and Western blot were performed as previously described (Laemmli, 1970 and Sambrook *et al.*, 1989). Briefly, recombinant protein extracted from infected Sf9 cells with recombinant baculovirus and from infected MDBK cells with BoHV-1 of Abu Hammed strain were electrophoresed on a 10% gel and then stained with Coomassie Brilliant Blue (R-250) [ICN]. For Western blotting assay, the separated protein in SDS-PAGE was electroblotted onto the nitrocellulose membranes, then blocked with blocking buffer [ROTH, Roti®] overnight at 4°C, washed three times with 0.05% Tween in PBS and incubated for 1 hr at room temperature with anti-BoHV-1 serum and washed with 3 times in PSB pH 7.4 containing 0.05 %Tween 20 [Serva]. The membrane was incubated with HRP peroxidase conjugated goat ant-bovine IgG

(H+L) [KPL] diluted 1/30,000 for 1 hour then washed and the reaction was detected using DAB substrate.

**Enzyme linked immunosorbent assay (ELISA):**

Indirect ELISA was conducted after some modifications in method described by (Mackow *et al.*, 1989). Optimiztion of the ELISA test was performed to provide a minimal background. Lysate from recombinant baculovirus infected SF9 cells, diluted 1:300 in 1X PBS containing 0.001% Triton X-100 as detergent. The 96- well ELISA plate [Nunc] was coated with 100µl/ well of recombinant protein antigen and kept at 4°C overnight. Plate were decanted, washed 3 times in 1X PSB pH 7.4 containing 0.05 %Tween 20; blocked with block buffer [3% bovine serum albumin (BSA) in 5% milk buffer] for overnight at 4°C. Plates were decanted, washed as before. Each bovine serum sample was diluted to 1:100 in 1X PBS containing 1% BSA and incubated at 37°C for 1h. The test included a positive and negative sera as well as a blank control. Plate was decanted and washed as before. HRP peroxidase conjugated goat ant-bovine IgG [KPL] was diluted to 1:2000 in 1X PBS buffer and added at 100 µl/well and the plate incubated for 1h at 37oC. TMB substrate indicator mixture was added, incubated in a dark place at room temperature for 15 minutes to allow color development and then stoped by stopping buffer. Optical densities at 630 nm wavelength were determined by using a microplate ELISA reader.

## RESULTS

**1. PCR analysis of recombinant baculovirus:**

PCR analysis using pH primers allow verification of recombinant baculoviruses containing gE gene of BoHV-1 and also determine the wild type contamination by yielding one single band of the proper calculated size about 2182 bp [316 bp + size of gE gene in recombinant virus, 1866 bp] and 839 bp in case of wild-type baculovirus. While, PCR analysis using specific gE primers amplified specific fragment of 1866 (Fig.1).

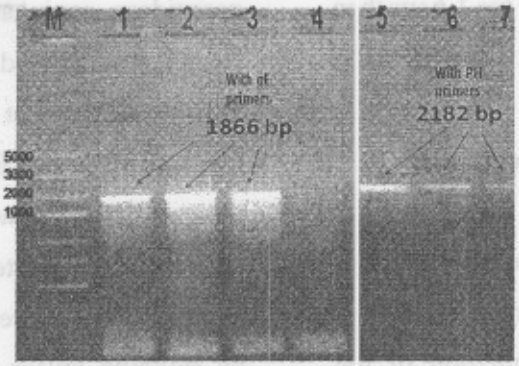


**Fig (1):** PCR screening of plaque purified Recombinant baculovirus BoHV-1 / gE DNA using the gE primers and polyhedrin primers, separated on 1% agarose gel stainted with Ethidium bromide. Lanes: (M) Gene Rular Express DNA ladder [Fermentas]; Lanes: (1,3) Recombinant baculovirus BoHV-1 / gE shows specific bands of 1866 bp [using gE primers]; Lane: (4) non- infected SF9 cell culture supernatant control [using gE primers]; Lanes: (5,7) Recombinant baculovirus BoHV-1 / gE shows specific bands of 2182 bp [ 1866+316 using pH primers].

**2. Fluorescent antibody technique (FAT)**

The in situ immunofluorescence staining studies revealed that gE recombinant protein is authentic and accumulated mainly in the cytoplasm of the infected Sf9 cells. indirect immunofluorescence observed throughout the cytoplasm of the recombinant virus infected Sf9 cells suggesting that large quantities of the expressed gE protein were produced (Fig.2).

