Expression of the bovine coronavirus spike glycoprotein subunits in insect cells using recombinant baculoviruses

(Received: 02.03.2007; Accepted: 09.04.07)

H. M. Amer; H. A. Hussein*; I. M. El-Sabagh;A. A. El-Sanousi;M. S. Saber and M. A. Shalaby*
Department of Virology, Faculty of Veterinary Medicine, Cairo University, 11221 Giza, Egypt
* Corresponding authors.

ABSTRACT

In the present study, the bovine coronavirus (BCV) spike glycoprotein cleavage products (S1 and S2) were individually expressed in Spodoptera frugiperda (Sf9) insect cells, using a baculovirus expression system. The coding sequence of S1 and S2 gene fragments were amplified by RT-PCR and cloned into the baculovirus shuttle vector pBlueBac4.5/V5-His TOPO ® TA. The cloned fragments were inserted into the genome of Autographa californica nuclear polyhydrosis virus (AcMNPV) under control of the polyhedrin promoter, through a process of homologous recombination between the shuttle vector and a linearized replication-defective baculovirus DNA (Bac-N-Blue™). Recombinant baculoviruses were selected by plaque purification; verified for the presence of target sequences using PCR and propagated for generation of high-titer viral stocks. Infection of insect cells with the recombinant baculoviruses revealed high-level expression of the target proteins as indicated by immunofluoresent test and solid phase ELISA using BCV-specific polyclonal antiserum.

Keywords: *BCV, glycoproteins, recombinant, baculovirus*

INTRODUCTION

Bovine coronavirus (BCV) is one of the leading causes of neonatal calf diarrhea worldwide. It may be responsible for 11-81% of the reported cases and one-quarter of all deaths caused by the disease syndrome (Saif and Heckert, 1990). BCV particles are spherical, enveloped with a diameter of 80-160 nm and possessing a large (30 kb), single-stranded, non-segmented RNA genome of positive polarity (Regenmortel *et al*., 2000). The virion is comprised of four major structural proteins, three are surface tran-membrane glycoproteins: integral membrane (M); spike (S) and haemagglutinin-esterase (HE), whereas the fourth is an internal nucleocapsid (N) phosphoprotein (Lai and Cavanagh, 1997).

The spike glycoprotein is the predominant structure of viral envelope forming the characteristic petal-shaped projections protruding from the surface of BCV particles (Lai and Cavanagh, 1997). The protein is digested post-translation at amino acid residues 768-769, producing N-terminal S1 subunit, which forms the globular portion of spikes and C-terminal S2 subunit that forms the stalk portion (Struman *et al*., 1985). Several biological functions have been attributed to S glycoprotein from which cell-binding and cell-fusion are the most important for biology and pathogenesis of the virus (Holmes and Lai, 2001). In addition, S protein (particularly its globular portion; S1) is the major target of host's immune response, where neutralizing antibodies and cell mediated

immunity are potentially induced (Holmes *et al*., 1986 and Deregt *et al*., 1987). Immunization of animals with S glycoprotein in the absence of any other virus component persuades a complete protection against coronavirus infections (Torres *et al*., 1995).

Protection against BCV-enteritis is basically dependent on the presence of adequate levels of specific antibodies in the gut lumen of newly born calves (Rodak *et al.*, 1982). Therefore, the immune status of susceptible calves may be raised either by parental vaccination of pregnant dams to increase the level of passively acquired immunity or by oral vaccination of newly born calves to stimulate the active immunity (Clark, 1993). Active immunization of calves with live attenuated BCV vaccines has not proved any effectiveness in the field trials (Thurber *et al.*, 1977). Although vaccination of pregnant animals with inactivated vaccines has been used successfully to reduce the incidence and severity of infection (Heckert *et al.*, 1991), several researchers are still arguing that the induced antibody level in lacteal secretions of immunized dams is not satisfactory for protection against field infection (Rodak *et al*., 1982 and Möstl and Bürki, 1987).

Modern strategies of vaccine production have been introduced and developed in the last two decades due to the rapid advance in molecular biology techniques. Baculovirus expression system has become among the most popular systems for production of recombinant proteins used extensively in formulation of subunit vaccines and development of diagnostic tests (Luckow and Summers, 1988). In the present study, we describe the construction of recombinant baculoviruses containing BCV S1 and S2 gene fragments and characterize their expressed products. The produced recombinant proteins will serve in the future as invulnerable tools for development of subunit vaccines for immunization of pregnant cows and buffalos as an alternative to the currently available vaccine candidates; and production of improved diagnostic tools for rapid and accurate diagnosis of BCV infection in field cases, beside their potential use in the basic structural, functional and immunological studies of BCV.

MATERIALS AND METHODS

Cells, viruses and antibodies

The Mebus strain of BCV (Mebus *et al.*, 1973) was kindly provided by L.J.Saif (Ohio Agricultural Research and Development Center, Ohio State University, Wooster, Ohio, USA) and was propagated for four successive passages in MDBK cells grown in minimal essential medium (MEM) containing 40 µg/ml gentamycin and 10% fetal bovine serum. *Spodoptera frugiperda* (Sf-9; Invitrogen, San Diego, CA) were grown in monolayer cultures in TNM-FH medium (Gibco BRL, Life Tech, Grand Island, NY) supplemented with 0.25 µg/ml amphotericin-B, 100 U penicillin; 100 µg/ml streptomycin; 0.1 mg/ml Pluornic polyol F-68 (Sigma chemical co., St. Louis, Mo, USA) and 10% fetal bovine serum at 27ºC (Summers and Smith, 1988). Polyclonal rabbit anti-BCV antiserum was prepared as previously described (Langone and Van Vunkis, 1986).

