EXPRESSION OF RECOMBINANT US9 PROTEIN OF BOVINE HERPESVIRUS TYPE1.1 (BOHV-1.1) IN INSECT CELL LINE AND EXAMINE ITS APPLICATION IN AN IMMUNO ASSAY FOR THE DIAGNOSIS OF BOHV-1.1 INFECTED CATTLE

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Infectious Bovine Rhinotracheitis (IBR), is a serious viral disease caused by bovine herpesvirus type1 (BoHV-1) which dramatically influence the upper respiratory tract of the infected animals. It is a major viral pathogen of cattle that causes significant economic losses to dairy production **(**Meurens *et. al.*, 2004; Robinson *et. al.*, 2008; Jaysukh *et. al.*, 2018). Bovine herpesvirus is an economically important disease of bovine and causes infertility, abortions, respiratory and gentile tract infection **(**Jaysukh *et. al.*, 2018).

High density of dairy cows promotes viral spread and increases the chances that healthy susceptible one will come into contact with infected animals (Chandranaik *et. al.*, 2014).

The BoHV-1 classified as a member of the family *Herpesviridae*, subfamily *Alphaherpes virinae*, genus *Varicellovirus* which considered as the prototype herpesvirus of ruminants (Schwyzer and Ackermann, 1996).

The BoHV-1 genome encodes 73 recognized open reading frames (ORFs) within 135,301 bp double-stranded DNA molecule, which has been completely sequenced (GenBank accession number AJ004801, strain Cooper) (Schwyzer and Ackermann 1996; Muylkens *et. al.*, 2007; Robinson *et. al.*, 2008). BoHV-1 genome encodes for at least 69 proteins among them 10 genes encoding for glycoproteins in which they are useful targets for diagnosis, prevention or antiviral treatment (Momtaz *et. al.*, 2009). The *Us9* is one of the genes that encoding an enveloped tegument glycoprotein. *Us9* and its homologue in alpha herpesviruses are necessary for the viral anterograde spread from the presynaptic to postsynaptic neurons.

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US9 is un glycosylated type II tail anchored membrane protein with a short ecto/luminal domain at the carboxyl terminus and contains a long cytoplasmic domain adjacent to a putative transmembrane domain at the amino terminus (Lyman *et. al.,* 2009). It consists of 144 amino acids encoded by a fragment of 435 bp. It has a profound effect on the neuro virulence properties of the virus and is also required for the entry and transport of envelope glycoproteins in which glycoprotein E (gE) and US9 tegument are conserved in all members of the neurotropic Alpha herpesviruses (Chowdhury *et. al.,* 2011). Many reports have showed that gE and US9 tegument glycoprotein are essential for the anterograde axonal transport of the virus and/or anterograde transsynaptic spread. Deletion of either gE or US9 has a profound effect on the neurovirulence properties of the virus. Furthermore, US9 is a required protein for the entry and transport of envelope glycoproteins in axons (Chowdhury *et. al.,* 2006). Because of its location in the virion envelope and on the surface of infected cells, it has considered as an important candidate targeted for molecular and immunological assays (Schwyzer and Ackermann 1996). The baculovirus expression vector system (BEVS) has gained particular prominence for producing such eukaryotic targets (Kost *et. al.,* 2005). Recently, several recombinant proteins have been produced using baculovirus expression system, including protein-based human and veterinary vaccine (El-Kholy *et. al.,* 2013). It is suited for producing foreign eukaryotic or prokaryotic proteins using BEVS because of different advantages of the system including, post-translational modification and high capacity for multiple genes or a large insert (Murphy *et. al.,* 2004). Moreover, baculovirus expression system has showed the ability for high yield protein expression using two strong prompters' very late promoters denoted pol and p10. Among the natural host cells, Sf21 and its clone Sf9 derived from the ovarian tissue of *Spodoptera frugipereda* are the most popular host suitable for protein expression using baculovirus system (Yu-chen HU 2005; Trowitzsch *et. al.,* 2010). Some BoHV-1 glycoproteins encoding genes have been expressed using baculovirus expression system and successfully used as a coating antigen in an indirect enzyme linked immunosorbent assay (ELISA) such as; the full length of *gD* gene (Abdelmagid *et al.,* 1998) and the *gE* gene of Egyptian BoHV-1.1 strain "Abu-Hammad"(El-Kholy *et. al.,* 2013). Hence, the aim of this study is to examine the specificity of the US9 tegument glycoprotein of BoHV-1.1 Egyptian strain "Abu-Hammad "as a coating antigen in a simple indirect ELISA, in the way for effective, fast and cheap diagnosis of BoHV-1 infected cattle using serological methods.