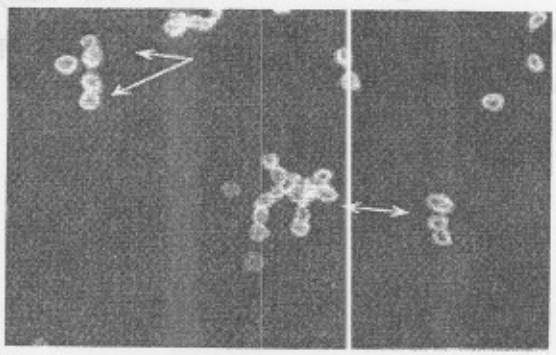

**Fig (2):** Immunofluorescence of SF9 cells infected with rBac/BoHV-1gE.

**2. SDS-PAGE and Western blot**

The recombinant baculovirus expressing gE protein at 48,72, and 96 hours postinfection produced a high reactivity band of approximately 80 KDa in coomassie Brilliant Blue-stained 10% SDS-PAGE gel and immunoblotted membrane (Fig:3 and 4).

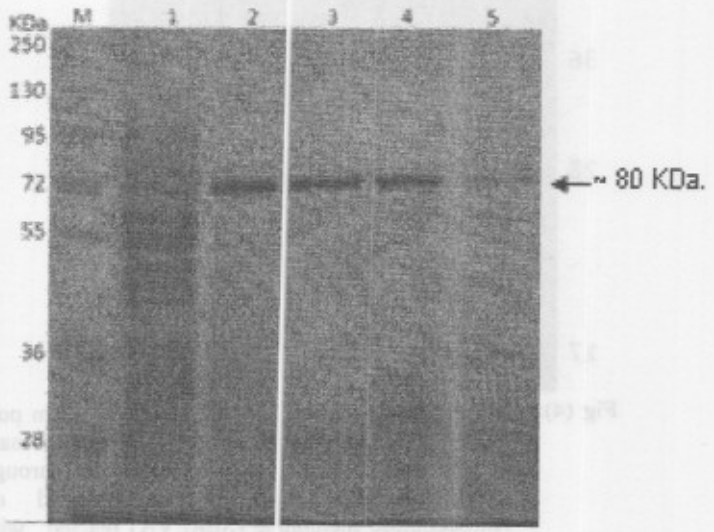


**Fig (3):** SDS-PAGE analysis of lysate recovered from post infection (PI) of SF9 cells infected with recombinant baculovirus. Samples were electrophoresed through 10% SDS-PAGE and stained with 0.25% coomassie Brilliant Blue R250, 4.5% methanol, and 4.5% acetic acid. lanes: (M) PageRular™ Pre-Stained protein ladder [Fermentas]; lane: (1) non-infected SF9 cell lysates; lanes: ( 2-5) cell lysates harvested at 96, 72, 48 PI, respectively. (size is ~ 80 kDa).

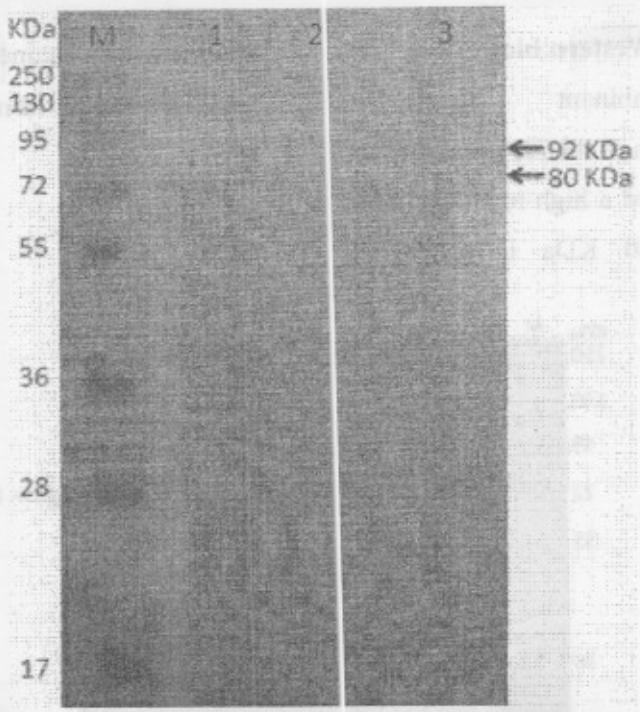


**Fig (4):** Western blot analysis of lysate recovered from post infection (PI) of Sf9 cells infected with recombinant baculovirus. Lysate was electrophoresed through 10% SDS- PAGE , semi-dry electroblotted on Nitrocellulose membrane (SEREVA) reacted with anti- BoHV-1 serum. Lanes: (M) PageRular™ Pre-Stained protein ladder [fermentas];(1) positive rBac/BoHV-1gE; (2) non –infected SF9 cell lysate; (3) MDBK infected with local strain of Abu Hammad (IBR).