Amplification of S1 and S2 gene fragments

Total RNA was extracted from BCV-infected MDBK cells by the guanidium-thiocyanate method using TRIZOL reagent (Gibco BRL) according to the manufacturer's instructions. The RNA was denaturated by incubation with dimethyl sulphoxide (DMSO) at 70°C for 10 minutes. The oligonucleotide primers used for cDNA synthesis and PCR were chosen from the BCV-ENT gene sequence (GenBank accession number, AF391541). Primers S1-F (5'-GGCTGCATGATGCTTAGACC-3') and S1-R (5'-GTAACCACTACCTACTGTGAGATCAC-3') were designed to amplify a fragment of 2298

bp that constitutes the S1-glycoprotein gene, while primers S2-F (5'-CTAACTCTTCCATGGAACCAGCATTGC-3') and S2-R (5'-CAAAGACGAACTTAATCTTCATGTGATG-3') were used for amplification of the S2-glycoprotein gene (2019 bp). The first strand synthesis was carried out in a volume of 20 µl containing 10 mM Tris-HCl (pH 8.3); 50 mM KCl, 2.5 mM Mg Cl_2, 500 µM each of the four dNTPs (Promega, Madison, USA); 1µl Reverse-iT® RTase (ABgene, Epson, Surrey, UK); 1µM of the downstream primer and 10 µl of the RNA, for 45 min at 47°C, and the reaction was stopped by heating at 95ºC for 2 minutes. The reaction of the second strand synthesis and amplification was performed using Gene-Amp 9700 (Applied Biosystem Inc., Foster City, CA, USA), where 5 µl of the reverse transcription product was added to a PCR mixture containing 25 µl 2x ReddyMix® PCR master mix (ABgene); 0.2 µM of both forward and reverse primers, and the volume were adjusted to 50 µl with nuclease free water. The PCR cycle profile was composed of: initial denaturation at 94°C for 2 min.; 40 cycles of 94°C for 1 min., 52°C for 1 min. and 72°C for 3 minutes; and final extension at 72°C for 10 min.

Construction of recombinant transfer vectors

To construct baculovirus transfer vectors containing S1 and S2 gene fragments, the RT-PCR product of both genes was firstly analyzed by agarose gel electrophoresis and gel-extracted using Montáge DNA gel extraction kit (Millipore, Concord Road Billerica, MA, USA), then cloned into the pBlueBac4.5/V5-His TOPO® TA cloning vector (Invitrogen). The cloning process was performed as directed by the manufacturer, according to Sambrook *et al*., (1989). Briefly, 4 µl of the gel-extracted DNA were mixed with 1 µl of the cloning vector and 1 µl of salt solution (1.2 M NaCl and 0.06 M $MgCl_2$). The mixture was incubated for 5 min. at room temperature, then 3 µl were gently swirled in a vial of competent TOP-10 cells. The cells were incubated on ice for 30 min.; heat shocked at 42ºC for 30 sec. and incubated on ice again. After addition of 250 µl of SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose), the transformed cells were incubated at 37ºC for 1 hr in a shaking incubator (200 rpm/min). Cells were spread onto Lauria-Bertani (LB) agar plates containing 50 µg/ml ampicillin (Sigma) and incubated at 37ºC overnight. A suitable number of colonies were picked up and allowed to propagate in 3 ml LB broth containing 50 µg/ml ampicillin for 12-16 hr in a shaking incubator (200 rpm/min). For identification of colonies that harbor recombinant plasmids carrying the target genes in correct orientation, 5-10 representative colony cultures were centrifuged at 5000 rpm for 5 min. and cell pellets were resuspended in 100 µl TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0). The recombinant plasmids were extracted by boiling for 10 min., then centrifuged for sedimentation of cell debris and collection of the supernatant were performed. A modified colony PCR procedure was employed by mixing 5 µl of the plasmid extract; 25 µl 2x ReddyMix® PCR master mix (ABgene); 0.2 µM of the forward primer (S1F or S2F) and 0.2 µM of the reverse primer (5`-GGAGATAATTAAAATGATAACCATCTCGC-3`). The amplification reaction was conducted as follows: 94°C for 2 minute; 30 cycles at 94°C for 1 min., 55°C for 1 min. and 72°C for 3 min.; and final extension of 72°C for 7 min.

Generation of recombinant baculoviruses

Baculoviruses each carrying S1 and S2 gene fragments were produced by homologous recombination between the transfer vector and linearized *Ac*MNPV DNA (Bac-N-Blue, Invitrogen) in insect cell culture (Kitts and Possee, 1993). Plasmid DNA was extracted from the transformed bacteria using QIAprep®

Miniprep plasmid extraction kit (QIAgen, Hilden, Germany) according to the manufacturer's instructions. Monolayer cultures of Sf-9 cells (1 X 10^6 cells per 35 mm dish) were transfected with a mixture of 0.5 µg Bac-N-Blue DNA; 8 µg of plasmid DNA and 20 µg Cellfectin® (Invitrogen) in unsupplemented Grace`s medium. The transfection mix was removed after 4 hr of incubation at 27°C and the cells were further incubated with 1 ml of fresh complete medium. The culture supernatant was harvested after 72 hr, and recombinant viruses were plaque-purified. Plaque assay was performed as described by Day *et al.,* (1995), in 35 mm dishes with 1% agarose overlay containing 150 µg/ml X-gal. Recombinant plaques appeared after 7-14 days of incubation as deep blue spots in the gel that was easily identified and picked-up. Harvested plaques were screened by PCR to confirm their purity utilizing two primers that flank the polyhedron region of baculovirus: BPH-F (5`-TTTACTGTTTTCGTAACAGTTTTG-3`) and BPH-R (5`-CAACAACGCACAGAATCTAGC-3`).The urified plaques were then amplified through successful passaging in insect cells and virus stocks with high titers (exceeding 10^7 PFU/ml) were used in protein expression studies.