MATERIALS AND METHODS

Virus and Insect cells

The Egyptian BoHV-1 (Abu-Hammad) strain was used in this study (Hafez *et. al.,* 1976). The purified genomic DNA of BoHV-1 was kindly

provided by Dr. Alaa El-kholy, Veterinary Serum and Vaccines Research Institute, Abbasia, Cairo, Egypt. Sf9 insect cells, a clonal isolate of *Spodopterafrugiperda* Sf21 cells (IPLB-SF21-AE), was used for virus propagation and recombinant protein production using baculovirus expression system. Cells were maintained in Excell-420 serum-free medium (Sigma Aldrich) at 27°C with an antibiotic-anti mycotic mixture.

PCR amplification of *Us9* ORF

One primer pair was designed for PCR amplification of *Us9* ORF according to the published sequence of Bovine herpesvirus type 1.1 (accession number AJ004801). The forward primer denoted *Us9*-F 5'-ATAGGATCCATGGAGAGTCCACGC AGCGCT-3' contains *Bam*HI site in its 5' end. The reverse primer denoted *Us9*-R 5'-ATAGAATTCTCAGGGCCGGGGCAC TACCTC-3' contains recognition site for *Eco*RI in its 5' end. The restriction enzyme sites (underlined) were added to both primers for subsequent cloning procedure. The reaction was performed in a volume of 50 µl contains; 1µl of the appropriate primers (10pmol/µl), 2 µl $MgCL_2$ (25mM), 1 µl of dNTPs mixture solution (10mM), 3 µl of DNA template (300 ng), (5u/µl) Taq DNA polymerase 10µl of 5x green Go Taq Flexi buffer (Promega). The PCR program used for *Us9* ORF amplification was as follow; an initial cycle of 95°C for 3 min., a total of 35 cycles includes; denaturation at 95°C for 1 min., primers annealing at 61°C for 1 min., polymerization at 72°C for 1 min. and a final extension cycle at 72°C for 7 min. The PCR product was analyzed by electrophoresis in1% agarose gel (Sigma, USA) containing 0.5 µg/ml ethidium bromide in 1X TAE buffer and visualized under UV light using gel documentation. Subsequently, PCR product was purified from Agarose gel using Qia quick gel extraction kit (Qiagen) for downstream work.

Generation of the recombinant bacmid

The purified PCR product of *Us9* ORF was directly cloned into pGEM®-T Easy vector and about 50 ng of the corrected construct was subsequently transformed into DH5α *E.coli*competent cells by heat chock at 42ºC for 50 seconds. The transformed bacteria were selected by screening the colonies on LB-Agar plates containing 50µg/ml ampicillin. The suspected colony was further analyzed by restriction enzymes digestion and PCR. In order to clone the *Us9* ORF fragment into pFastBac™1 vector, the PCR product was double digested with *Eco*RI and *Bam*HI and ligated into pFastBac™1 vector (cat. no. 10359-016 in Bac-to-Bacbaculovirus Expression System kit, ThermoFisher), which was previously digested using the same restriction enzymes. The DH5α *E.coli* competent cells were used for transformation of the mixture, then the transformed bacterial cells were selected

by screening the colonies on LB-plates containing 50µg/ml ampicillin. The suspected colony was further analyzed by restriction enzymes digestion. To generate the recombinant bacmid, 1ng of the purified pFastBac-vector DNA was transformed into 100µl DH10Bac chemically component cells according to the manufacture instruction supplied with the Bac-to-Bac Baculovirus Expression System (Thermo Fisher). Screening was performed using LB Agar plates containing 50µg/ml kanamycin, 7 µg/ml gentamycin, 10 µg /ml tetracycline, 100 µg /ml X-gal, and 40 µg/ml IPTG to select for DH10Bac transformants. The suspected colony was further analyzed by PCR using pUC/M13 forward primer 5-'CCCAGTCACGACGTTGTAAAACG-3' and pUC/M13 reverse primer 5'-AGCGGATAACAATTTCACACAGG-3' that hybridize to sites flanking the mini-*att*Tn7 site within the *lacZ*α-complementation region to facilitate PCR amplification of the target gene. The PCR analysis of the recombinant colonies was carried out in a total volume of 20µl containing 1µl of the appropriate primers (10 pmol/µl), 10µl Amerald mixture solution and 1µl of DNA template (20ng). The following cycling parameters were used; one cycle at 95°C for 3 min. as initial followed by a further 30 cycles: denaturation at 95°C for 45 sec., primer annealing at 55°C for 45 sec., and primer extension at 72°C for 4 min. and the final extension cycle at 72°C for 7 min. The PCR product was analyzed by electrophoresis in 0.7% agarose gel in 1X TAE buffer and visualized by ethidium bromide staining under UV light using gel documentation.