**3. Enzyme linked immunosorbent assay (ELISA):**

The recombinant baculovirus expressing gE protein was used as coating antigen in an indirect ELISA as described by (Mackow *et al.* 1989). The different preparations (cell lysate and supernatant) of infected Sf9 with recombinant baculoviruses harvested at 72 hr was tested by an indirect ELISA, revealed a high intensity reaction confirming the presence of the expressed gE protein in the cell lysate and supernatant. However, the cell lysate preparation revealed a high reactivity than supernatant.

**Table (1):** Optimization of antigen concentrat on by using indirect ELISA:-

| Bovine serum dilution | ELISA (mean absorbance values) | | | |
|---|---|---|---|---|
| | Virus (u ndiuted) | lysate dilutied1:10 | lysate dilutied1:20 | lysate dilutied1:40 |
| Control positive serum (1:100) [of BoHV-1 gE antibody test kit ;**IDEXX**] | 2.047 | 2.555 | 2.409 | 2.424 |
| Control negative serum (1:100) [of BoHV-1 gE antibody test kit; **IDEXX**] | 0.243 | 0.999 | 0.910 | 0.990 |
| Fetal calf serum (1:100) **[PAA]** | 0.109 | 0.471 | 0.388 | 0.438 |
| Anti-BoHV-1 serum (1:100) [previously tested] | 1.176 | 1.160 | 1.561 | 1.752 |
| Blank (1X PBS) | 0.089 | 0.077 | 0.085 | 0.100 |

**Table (2):** testing of serum samples using recombinant baculovirus expressing gE protein as coating antigen in an indirect ELISA

| **Bovine Sera** | ELISA values* | **Bovine Sera** | ELISA values* |
|---|---|---|---|
| **$C_{+ve}$** | 1.682 | **Sample 11** | 1.509 |
| **$C_{-ve}$** | 0.326 | **Sample 12** | 1.353 |
| **FCS** | 0.137 | **Sample 13** | 1.271 |
| **Blank** | 0.110 | **Sample 14** | 1.527 |
| **Sample 1** | 1.364 | **Sample 15** | 1.099 |
| **Sample 2** | 1.653 | **Sample 16** | 1.320 |
| **Sample 3** | 1.183 | **Sample 17**** | 0.439 |
| **Sample 4** | 1.380 | **Sample 18**** | 0.553 |
| **Sample 5** | 1.412 | **Sample 19**** | 0.368 |
| **Sample 6** | 1.394 | **Sample 20**** | 0.504 |
| **Sample 7** | 1.122 | **Sample 21**** | 0.452 |
| **Sample 8** | 1.366 | **Sample 22**** | 0.447 |
| **Sample 9** | 1.625 | **Sample 23**** | 0.503 |
| **Sample 10** | 1.349 | **Sample 24**** | 0.424 |

* ELISA values were expressed as the mean absorbance at wave length ($\lambda$ = 630Ao)
C +ve control positive serum of BoHV-1 gE antibody test kit ; [IDEXX]
C-ve control negative serum of BoHV-1 gE antibody test kit ; [IDEXX]
Samples (1-16) obtained at 3 weeks after booster vaccination from calves vaccinated with killed BoHV-1 vaccine.
Samples (17-24)** obtained at 3 weeks after booster vaccination from calves vaccinated with BoHV-1 deleted vaccine. $\leq$ 0.6 of OD value representing negative reaction.

## DISCUSSION

In this the study, to prepare a rapid, safe and inexpensive gE based ELISA test for diagnosis of BoHV-1 by detecting antibody against BoHV-1/gE in cattle sera, gE recombinant protein based on baculovirus expression vector system (BEVS) was antigenically and molecularly characterized to be used as coating antigen. The recombinant baculovirus expressing the gE protein of BoHV-1was constructed and developed through a research project sponsored by the National Academy for scientific Research and Technology and assured by PCR analysis and indirect fluorescent antibody technique (FAT) and then used to be characterized

PCR analysis represents and allows a quick and safe method to verify the presence of the interest gene in a putative recombinant virus. PCR analysis was conducted using using polyhedren primers which flank the polyhedrin region and are compatible with all polyhedrin promotor-based baculovirus transfer vectors showing a fragment of 1866 bp, which is the full length of gE, plus 316 from vector with a total of 2182 bp (Webb *et al.*, 1991) demostrated recombinant baculovirus from the wild type baculovirus DNA which produced a fragment of 839 bp. Also other set of specific gE primers are applied to recombinant baculovirus DNA verifying the presence of the interest gE gene.

