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BACULOVIRUS EXPRESSION OF THE BOVINE CORONAVIRUS NUCLEOCAPSID PROTEIN IN *SPODOPETRA FRUGIPERDA* INSECT CELLS

Amer, H.M.; Hussein, H. A.; El-Sabagh, I. M.; El-Sanousi, A. A.; Saber, M. S. and Shalaby, M. A.

In the current study, cloning and expression of the bovine coronavirus (BCV) nucleocapsid protein was carried out in a baculovirus expression system. The specific RT-PCR product of N gene was cut and extracted from gel using DNA gel extraction kit (Millipore). Eluted DNA was successfully cloned in pBlueBac4.5/V5-His TOPO TA baculovirus transfer vector and transformed in chemically competent *E. coli*. A modified colony PCR assay was utilized to identify the positive bacterial colonies that harbor the recombinant plasmids carrying N gene in correct orientation. Generation of recombinant baculoviruses was achieved by co-transfection of *Spodopetra frugiperda* (Sf-9) insect cells with a linearized replication-defective baculovirus DNA (Bac-N-Blue™) and the transfer vector. The recombinant baculoviruses were plaque purified; verified for the presence of target sequences using PCR and propagated for generation of high-titer viral stocks. In vitro expression studies utilizing the recombinant baculoviruses were conducted and revealed high-level expression of N protein as indicated by its distinct reactivity in immunofluoresence, solid phase ELISA and Western blot.

Key words:

Department of Virology, Faculty of Veterinary Medicine, Cairo University, 11221 Giza, Egypt

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INTRODUCTION

Bovine coronavirus (BCV) is the causative agent of several enteric diseases which affect cattle and buffaloes including neonatal calf diarrhea (NCD), winter dysentery (WD) and chronic shedding in adult cattle **(Saif and Heckert, 1990 and Clark, 1993)**. The virus also infects the respiratory tract of growing and feedlot calves causing mild to severe pneumonia **(Thomas et al., 1982 and Storz et al., 2000)**.

The genome of BCV is a single-stranded, non-segmented RNA molecule of positive polarity. It represents one of the largest genomes among RNA viruses with an average length of 31 kb **(Yoo and Pei, 2001)**. Five major structural proteins are encoded by the viral genome, which are: the nucleocapsid protein (N; 50-52 kDa), the integral membrane protein (M; 22-26 kDa), the Spike protein (S; 180-220 kDa), the haemagglutinin-esterase protein (HE; 124-140 kDa) and the small membrane protein (E; 9-12 kDa) **(Lai and Cavangh, 1997)**.

The nucleocapsid protein is the only structural protein that locates internally in association with the virus genome to protect it from environmental conditions and the effect of ribonucleases **(Cavangh, 1997)**. In addition, it plays an important role in regulation of RNA synthesis and transcription process **(Zhou et al., 1996)**.

As a result of failure or unsatisfaction of the conventional BCV vaccines in protection against field infections, the need for development of modern subunit vaccines has been aroused. Inclusion of N protein as a component of the proposed subunit vaccines is strongly recommended since it induces a strong cell-mediated immune response **(Boots et al., 1990)**, besides; it encompasses multiple non-conformational epitopes which are responsible for partial protection against BCV infection *in vivo* **(Derget and Babuik, 1987 and Regnmortel et al., 2000)**.

In the current report, we made use of the potential of baculovirus expression system to express high levels of the BCV N protein. The use of such expression system has many advantages that guarantee production of biologically and immunologically competent protein which could be utilized in formulation of subunit vaccine candidates and/or production of

diagnostic reagents, such as: 1) Permission of proper protein folding, oligomerization and solubilization and 2) Induction of the post-translational modifications in a manner that is nearly resembling those occur in mammalian cells. In addition, it possesses little or even no safety considerations either during preparation of the recombinant protein or during its use in practice **(O`Reilly et al., 1992).**

MATERIALS AND METHODS

Virus and Cell lines:

Bovine coronavirus (Mebus strain) was propagated in MDBK cells grown in minimal essential medium (MEM) containing 40 µg/ml gentamycin and 10% fetal bovine serum. Monolayer cultures of *Spodoptera frugiperda* (Sf-9) cells were maintained in Grace's insect medium (Gibco BRL, Life Tech, Grand Island, NY) supplemented with 10% fetal bovine serum, 1% Pluornic polyol F-68 and 100 U penicillin, 100 µg streptomycin, 0.25 µg amphotericin-B per ml **(Summers and Smith, 1988).**