Propagation of vAc-US9 in Sf9 cells

To produce recombinant baculoviral stocks in Sf9 cells, an amount of 8x105 Sf9 cells were transfect in a 6-well plate (35mm) with 1 to 2 µg of the recombinant bacmid DNA using 8µl of cell fection II reagent. The cells were incubated at 28°C for 72 h until signs of viral infection were seen. Once the transfected cells were demonstrated signs of infection, the medium containing the budded virus (BVs) were collected by centrifugation. The Sf9 cells were infected with the clarified supernatant in serial infection process to generate a high-titer P4 viral stock according to (Luckow and Summers1988).

Indirect Immunofluorescence Assay (IFA)

The distribution of the expressed US9 protein in infected Sf9 cells were screened by IFA technique as described in (Abdelmagid *et. al.,* 1998) after some modifications as in (El-Kholy *et. al.,* 2013). The Sf9 cells were incubated with the recombinant virus harbouring the *Us9* gene that denoted (vAc-US9) at 27°C for 48h, then cells washed using phosphate buffer saline (PBS, PH 7.6) and subsequently reacted with anti BoHV-1 positive serum at 27°C for 1h. The cells were probed after washing in PBS with Fluorescein isothiocyanate (FITC) diluted to 1:50 in PBS. Subsequently, infected cells were examined using inverted

fluorescent microscope after mounting with glycerol.

SDS-PAGE and western blot analysis

The Sf9 cells were infected with the generated P4 viral stock of vAc-US9 using multiplicity of infection (m.o.i) of 5 in 6-well plate, and the total proteins were extracted from the cell lysates using 400 µl 1x SDS-PAGE buffer. SDS-PAGE and Western blot assays were performed for the total proteins extraction according to the method described by (Laemmli, 1970; Sambrook and Russell, 2000), to determine and identify the expressed recombinant US9 protein. Protein components of the crude cell lysate (vAc-US9) and the mock cell lysate were electrophoresed through 12% SDS-PAGE gel and transferred onto PVDF membrane using semi dry transfer cell (TRANS-BLOT®SD). The electro blotting was run for 30 min at 20 V. The membrane was blocked in 5% skimmed milk dissolved in PBS-T at room temperature (RT) for60 min. Then it was incubated with the primary anti-BoHV-1 antibody with dilution of 1:1000 for 90 min at RT. After washing three times with 0.1% Tween-20 in 1x PBS (phosphate buffer saline) each for 10 min., the membrane was incubated with BoHV-1 antisera as the secondary antibody conjugated with Horse- Radish Peroxidase (Bio-Rad, Milano, Italy) at RT for 60 min. The peroxidase activity was developed by adding a substrate AEC solution and the membrane was visualized.

Indirect US9-based ELISA

Cell lysate infected with the recombinant virus vAc-US9 was used as a coating antigen in an indirect ELISA test according to the method described by (Voller *et. al.,* 1976) with some modifications described by (El-Kholy *et. al.,* 2013). Cell lysate was harvested and diluted 1:100 in PBS (0.05% Triton X-100). ELISA 96-well plate was coated with 100 µl of diluted protein lysate either cell lysate (pellet) or culture filtrate (supernatant) per well. The plate was incubated with this coating antigen at 4°Covernight. The plate was washed using 300 µl PBS containing 0.1% Tween 20 (PBST), then incubated at 37°C for 2 h with 100 µl of blocking buffer (PBS, pH 7.4 containing 5% non-fat milk and 3% bovine serum albumin, BSA). After washing, the plate was incubated with 100 µl of anti BoHV-1 serum diluted to 1:100 at 37°C for 2 h. Then 100 µl of diluted anti-bovine horse radish peroxidase conjugated IgG (1/1000) were added after washing the plate to each well and reincubated at 37 °C for 1 h. To allow colour development 100 µl of TMB substrate indicator mixture were added to each well and the plate was incubated in a dark place at room temperature for 15 min. Then, 100 µl stopping buffer were added to each well to stop colour development. Finally the absorbencies