Expression of a biologically active recombinant protein was observed in situ immunofluorescence (IF) demonstrating that the gE recombinant protein accumulated in cytoplasm of insect cells infected with the recombinant virus. It could be concluded that the gE recombinant protein was synthesized by the recombinant virus and translocated in insect cells as appropriate as would be expected for the authentic protein in their own host cells.

Immunological and biochemical analysis in denaturing polyacrylamide gels, revealed that a high reactivity band appeared as predicted from its amino acid sequence (Rebordosa *et al.*, 1994; Leung-Tack *et al.*, 1994). SF9 lysates infected with the recombinant baculovirus expressed a protein of approximately 80 KDa that had the same mobility in SDS-PAGE stained with coomassie Brilliant Blue R250. While, MDBK infected with BoHV-1 of Abu Hammad strain expressed a protein of approximately 92 KDa which had the same mobility in SDS-PAGE suggesting that The reduced sized protein can be caused by many factors, as degradation of the in vitro expressed gE protein by any cellular protease enzyme. But the most reliable cause of this degradation is the migration of the expressed protein under reducing condition in the presence of 2-mercaptoethanol which affect the sulfer containing amino acids (mayo and cooper, 1973).

The diagnostic utility of crude lysate from infected insect cells, containing the gE recombinant protein, was determined by an indirect ELISA. The gE recombinant protein was used a coating antigen in ELISA plates.

Different dilutions of the antigen in PBS containing 0.001% Triton X-100 as a nonionic detergent that has been known to bind preferentially to hydrophobic proteins and it breaks up aggregations of the expressed proteins in crude lysate (El-Kholy *et al.*, 1999), was established for optimal conditions of gE based ELISA for detection of anti-BoHV-1/ gE antibodies in cattle serum and differentiate infected cattle with wild type of BoHV-1 from vaccinated cattle with gE-negative BoHV1 vaccines.

The crude lysate from Sf9 cells infected with the recombinant baculovirus, containing the recombinant protein proved to be sensitive, efficient ELISA antigen for detection of anti BoHV-1 gE antibodies in cattle sera and not interfere with ELISA test.

## CONCLUSION

The current study has succeeded in using of the baculovirus expression vector system (BEVS) for characterization and production of of rBac/BoHV-1gE protein. The high level of expression of gE protein of BoHV-1 by the recombinant baculovirus provides sufficient material for development of diagnostic ELISA kit to detect anti-gE (BoHV-1). Furthermore, the establishment of the baculovirus expression vector system in our laboratory offers the opportunity to production of mAbs against gE epitopes of BoHV-1 in the future.

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# توصيف فيروس باكيلو مدمج يعبر عن البروتين gE لفيروس الهيربس البقرى-١

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في هذه الدراسة تم التوصيف الانتيجينى والجزيئي للجليكوبروتين ايه gE لفيروس الهيربس البقرى-١ لعتره اللقاح المحلى أبو حماد والمدمج وراثيا في فيروس الباكياو لاستخدامه كاداه مشخصه في اختبار الاليزا. وذلك من خلال تمرير فيروس الباكيلو المدمج وراثيا بالجين المشفر للجليكوبروتين ايه gE في الخلايا الحشرية Sf9 وعزل حمضه النووي واختباره باستخدام تفاعل البلمره المتسلسل للتأكد من إدماج جين الجليكوبروتين ايه gE في جينوم فيروس الباكيلو. و قد تم توصيف بروتين ال gE المدمج باستخدام اختبارات متعددة مثل اختبار الفلورسنت المناعي غير المباشر و اختبار الإليزا و اختبار الفصل الكهربي للبروتينات SDS-PAGE، بالإضافة إلى اختبار اللطع المناعي Western blot. و تم استخدام بروتين إل gE المدمج كأنتيجين مبطن في اختبار الاليزا غير المباشر للكشف عن الأجسام المناعية المضادة لفيروس الهيربس البقرى-١ فى عينات السيرم البقرى. وقد أثبتت الدراسة فعالية هذا البروتين كانتيجين مبطن لإطباق الاليزا، بالاضافه انه أمكن بواسطته التفريق بين سيرم العجول المحصنة باللقاح المدلى وسيرم العجول المحصنة باللقاح الخالي من الجليكوبروتين ايه gE لفيروس الهيربس البقرى-١ .