RT-PCR:

Viral RNA was isolated from the BCV-infected MDBK cells using TRIZOL reagent (Gibco BRL) and incubated with dimethyl sulphoxid (DMSO) at 70°C for 10 minutes. The entire sequence of BCV-N gene was amplified in a single step RT-PCR using the sense primer BCV-NF (5'-GCTTGGACACCGCAT-TGTTG-3') and the antisense primer BCV-NR (5'-CACCAGGTGCC-GACATAAGG-3'). The RT-PCR mixture was prepared by combining 10 µl of the treated viral RNA extract, 25 µl of 2x ReddyMix® PCR master mix, 1µl of Reverse-iT® RTase (ABgene, Epson, Surrey, UK) and 0.2 µM of both primers. The volume was adjusted to 50 µl with nuclease free water. The reaction was conducted in a Gene-Amp 9700 PCR thermal cycler (Applied Biosystem Inc., Foster City, CA, USA) using the following cycling protocol: First strand synthesis at 47°C for 30 min; reverse transcriptase inactivation and initial denaturation at 94°C for 2 min; 35 cycles of 94°C for 45 seconds, 55°C for 45 seconds and 72°C for 2 minutes; and a final extension step at 72°C for 10 minutes.

Construction of plasmid nsertion vectors:

The specific RT-PCR product was extracted from agarose gel using Montáge DNA gel extraction kit (Millipore, Concord Road Billerica, MA, USA) and cloned into the pBlueBac4.5/V5-His TOPO® TA baculovirus cloning vector (Invitrogen, San Diego, CA). The cloning process was performed as directed by the manufacturer, where 4 µl of the gel-extracted DNA were mixed with 1 µl of the cloning vector and 1 µl of salt solution (1.2M NaCl and 0.06M $MgCl_2$), and the mixture was incubated at room temperature for 5 minutes. In a vial of competent TOP-10 bacterial cells, 3 µl of the mixture were added carefully and the vial was incubated on ice for 30 minutes. The competent cells were heat shocked at 42°C for 30 seconds and kept on ice before being incubated with 250 µl 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) at 37°C for 1 hour in a shaking incubator (200 rpm/min). Cells were spread on Lauria-Bertani (LB) agar plates containing 50 µg/ml ampicillin (Sigma chemical co., St. louis, Mo, USA) and kept overnight at 37°C. The developed colonies were picked up and allowed to grow in LB broth with ampicillin for 12-16 hours in a shaking incubator. Positive colonies that harbor recombinant plasmids with the target gene in correct orientation were identified by colony PCR utilizing the forward gene-specific primer (BCV-NF) and a reverse vector primer (5`-GGAGATAATTAAAATGATAA CCATCTCGC-3`).

DNA transfection and screening of recombinant baculoviruses:

A Monolayer culture of Sf-9 cells ($1x10^6$ cells per 35 mm dish) was co-transfected with 0.5 µg of *Ac*MNPV linearized DNA (Bac-N-Blue, Invitrogen) and 8 µg of plasmid DNA, extracted from the transformed bacterial cells using QIAprep® Miniprep plasmid extraction kit (QIAgen, Hilden, Germany), in the presence of 20 µg Cellfectin® reagent and unsupplemented Grace`s medium (Invitrogen). After 4 hr of incubation at room temperature on a rocking platform (2 side-to-side motion/minute), 1 ml of complete Grace's medium was added and plates were incubated at 27°C. Three days later, the culture supernatant was harvested and recombinant baculoviruses were

purified by plaque assay as described by Day *et al.*, (1995). Harvested plaques were amplified by passage in Sf-9 cells and then analyzed by PCR to identify the pure plaques that contain recombinant baculoviruses uncontaminated with the wild-type virus. PCR analysis was typically performed using the forward primer BPH-F (5`-TTTACTGTTTTCGTAACAGTTTTG-3`) and the reverse primer BPH-R (5`-CAACAACGC-ACAGAATCTAGC-3`). Pure recombinant baculoviruses were propagated and titrated on Sf-9 cells, where the high-titer viral stocks were used in protein expression studies.