Expression of S1 and S2 proteins in insect cells

Sf-9 cells, at a density of 3X10^6 cells per 25 cm^2 culture flask, were infected with the recombinant as well as wild-type baculoviruses at a multiplicity of infection (m.o.i) of 10. After 48 and 96 hr of incubation, cells were harvested and centrifuged at 2000 rpm for 10 min. (4ºC). The supernatant fluid was collected separately, while the cell pellet was washed twice with PBS. The cells (10^6 cells) were incubated with 100 µl of lysis buffer (1% Triton X-100 and 1 µg/ml Pepstatin A in PBS) for 45 min. on ice. Complete cell lysis was achieved by applying two cycles of freezing and thawing. Cell debris was sedimented by centrifugation at 4000 rpm for 10 min. (4ºC) and the supernatant (cell lysate) was collected. The debris was also resuspended in PBS; mixed vigorously and stored until use.

Enzyme linked immunosorbent assay

High protein-binding ELISA plates (BD Falcon™, Franklin Lakes, NJ, USA) were coated with the different preparations (culture supernatant; cell lysate and cell debris) of Sf-9 cells infected with recombinant and wild-type baculoviruses as well as uninfected cells. The plates were blocked with 5% non-fat dry milk (NFDM) in PBS. The plates were then incubated with 100 µl / well of the rabbit polyclonal anti-BCV serum (diluted 1/50 in PBS containing 0.5% NFDM) at 37°C for 2 hr. After washing the plate three times with PBS containing 0.05% Tween-20, 5 min. each, 100 µl of the peroxidase-labeled anti-rabbit IgG (Sigma) were added to each well. The plate was incubated at 37°C for 1 hr, and then washed three times with PBS-Tween. Color development was achieved by addition of H_2O_2/ABTS solution for 30 min. in a dark place, and then stopped by SDS 0.5%. Absorbencies were read using the automated ELISA reader at a wave length of 405 nm.

Immunofluorescence analysis of protein expression

Monolayer cultures of Sf-9 cells were prepared on cover slips at a density of 7.5 X 10^5 cells and infected with the recombinant baculoviruses at m.o.i of 10. Wild-type infected and non-infected cells were kept as negative controls. After 48 hr of incubation at 27°C, cultures were rinsed twice with PBS; air dried and fixed with chilled acetone (80%) for 20 minutes at 4ºC. Acetone was then discarded and cover slips were allowed to dry at room temperature. The cover slips were overlaid with 100 µl of rabbit polyclonal anti-BCV serum (diluted 1/20 in PBS) and incubated at 27ºC in a

humid chamber for 1 hour. After three times washing with PBS, 5 minutes each, 100 µl of the fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit IgG (Sigma) were added to each cover slip and incubated at 27ºC for 1 hour. Following another 3 washing cycles, the cover slips were removed and inverted on a microscopical slide covered with one drop of mounting buffer (50% glycerol in PBS). Positive fluorescence reactions were observed using a fluorescent microscope.

RESULTS

Cloning of S1 and S2 gene subunits in baculovirus transfer vector

The amplification products of S1 and S2 gene fragments (Fig. 1) were successfully inserted in the baculovirus transfer vector [pBlueBac 4.5/V5-His-TOPO®]. Transfo-rmation of competent TOP10 *E. coli* with the cloned vectors resulted in development of a satisfactory number (over 50) of recombinant bacterial colonies on the LB agar plates containing ampicillin. Only the distinct and well-spaced colonies were picked up and cultured in LB broth. The majority of these colonies (61% for S1 and 89% for S2) grew well in broth culture and showed a marked turbidity within 16-24 hr of incubation. The culture of five representative colonies for each gene was selected to identify those carrying recombinant plasmids with correct gene orientation. A modified method of colony PCR, utilizing forward primer of the target gene and reverse primer of the vector, was exploited for such purpose. Two positive colonies were identified for S1 gene subunit, while all tested colonies for S2 were completely negative. This required testing of another five colonies to define only one positive colony for S2 gene subunit.

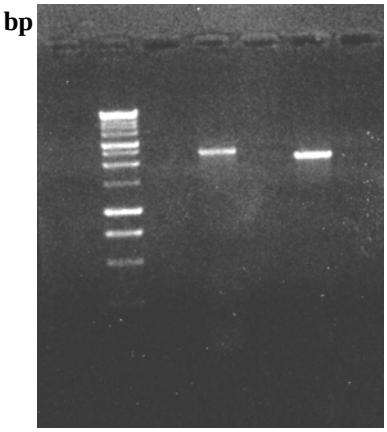

Fig. (1): RT-PCR products of S1 (2298 bp) and S2 (2019) gene sequences of BCV in ethidium bromide stained agarose gel electrophoresis, along with 1 kbp DNA ladder (M).

Production of recombinant baculoviruses

Synthesis of recombinant baculoviruses was manifested by sequential development of specific signs of infection in insect cells (Fig. 2). Signs appeared so early, after 24 hr of transfection, and were characterized by enlargement of cell diameter and increase in the nuclear size. After 48 hr of transfection, the cells began to stop growing with few cells were detached from the culture vessel surface. At the late stages of infection (over 72 hr), complete detachment and lysis of cells were clearly seen.