were read at 450 nm wavelength filters in an automated ELISA reader. The optical densities of the infected cells and non-infected (mock) cells were recorded at OD of 0.5 which was measured as interrupted point. All reads that were higher than 0.5 considered as positive samples, and reads less than 0.4 considered as negative samples for all tested samples.

RESULTS AND DISCUSSION

PCR amplification of *Us9* gene

In this study, a recombinant baculovirus containing the enveloped tegument glycoprotein (*Us9*) of BoHV-1.1 Abu-Hammad strain was constructed and used to express full length *Us9* in insect cells. The expressed protein was used to develop a simple indirect ELISA based on a recombinant *Us9* antigen expressed using baculovirus system. The BoHV-1 of the Egyptian Abu-Hammad BoHV-1 strain has revealed close identity to the reference Cooper1 BoHV-1.1 strain (El-Kholy and Abdelrahman, 2006). For this, one pair of specific primers was used to amplify the coding sequence of *Us9* gene based on BoHV-1 genome sequence published in the GenBank (accession number NC-001847.1), in which the sequence begins at nucleotide number 123548 and ends at nucleotide number 123982 of the BoHV-1 genome. The generated PCR amplicon was 435bp according to the corresponding size of *Us9* as shown in Fig. (1).

Generation of the vAc-US9 recombinant bacmid

Recombinant bacmid harbouring the BoHV-1 *Us9* gene under the control of polyhedrin promoter (polh) was generated using Bac-To-Bac baculovirus expression system. The recombinant plasmid carrying the *Us9* fragment downstream of polh promoter was transformed into DH10Bac cells to facilitate transposition process into *att*Tn7 region. To verify the correct bacmid construct, PCR analysis was performed using pUC/M13 specific primers flanking the transposition site of the BoHV-1 *Us9* cassette. The PCR analysis revealed a single amplicon at the calculated size of about 2735 bp corresponding to the BoHV-1 *Us9* (435 bp) plus the pFastBac1 cassette of 2300 bp (Fig. 2). No PCR amplicon of 300 bp was observed in the same lane of the recombinant bacmid. These results confirm the purity of the DH10Bac colony contains the recombinant bacmid. The generated recombinant virus was denoted as vAc-US9.

In this study, an advanced method for the detection of BoHV-1-infected animals was developed using US9 as an enveloped tegument glycoprotein. US9 is required protein important for the entry and transport of envelope glycoproteins in axons (Enquist, 2002; Tomishima and Enquist, 2001; Tomishima, *et. al.,* 2001). US9 tegument glycoprotein is a type II tail anchored membrane protein in BoHV-1 and conserved in alphaherpesviruses (Chowdhury *et. al.,* 2006). Since it relies

in the envelope of BoHV-1 virion, *Us9* can be a good candidate to have a pathogenicity action against the host immune system (Schwyzer and Ackerman, 1996). Infection of primary neuronal cultures with PRV *Us9* mutants demonstrated that in the absence of *Us9*, the entry and transport of envelope glycoproteins in axons are affected (Enquist, 2002; Tomishima and Enquist, 2001). In order to examine the properties of US9 as a candidate antigen for BoHV-1-infection diagnosis, the *Us9* coding sequence was expressed using baculovirus expression system. Subsequently, the antigenic properties of the US9 antigen was verified against BoHV-1 specific antibodies. For this, a recombinant baculovirus harbouring the coding sequence of BoHV-1/*Us9* gene of 435bp was amplified and cloned into baculovirus vector system and expressed in insect cell culture.

Screening of the infected Sf9 cells with the vAc-US9

Signs of viral infection have been detected in Sf9 insect cells transfected with the recombinant virus vAc-US9 about 48-72 hours post infection (h.p.i) compared to non-infected cells (mock infection). Cytopathic effect of infected Sf9 cells at different times post-infection was clearly observed. Most of all cells rounded and increased in size and grainy nuclei appearance was also observed in some cells confirmed the successful transfection with the vAc-US9 virus (Fig. 3).