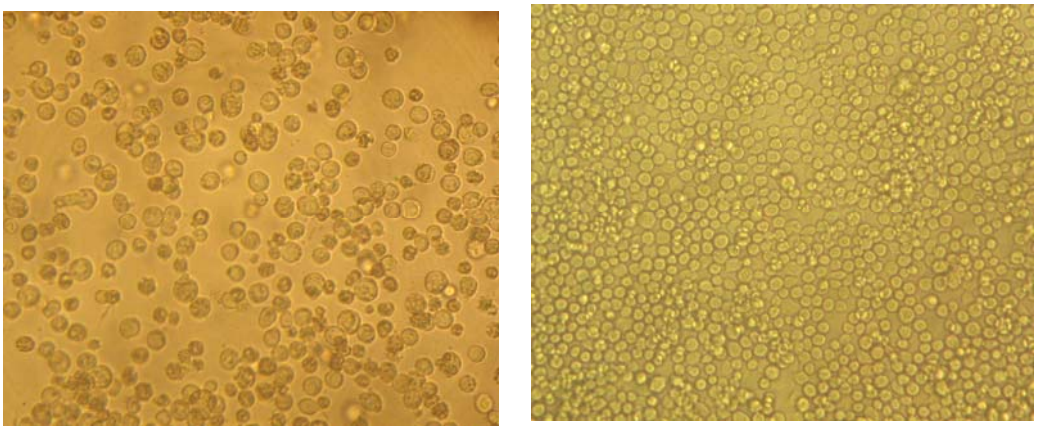

Fig. (2): Signs of replication of recombinant baculoviruses in Spodoptera frugiperda (Sf-9) cells [Left] along with the non-transfected control cells [Right].

The recombinant baculoviruses were purified from the culture fluid collected 72 hr post transfection using the plaque purification technique. Plaques appeared after 7-14 days of incubation in soft agarose medium as rounded, opaque areas in the tissue culture plate with a variable size range. Due to the presence of *Lac Z* gene in the transfer vector, the recombinant plaques were deep blue in color when the chromogenic substrate X-gal was added in the medium. Microscopically, the plaques appeared as empty areas in the cell monolayer and surrounded with infected cells, which were larger in diameter, displayed a marked increase in the nuclear size and showed signs of cell lysis (Fig. 3). Plaques were harvested and propagated in insect cells for production of a satisfactory virus (P1) stock.

Isolation of the pure, recombinant plaques from those mixed with the wild-type baculovirus was based on the use of PCR analysis of the P1 virus stocks. Two primers flanking the polyhedrin region of baculovirus genome were utilized for such purpose. When these primers were used for amplification of the target gene inserts, they yielded PCR products representing the size of the inserted gene + 435 bp additional sequence originated from the polyhedrin flanking region, whereas amplification of the polyhedrin region in the wild-type baculovirus produced a fragment of 839 bp. Results of PCR analysis of seven representative plaques (five for S1 and two for S2) identified two pure clones for each S-gene subunit (Fig. 4). One representative clone was selected for each gene, propagated for several successive passages and titrated by plaque assay.

Synthesis of the S1 and S2 polypeptides in insect cells

The expression of S1 and S2 proteins in insect cells infected with recombinant baculoviruses was characterized by indirect immuno-fluorescence and solid phase ELISA. Results of immunofluorescence utilizing BCV-specific polyclonal antibodies indicated the presence of diffuse intra-cytoplasmic and peripheral fluorescent granules in infected cells, which were completely missing in the wild type infected cells (Fig. 5). ELISA showed high antigenic reactivity regarding the cell debris preparation of infected cells. No similar reactivities were identified for the other preparations (culture supernatant and cell lysate), and in all preparations of wild type-infected. To assess secretability of the recombinant proteins from infected cells, ELISA was applied on the culture supernatant, cell

lysate and debris of infected cultures at two time points (48 and 96 hr). S1 protein was only recognized in the cell debris after 48 hr of incubation and significantly decreased after 96 hr, which indicates secretion of such protein before that time (96 hr). On the other hand, S2 protein was recognized in debris of infected cells throughout the entire time period. Unfortunately, the expressed proteins have not been detected in the culture supernatant in different time points probably as a result of their extensive dilution in the culture medium (Table 1).

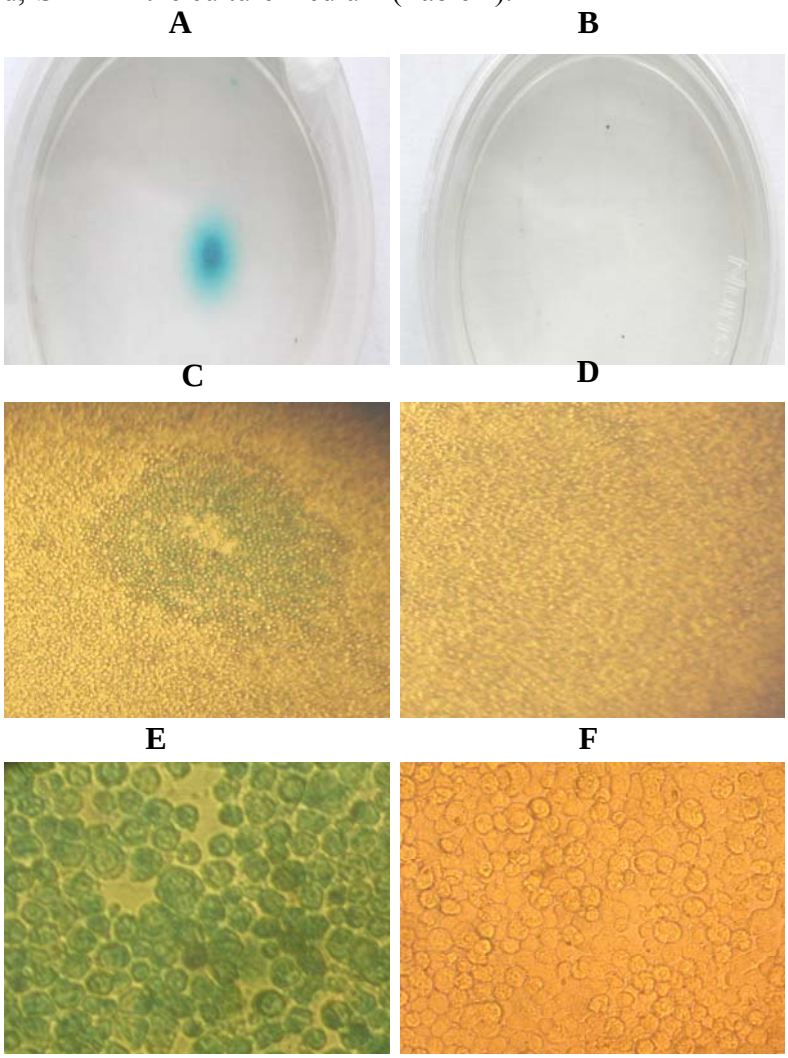

Fig. (3): Plaque purification of the recombinant baculoviruses. Monolayer cultures of Sf-9 cells were infected with the transfection supernatant and overlaid with soft agarose medium containing X-gal. The developed plaques appear as deep blue rounded spots in the agarose overlay (A), The plaques appeared microscopically under low power 10 X (C) and high power 40 X (E) as empty zones in the cell monolayer surrounded by infected cells that are larger in diameter, bluish in color and show signs of cell lysis. Photos (B), (D), and (F) represent the corresp-onding control non-infected Sf-9 cells.