The baculovirus expression system has been used extensively for expression of different eukaryotic genes such as; Lymphocytic Chronic meningitis (LCMV), Glycoprotein precursor (GPC), Heamaglutinin gene of Influenza A virus (HA) (Matsura *et. al.*, 1987), G protein-coupled receptors (GPRCs) (Massotte, 2003), gD of BoHV-1 (Abdelmagid *et. al.*, 1998) and glycoprotein E of BoHV-1 (El-Kholy *et al.*, 2013). In the current study, the glycoprotein tegument US9 of BoHV-1 was successfully expressed in Sf9 cells using the generated recombinant virus vAc-US9 which was subsequently detected using immunofluorescence assay and western blot. The BoHV-1/ US9 ORFs consisted of 144 amino acids and the corresponding predicted molecular mass is considered to be about 14.77kDa. However, protein analysis of the expressed US9 protein using SDS-PAGE and western blot showed a clear protein band at approximately 32-34 kDa. The predicted amino acid sequence alignment of BoHV-1/ US9 sequence revealed that there are several phosphorylation motifs conserved in this sequence (Chowdhury *et. al.*, 2006). Consistent with their analysis, they considered the BoHV-1/US9 as phosphoprotein in which the additional 10 a.a. and the extra phosphorylation sites was contributed to the increase in its molecular weight compared to its predicted molecular weight. These reports in accordance with

our results suggested posttranslational modifications, mainly phosphorylation of the expressed US9. Similar incomplete processing in insect cells has been observed also with *gB* (El-Kholy *et. al.,* 2013), *gD* (Van Drunen Little-van den Hurk, *et. al.,* 1993), *gE* (Yoshitake *et. al.,* 1997).

Immunofluorescence assay (IFA)

The expression of US9 protein in infected Sf9 cells was detected using the indirect immunofluorescence assay (IFA). The reaction showed specific reactivity with the anti-BoHV-1 positive serum for vAc-US9-infected cell, while no reactivity could be observed in non-infected cells (mock-infection). The infected cells exhibited fluorescence of expressed protein in positively stained cells (Fig. 4). These confirms successful expression and correct folding of US9 in Sf9 cells.

Therefore, using indirect immunofluorescence assay (IFA), the infected cells exhibited cell greenish fluorescence confirmed the successful expression and correct folding of US9.

SDS-PAGE and western blot

As shown in Fig. (5A) analysis of the total protein extracts from vAc-US9-infected Sf9 cells showed a clear protein band at 32-34 kDa. In a time course of 24-72 hpi on SDS-PAGE, the higher protein intensity was showed at 72 h.This time point was used for protein immunoassay detection of the expressed protein using western blot. As shown in Fig. (5B) a clear signal was observed at 72 hpi in PVDF membrane incubated with the BoHV-1 specific antibodies at the same molecular mass of 32-34 kDa. This result confirmed the successful folding of the expressed US9 protein using baculovirus system which could be correctly detected using BoHV-1 antisera.

Indirect US9- based ELISA test

The indirect US9-based ELISA was performed in order to verify the antigenic properties of the US9 antigen expressed by the recombinant virus in Sf9 insect cells. Bovine serum samples were used to examine the sensitivity of specific antibodies against BoHV-1-US9 using the lysate of infected cells using vAc-US9 either cells pellet or cells filtrate (supernatant), which was used as a coating antigen in indirect enzyme-linked immunosorbent assay (ELISA). As shown in Table (1) all serum samples isolated from BoHV-1 vaccinated cattle were seropositive by indirect ELISA-US9 test in which S_2 and S_3 shows high reactivity with anti BoHV-1 serum estimated by ELISA values with a mean of 0.215 (supernatant) and 0.216 (pellet) for S_2, and 0.198 (supernatant) and 0.227 (pellet) for S_3.