DISCUSSION

Spike glycoprotein is the major immunogenic protein of BCV (Lai and Cavanagh, 1997). Several neutralizing and non-neutralizing antigenic determinants were identified on the surface of S-glycoprotein using monoclonal antibodies (Mabs) (Derget and Babuik, 1987 and Vautherot *et al.*, 1990). Besides, S-glycoprotein induces a strong cell-mediated immune response. Immunization of animals with S glycoprotein alone produces a complete protection against several corona-viruses (Torres *et al.*, 1995). Exploitation of the advanced tools of recombinant DNA technology in cloning and expression of several coronavirus proteins (S in particular) has opened new avenues in the development of

new vaccine candidates and improved diagnostic utilities (Yoo *et al.,* 1990 and Pfleiderer *et al.*, 1990).

During the past two decades, a wide variety of expression systems have been developed for production of recombinant proteins *in vitro.* Because the different coronavirus proteins undergo post-translational modifications like glycosylation, phosphorylation, palmitolation and acylation, the eukaryotic expression systems were preferentially used for production of coronavirus antigens (Yoo *et al*., 1990; 1992 and Reddy *et al*., 2000). Baculovirus gene expression system is a popular eukaryotic system used for producing extremely large quantities of recombinant proteins (up to 600 mg/liter) in insect host cells. These proteins are soluble, correctly folded and biologically active (O'Reilly *et al*., 1992). In addition, baculoviruses are known to infect arthropods only with a narrow host range (Friesen and Miller, 2001), which requires little or even no safety considerations either during preparation of the recombinant proteins or during their use in practice.

The transfer (Shuttle) vector is probably the most important element in the process of developing recombinant baculoviruses (Galleno and Sick, 1999). A wide variety of baculovirus transfer vectors are now available for expression of recombinant proteins in lepidoptrean insect cell lines. The pBlueBac4.5/V5-His TOPO® vector, from Invitrogen, was selected in the current study for cloning the target gene fragments as a result of its favorable structural components including: 1) TOPO cloning site for highly efficient, rapid and simple cloning of the Taq-polymerase amplified PCR products; 2) A C-terminal peptide encoding the V5 epitope and a 6X His tag for detection and purification of the expressed protein, when needed; 3) The SV40 polyadenylation signal for increased termination efficiency and mRNA stability; and 4) The early-to-late (ETL) promoter for expression of β-galactosidase in recombinant baculoviruses and development of easily distinguished blue plaques.

The entire sequence of S-gene extends for 4089 nucleotides starting from nucleotides 3301 to 7389 distal to the 3\ end of the viral genome (Parker *et al*., 1990b). This long sequence is difficult to be cloned in the different available baculovirus cloning vectors; therefore, the regions corresponding for both S protein cleavage products (S1 and S2) were amplified, cloned and expressed individually.

The primers designed for amplification of S1 and S2 gene sequences were adapted to certain modifications to permit expression of integral proteins. Due to the lack of a translation stop codon for S1 gene sequence, its reverse primer was arranged to be cloned-in-frame with the stop codon of transfer vector. In contrary, S2 has no initiation codon for expression and required inclusion of a start codon with its related optimal sequences in the S2 forward primer (Kozac, 1987).

Table (1): Results of indirect solid phase ELISA as expressed by optical density (OD) values for the different recombinant and wild-type baculovirus-infected cell preparations at two time points (48 and 96 hr).

Recombinant virus clone	Debris		Cell lysate		Culture Supernatant	
	48 hr	96 hr	48 hr	96 hr	48 hr	96 hr
S1	0.602	0.257	0.145	0.163	0.172	0.155
S2	ND	0.628	ND	0.155	ND	0.140
Wild type virus	0.199	0.170	0.146	0.160	0.148	0.164

ND: not done.

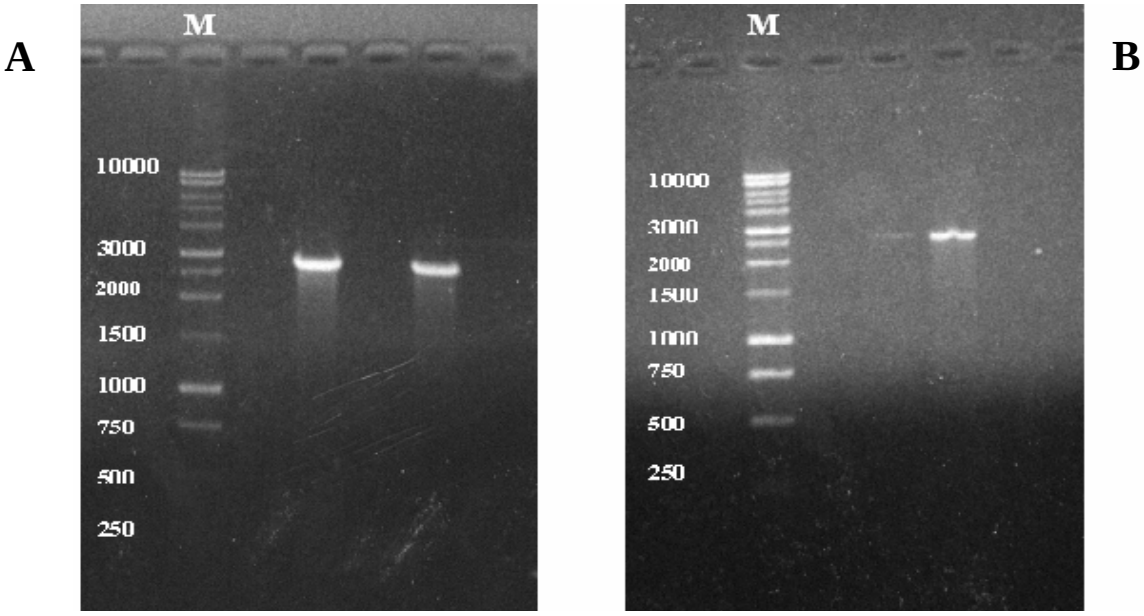

Fig. (4): PCR products of the pure recombinant plaques carrying: (A) S1 gene (2633 bp) and (B) S2 gene (2454 bp) of BCV in ethidium bromide stained agarose gel electrophoresis, along with 1 kbp DNA ladder (M).

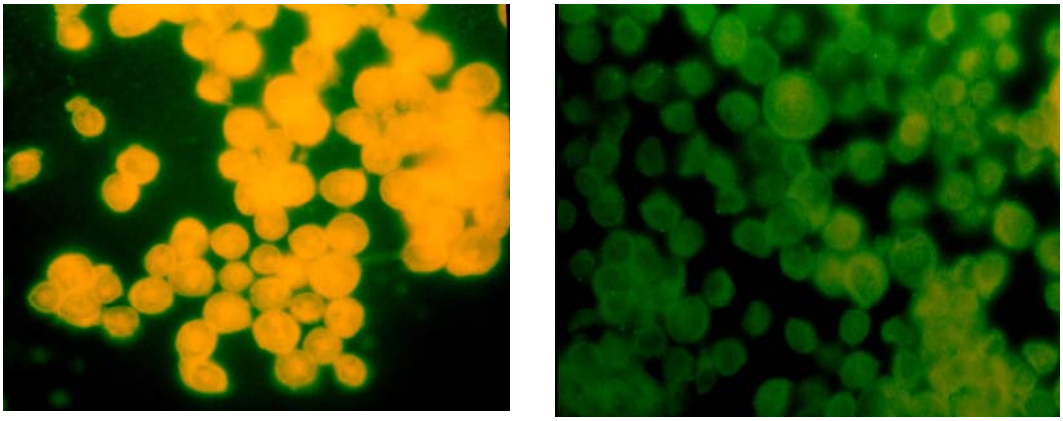

Fig. (5): Immunofluorescent identification of the S1 protein expressed in insect cells infected with the recombinant baculoviruses. Distinct intra-cytoplasmic and peripheral fluorescent granules are shown in the majority of infected cells under fluorescent microscope (A), in comparison with the control wild type infected cells (B), Power 10 X .

Numerous improvements were introduced to the baculovirus expression system to facilitate the generation and screening of recombinant baculoviruses. One of the most widely acceptable improved systems is Bac-N-Blue™, Invitrogen. This involves a triple-cut of the wild-type baculovirus genome using *Bsu36I* restriction enzyme for complete linearization and removal of essential sequences for virus replication (Kitts and Possee, 1993 and Zhao *et al.*, 2003). The choice for using such system in generation of recombinant baculoviruses carrying BCV target genes was potentiated by

its superior features including: very high recombination efficiency (more than 90%), low background of non-recombinant viruses, easier and more practical identification of recombinant viruses through development of characteristic blue plaques (Galleno and Sick, 1999).

Characterization of the expressed BCV target proteins (S1 and S2) in insect cell cultures was performed using solid phase ELISA and indirect fluorescent antibody technique. All of the expressed target proteins reacted specifically with the BCV-polyclonal antiserum in FAT and ELISA, which indicated the proper expression of the target proteins in insect cells.

S-protein of BCV carries 17-amino-acid N-terminal signal sequence that is proteo-lytically spliced during the process of virus maturation. This signal sequence promotes translocation of S-protein from the cellular membranes during the exocytic pathway (Abraham *et al.*, 1990). Since the N-terminal portion of S protein (S1) contains this signal sequence, with no membrane anchor domain in the C-terminus, the expressed protein should be in theory secreted in the culture medium soon after synthesis and maturation. Efficiency of protein glycosylation also plays an important role in the proper secretion of expressed proteins (Jarvis and Summers, 1989). In the current study, the secretability of S1 was examined by detecting the expressed protein intra- and extra-cellular at two time points (48 and 96 hr post-inoculation) using solid phase ELISA. The results confirmed that the signal sequence of BCV-S protein was efficiently recognized in insect cells and directed membrane translocation and secretion of the expressed protein as indicated by the presence of the protein intracellularly after 48 hr, and its disappearance after 96 hr of incubation (Table 1). These observations also provide the evidence that the expressed S1 protein is probably fully glycosylated and biologically active.

On the other hand, S2 polypeptide located at the carboxy terminal portion of S glycoprotein, contains a membrane anchor domain but lacks the membrane translocation signal, which predicts the retaining of such protein within infected cells (Yoo *et al.*, 1990). ELISA results also confirmed such idea by identifying the expressed S2 protein at both time points.

The present study describes the production and characterization of recombinant S1 and S2 polypeptides of BCV (Mebus strain) using the baculovirus expression system. The expressed proteins may be utilized for advanced structural, functional and immunological studies that illuminate many dark points regarding such proteins. Also, they can be exploited in development of a subunit vaccine for control of the BCV-induced disease problems in field and production of valuable diagnostic materials like monoclonal antibodies, hyperimmune serum, ELISA kits for rapid diagnosis of infection and identification of carrier animals. In addition, the use of baculovirus expression system in production of a wide range of biologically active proteins will provide the scientists with a great tool that could be used in many disciplines of medicine and science.