While S_1 and S_5 show lower reactivity estimated by ELISA-US9 mean values of 0.103 (supernatant) and 0.113 (pellet) for S_1, and 0.136 (supernatant) and 0.133 (pellet) for S_5 in comparison with C^+ (positive control for BoHV-1 antibody diluted to 1:100). In addition, one serum sample S_4 of the BoHV-1 vaccinates

shows a seronegative result estimated by ELISA values of 0.06 (supernatant) and 0.066 (pellet) in comparison with ELISA values of 0.075 (supernatant) and 0.06 (pellet) for C^- (Fetal Calf Serum without anti IBR) as negative control. In addition to Blank (diluting buffer) (1xPBS) only without serum. The indirect ELISA-US9 showed high signals in four of the five tested sera; S_1, S_2, S_3, and S_5 that were previously tested.

In the same context, a recombinant baculovirus expressing glycoprotein E (gE) of the Egyptian BoHV-1.1 was previously generated (El-kholy *et. al.,* 2013). The expressed protein was used as a coating antigen for the detection of anti-gE antibodies in experimental bovine sera. Results showed that gE-based indirect ELISA was able to detect anti-gE antibody in sera from gE+ vaccinated calves in all dilution samples (1:2-1:128) of anti-gE antibody.In the same context, the results of an indirect ELISA test using recombinant gE showed 98.41% sensitivity and 99.76 % specificity against BoHV-1-gE in bulk milk as a source of antibodies for IBR (Bertolotti *et. al.,* 2015). In the current study, results showed that the vAc-US9 based indirect ELISAwas able to detect anti-US9 antibody in sera from infected cattle in dilution sample of (1:100) of anti-US9 antibody. However, the sensitivity of anti-US9 antibody was higher in crude cell lysate infected with vAc-US9 compared to cell culture filtrate (supernatant), which confirm the high concentration of the expressed protein in Sf9 cells compared to cell culture filtrate.

Thus, the results of the simple indirect ELISA test revealed that, the expressed US9 tegument glycoprotein in crude lysate of vAc-US9-infected Sf9 cells was sensitive enough as an antigen to be recognized by serum samples specific for BoHV-1. In conclusion, this study we succeeded to prove the effective usage of BEVs for the production of recombinant baculovirus/BoHV-1/US9 protein. The crude lysate from the infected Sf9 cells with vAc-US9 as coating antigen proved to be sensitive and efficient in indirect ELISA test for detection of anti-US9 serum samples infected with BoHV-1. The reactivity of vAc-US9/BoHV-1 with anti BoHV-1 positive serum in indirect ELISA indicated that, the baculovirus expressed US9 was retained its antigenic properties as well as the native US9 of BoHV-1. These results suggested that the BoHV-1/US9 tegument glycoprotein can be used as a coating antigen in indirect ELISA as a diagnostic value for detection of BoHV-1infection among cattle in Egypt. Herpesviruses have a number of genes encoding glycoproteins located in the viral envelope which is important targets for host immune responses (El-kholy *et. al.,* 2013). Therefore, further studies using one or more of such glycoproteins may be used in order to examine their immunoassay capability for the diagnosis of BoHV-1.1 infected animals.

SUMMARY

In this study, a recombinant baculovirus expressing the US9 tegument protein of the Egyptian BoHV-1.1 Abu-Hammad strain (vAc-*Us9*) was constructed using Bac-to-Bac expression system. A coding fragment of 435bp of *Us9* gene was cloned into pFastBac-1 expression vector then transferred into the baculovirus expression vector system (BEVs) for expression f BoHV-1 US9 tegument protein. The PCR analysis of the recombinant virus confirmed the successful transposition of the expression cassette into the recombinant bacmid. The expressed US9 protein in infected cell culture was assayed using Indirect Immunofluorescence Assay (IFA) in addition to SDS-PAGE and western blot using crude cell lysate. The US9 protein expressed in infected cell culture supernatant as well as cell lysate were used as a coating antigen in an indirect enzyme linked immunosorbent assay (ELISA) to examine its sensitivity for the detection of the specific antibodies that generated against BoHV-1. The effectiveness of using of the expressed US9 protein as antigen for anti-US9 detection in serum samples infected with BoHV-1 was discussed in details.

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Table (1): Antigen sensitivity test using ELISA. The sensitivity of the expressed US9 protein as an antigen against anti-BoHV-1specific antibodies was tested using an ELISA assay. The table shows mean ELISA optical density value (OD) determined using wavelength 405nm.