ACKNOWLEDGMENT

This study was financially supported by the Egyptian National Academy of Science and Technology as a part of the research project (No. 34).

REFERENCES

Abraham, S.; Kienzle, T.E.; Lapps, W. and Brian, D.A. (1990). Deduced sequence of the bovine coronavirus spike protein and

identification of the internal proteolytic cleavage site. Virology, 176: 296-301.

Clark, M.A. (1993). Bovine Coronavirus. A review. Br. Vet.J., 149: 51-70.

Day, A.; Wright, T.; Sewall, A.; Price-Laface, M.; Srivastava, N. and Finlayson, M. (1995). Rapid procedures for the isolation and PCR analysis of recombinant baculovirus. In Methods in Molecular Biology, Vol. 39: Baculovirus expression protocols. Chapter 8, pp. 143-159. Edited by Richardson, C. D. Humana Press Inc., Totowa, New Jersey.

Deregt, D. and Babuik, L.A. (1987). Monoclonal antibodies to bovine coronavirus: Characteristics and topog-rahical mapping of neutralizing epitopes on the E2 and E3 glycoproteins. Virology, 161: 410-420.

Deregt, D.; Sabara, M. and Babiuk, L.A. (1987). Monoclonal antibodies to two bovine coronavirus glycoproteins neutralize virus infectivity. Adv. Exp. Med. Biology, 218: 473-474.

Friesen, P.D. and Miller, L.K. (2001). Insect Viruses. In Field's Virology, Chapter 20, Fourth edition, pp. 599-628. Edited by P. M. Howley; Knipe, D. M. et al., Lippincott-Raven Publishers, Philadelphia.

Galleno, M. and Sick, A.J. (1999). Baculovirus expression vector system. In Gene Expression Systems: Using nature for the art of expression. Chapter 12, pp. 331-363. Edited by J. M. Fernandez and J. P. Hoeffler. Academic Press. San Diego. London. Boston. New York. Sydney. Tokyo. Toronto.

Heckert, R.A.; Saif, L.J.; Myers, G.W. and Anges, A.G. (1991). Epidemiologic factors and isotype-specific antibody responses in serum and mucosal secretion of dairy calves with bovine coronavirus respiratory tract and enteric tract infections. Amer. J. Vet. Res., 52 (6): 845-851.

Holmes, K.V. and Lai, M.M. (2001). Coronaviruses. In Fields Virology, Fourth edition. Chapter 35, pp. 1163-1185. Edited by P. M. Howley; D. M. Knipe, *et al.* Lippincott-Raven Publishers, Philadelphia.

Holmes, K. V.; Welsh, R. M. and Haspel, M. V. (1986). Natural Cytotoxicity against mouse hepatitis virus-infected target cells. I. correlation of cytotoxicity with virus binding to leukocytes. J. Immunol., 136: 1446-1453.

Jarvis, D.L. and Summers, M.D. (1989). Glycosylation and secretion of human tissue plasminogen activator in recombinant baculovirus infected insect cells. Mol. Cell. Biol., 9: 214-223.

Kitts, P. A. and Possee, R.D. (1993). A method for producing recombinant baculovirus expression vectors at high frequency. Bio/Techniques, 14: 810-817.

Kozac, M. (1987). An analysis of 5`-Noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acid Res., 15: 8125-8148.

Lai, M.M.C. and Cavanagh, D. (1997). The molecular biology of coronaviruses. Adv. Virus Res., 48: 1-100.

Langone, J.J and Van Vunakis, H. (1986). Immunological techniques, Part I: Hybridoma technology and monoclonal antibodies. Methods Enzymol., 121: 1–947.

Luckow, V.A. and Summers, M.D. (1988). Trends in the development of baculovirus expression vectors. Bio/Technology, 6: 47-55.

Mebus, C.A.; Stair, E.L.; Rhodes, M.B. and Twiehaus, M.J. (1973). Neonatal calf diarrhea: propagation, attenuation, and characteristics of a coronavirus-like agent. Am. J. Vet. Res., 34 (2): 145-150.

Möstel, K. von and Bürki, F. (1987). Causal incidence of bovine coronaviruses with outbreaks of respiratory disease in calves and pathogenitical and immunological

considerations made. Deutche Tierärztliche Wochenschrift, 95: 19-22.

O`Reilly, D.R.; Miller, L.K. and Luckow, V.A. (1992). Baculovirus expression vectors: A laboratory manual. W. H. Freeman, New York.

Parker, M.D.; Yoo, D.; Cox, G.J. and Babuik, L.A. (1990). Primary structure of the S peplomer gene of bovine coronavirus and surface expression in insect cells. J. Gen. Virology, 71 (2): 263-270.

Pfleiderer, M.; Routledge, E. and Siddell, S.G. (1990). Functional analysis of the coronavirus MHV-JHM surface glycoproteins in vaccinia virus recombinants. Coronaviruses and Their Diseases, edited by Cavanagh, D. and Brown, T. D. K.., Plenum Press, New York, pp. 21-31.

Reddy, P.S.; Idamakanti, N.; Zakhartchouk, L.N.; Babuik, L.A.; Mehtali, M. and Tikoo, S.K. (2000). Optimization of bovine coronavirus haema-gglutinin-easterase glycoprotein expression in E3 deleted bovine adenovirus-3. Virus Res., 70: 65-73.

Regenmortel, M.H.V. Van.; Fauquet, C.M.; Bishop, D.H.L. *et al.*, (2000). Virus Taxonomy. Classification and Nomenclature of Viruses. pp. 195-202, 493-497. Seventh report of the International Committee on the Taxonomy of Viruses. Academic Press. pp 827-842, San Diego, San Francisco, NewYork, Boston, London, Sydney and Tokyo.

Rodak, L.; Babiuk, L.A. and Acres, S.D. (1982). Detection by radioimmunoassay and enzyme-linked immunosorbent assay of coronavirus antibodies in bovine serum and lacteal secretions. J. Clin. Pathol., 16 (1): 34-40.

Saif, L. J. and Heckert, R.A. (1990). Enteric coronavirus. In Viral Diarrheas of Man and Animals, L. J. Saif and K. W. Theil, eds, CRC Press, Boca Raton, Florida, pp. 185-252.

Sambrook, J.; Fritscgh, E. F. and Meniates, A. (1989). Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory Press, New York.

Sturman, L.S.; Ricard, C.S. and Holmes, K.V. (1985). Proteolytic cleavage of the E2 glycoprotein of murine coronavirus: Activation of cell fusing activity of virions by trypsin and separation of two different 90k cleavage fragments. J. Virology, 56: 904-911.

Summers, M.D. and Smith, G.E. (1988). A manual of methods for baculovirus vectors and insect cell culture procedures. Texas Agric. Exp. Stn. (Bulletin), B-1555.

Thurber, E.T.; Bass, E.P. and Beckenhauer, W.H. (1977). Field trial evaluation of a reo-coronavirus calf diarrhea vaccine. Can. J. Comp. Med., 41 (2): 131-136.

Torres, J. M.; Sanchez, C.; Sune, C.; Smerdou, C.; Prevec, L.; Graham, F. and Enjuanes, L. (1995). Induction of antibodies protecting against transmissible gastroenteritis coronavirus (TGEV) by recombinant adenovirus expressing TGEV spike protein. Virology, 213: 503-516.

Vautherot, J.F.; Madelaine, M.F. and Laporte, J. (1990). Topological and functional analysis of epitopes on the S (E2) and HE (E3) glycoproteins of bovine enteric coronavirus. Coronaviruses and Their Diseases, edited by Cavanagh, D. and Brown, T. D. K., Plenum Press, NewYork, pp. 173-179.

Yoo, D.; Graham, F.L.; Prevec, L.; Parker, M.D.; Benkö, M.; Zamb, T. and Babuik, L.A. (1992). Synthesis and processing of the haemagglutinin-esterase glycoprotein of bovine coronavirus encoded in the E region of adenovirus. J. Gen. Virology, 73: 2591-2600.

Yoo, D.; Parker, M.D. and Babiuk, L.A. (1990). Analysis of the S spike (peplomer) glycoprotein of bovine coronavirus

synthesized in insect cells. Virology, 179: 121-128.

Zhao, Y.; Chapman, D.A. and Jones, I.M. (2003). Improving baculovirus recombination. Nucleic Acid Res., 31 (2): e6.

الملخص العربي

إنتاج الجزيئان التركيبيان للبروتين السطحي (إس) لفيروس الكورونا البقري بواسطة فيروسات الباكولو المدمجة في الخلايا الحشرية

هيثم محمد عامر – حسين علي حسين – إبراهيم محمد الصباغ – أحمد عبد الغني السنوسي - محمد سامي صابر – محمد عبد الحميد شلبي

قسم الفيروسات – كلية الطب البيطري – جامعة القاهرة – الجيزة –ج.م.ع

في هذه الدراسة تم استخدام أسلوب التعبير الوراثي بواسطة فيروسات الباكولو المدمجة لإنتاج الجزيئين التركيبيين للبروتين السطحي(إس) في فيروس الكورونا البقري. و لبلوغ هذا الهدف تم استخلاص الحمض النووي الريبوزي للفيروس ثم مضاعفة الجينات الخاصة بالمقاطع البروتينية المستهدفة باستخدام اختبار تفاعل البلمرة المتسلسل المسبق بعملية نسخ عكسي RT-PCR . و قد تمت كلونة نواتج تفاعل البلمرة المتسلسل بعد عزلها من الجل في بلازميدات ناقلة بها مقاطع خاصة من فيروس الباكيلو ثم استخدمت هذه البلازميدات الناقلة في نفس الوقت مع جينوم فيروس الباكولو لحقن الخلايا الحشرية (Sf-9) في وجود مادة الليبوفكتين و ذلك للحصول علي فيروسات من الباكولو تحمل جينات البروتينات المستهدفة من خلال عملية عبور وراثي بين مقاطع متماثلة من كلا الطرفين. و لقد تم انتقاء فيروسات الباكولو المدمجة باستخدام أسلوب البليك Plaque assay و إكثارها في خلايا حشرية مناسبة ثم استخدم تفاعل البلمرة المتسلسل للتعرف علي الفيروسات المدمجة النقية. و لقد تم تمرير هذه الفيروسات للحصول علي مخزون وافر منها و معايرتها ثم استخدامها في عمليات إنتاج البروتينات المهندسة وراثيا لفيروس الكورونا البقري. و لتوصيف البروتينات المنتجة استخدمت اختبارات الإليزا و الفلورسنتي المناعي غير المباشر و التي أثبتت قدرة فيروسات الباكولو المدمجة علي إنتاج كميات كبيرة من بروتينات فيروس الكورونا البقري المستهدفة من الدراسة. و كخلاصة فإن هذه الدراسة تأسس لاستخدام تقنية إنتاج البروتينات المهندسة وراثيا من خلال فيروسات الباكولو المدمجة في معاملنا مستقبلا مما يفسح المجال لتقديم منتجات مؤثرة و فعالة في مختلف مجالات العلوم و التكنولوجيا. كما أنها تقدم حلا جزئيا للمشاكل المرضية المترتبة علي الإصابة بفيروسات الكورونا في الأبقار حيث أن المنتج النهائي للدراسة يسهم في إنتاج أنواع مستحدثة من اللقاحات و الوسائل التشخيصية المتطورة.