Serum Sera	ELISA Values					
	Supernatant		Mean	Pellet		Mean
C^+	0.119	0.100	0.109	0.100	0.106	0.103
C^-	0.077	0.074	0.075	0.060	0.060	0.060
Blank	0.087	0.079	0.083	0.065	0.066	0.066
S1	0.099	0.107	0.103	0.109	0.118	0.113
S2	0.188	0.242	0.215	0.227	0.206	0.216
S3	0.203	0.193	0.198	0.257	0.197	0.227
S4	0.079	0.075	0.077	0.062	0.067	0.066
S5	0.128	0.144	0.136	0.128	0.139	0.133

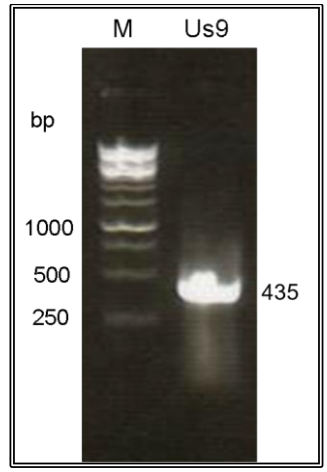

Fig. (1): Ethidium bromide stained agaros gel shows PCR amplification of *Us9* gene ORF ofBoHV-1. The PCR using Us9 specific primers gave the expected size of ~ 435 bp corresponding to the Us9 ORF. M: 1 kb DNA ladder.

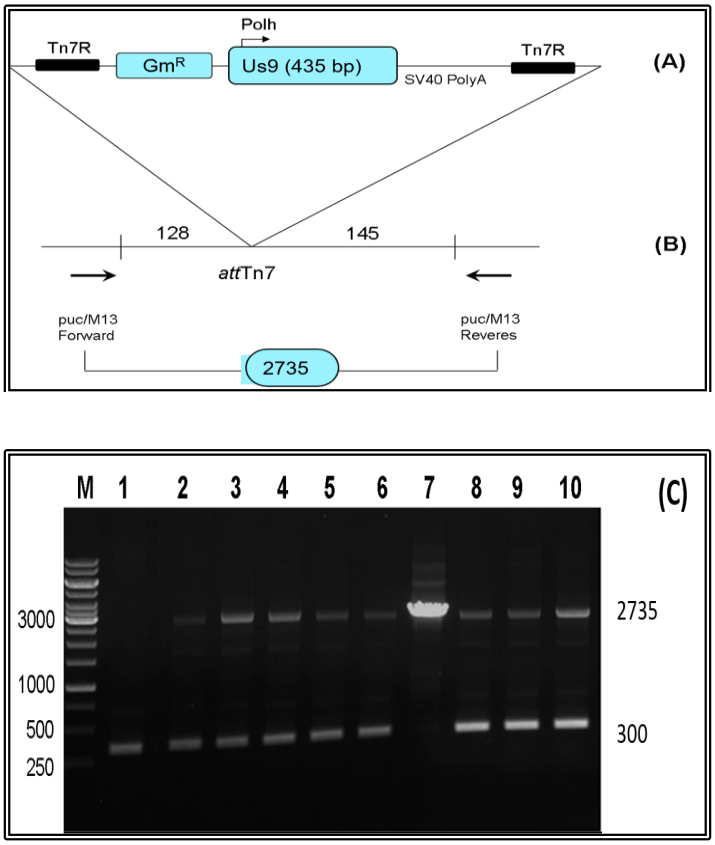

Fig. (2): Schematic representation of the recombinant virus vAc-Us9. (A) The bacmid cloning vector pFBD-Us9 harboring the BoHV-1 Us9 ORF under the control of polyhedrin promoter (Polh). (B) The generated vAc-US9 virus shows the *att*Tn7 transposition site and the M13 Forward/M13 Reverse primers used for PCR detection of the recombinant virus. (C)Agaros gel shows PCR detection of the recombinant bacmid using forward M13 and M13 reverse primers flanking the *att*Tn7 transposition site. Lane 1: Negative control (blue colony). Lane 2-6; 8-10: represent PCR products of colonies carrying both recombinant (2735 bp) and empty (300bp) bacmid. Lane 7: Positive colony represents the PCR product of the recombinant bacmid at 2735bp. M: 1 Kb DNA ladder. The fragments sizes (bp) are given to the left and to the right.

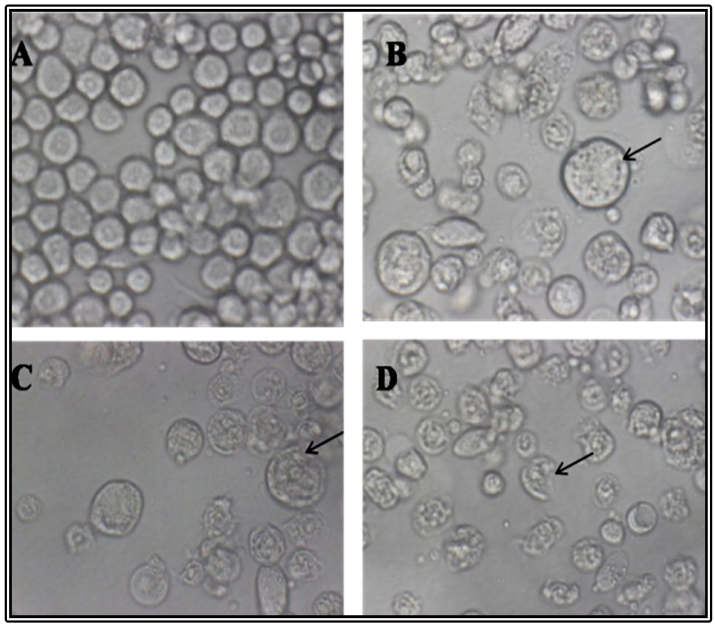

Fig. (3): Characteristics of viral-infected Sf9 cells using vAc-US9. A: represents non-infected Sf9 insect cells (mock infection). B, C and D in which the arrows show signs of viral infection in Sf9 cells through time points of 24, 48 and 72-hours post infection, respectively.

Fig. (4): Indirect immunofluorescence assay (IFA). The photo shows infected Sf9 cells using the recombinant virus expressing US9 protein (vAc-US9) which was reacted with anti BoHV-1 positive serum and probed with FITC-labelled anti-BoHV-1. Arrow refers to surface perinuclear fluorescence in positively stained cells against a negative background. Magnification is 300x.

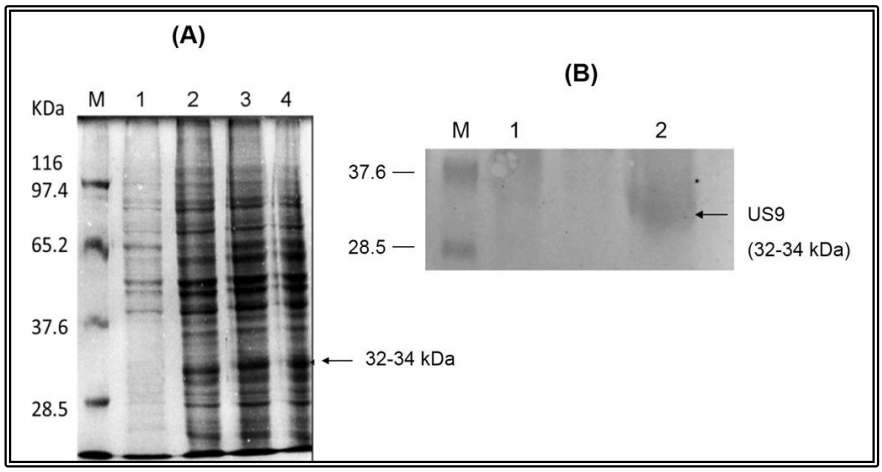

Fig. (5): Expression of US9 by the recombinant vAc-US9. Sf9 cells were infected at an m.o.i. of 5 withvAc-US9. Cellular proteins were extracted and harvested at appropriate time point's p.i., and separated with 12%SDS-PAGE. The polyclonal antibody against BoHV-1.1 (anti-US9) was used for western blot analysis. (A) SDS-PAGE analysis for US9 expressed protein using the recombinant virus vAc-US9. Lanes M is a broad range protein marker. Lanes 1 represent the total protein extracts from healthy Sf9 cells (mock infection). Lanes 2 represents total protein extracts from infected cells using the recombinant virus vAc-US9. Arrow shows protein band of 32-34 KDa corresponding to US9. (B) Western blot analysis of vAc-US9-infected Sf9 cells. (M) Page Ruler prestained protein ladder. (1) Normal Sf9 cells (mock infection), (2) Infected Sf9 cells using vAc-US9. Arrow shows signals at about 32-34 KDa corresponding to the US9 protein.