Infection and protein expression:
In vitro expression of N protein was carried out by infecting Sf-9 cells, at a density of $3x10^6$ cells per 25 cm^2 tissue culture flask, with the recombinant baculoviruses at a multiplicity of infection (m.o.i) of 20. The whole culture was harvested after 96 hr of incubation and cells were pelleted by centrifugation at 2000 rpm for 10 minutes. The cells were lyzed with PBS containing 1% Triton X-100 and 1 µg/ml Pepstatin A (100 µl / 10^6 cells). Complete cell lysis was achieved by applying two cycles of freezing and thawing. The cytoplasmic fraction of cells was separated from the cellular membranes by centrifugation of lysates at 4000 rpm for 10 minutes. Both fractions were utilized for characterization of the expressed protein. Wild-type-infected and non-infected cells were included in the expression study and served as controls.

Immunofluoresnce:
Indirect immuno-fluorescent technique was utilized for detection of protein expression in insect cells as previously described by **Ausubel et al., (1994)** with certain technical modifications. Briefly, Sf-9 cells were cultured on the surface of round glass slips in cell culture staining chambers (CCSCs) at a density of 7.5 X 10^5 cells. Separate cultures were infected with the recombinant and the wild-type baculoviruses at m.o.i of 10, while uninfected cultures were left as cell control. After 2 days of incubation, cells were washed with PBS and fixed with 80% chilled acetone. After complete dryness, the slips were covered with 100 µl of the BCV rabbit hyperimmune serum and incubated for 1 hour at 27°C, then washed with PBS for three successive times. Cover slips were

further incubated with 100 µl of the fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit IgG (Sigma) for another 1 hour before being washed with PBS three times again. The glass slips were removed from the CCSCs, covered with mounting buffer (50% Glycerol in PBS) and examined under the fluorescent microscope.

ELISA:

Solid phase ELISA was performed in polystyrene microtiter plates (BD Falcon™, Franklin Lakes, NJ, USA). Both fractions of cell lysates (cytoplasmic extract and cellular membranes) and the cell-free culture supernatant fluid collected from recombinant and wild-type baculovirus infected and non-infected flasks were used as coating materials. Coating process was carried out by dilution of the protein fractions in carbonate-bicarbonate buffer, pH 9.6 before being incubated in the ELISA plate for 1 hour at 37°C. 200 µl of the blocking buffer (5% non-fat dry milk in PBS) were added to each well of the plate and incubated overnight at 4°C. The plates were then incubated with the BCV rabbit hyperimmune serum (diluted in PBS containing 0.5% NFDM) at 37°C for 2 hour. After three successive washes with PBS containing 0.05% Tween-20 (PBS-T), 100 µl of the affinity-purified antibody peroxidase-labeled anti-rabbit IgG (Sigma) were added to each well and incubated at 37°C for 1 hour. After another cycle of wash with PBS-T, the ELISA plates were incubated with the substrate-indicator (H_2O_2/ABTS) mixture for 30 minutes in a dark place and then stopped by 5% SDS solution. Absorbencies were read using the automated ELISA reader at a wave length of 405 nm.

Western blot analysis:

Cellular extracts of recombinant and wild-type baculovirus-infected and non-infected cells were resolved by SDS-PAGE on 10% discontinuous gel. and transferred to nitrocellulose membrane by electroblotting in Tris-glycine buffer (25 mM Tris, 190 mM glycine and 20% methanol) **(Towbin et al., 1979)** using a Miniprotean II electrophoretic cell (BioRad, Hercules, California, USA) at 100 Volt for 1 hour. Membranes were blocked with 10% skimmed milk powder in PBS overnight at 4°C. The membranes were incubated with BCV rabbit hyperimmune serum (diluted 1/100 in blocking buffer) for 1 hr at room temperature and then washed three times with PBS-T. Peroxidase-labeled goat anti-

rabbit IgG (Sigma) diluted 1/2500 in blocking buffer was added to the membranes and incubated for 1 hr at room temperature before three other successive washing steps with PBS-T were applied. Blots were developed by reaction with hydrogen peroxide and 0.05% 4-chloro-1-naphthol substrate solution for 30 minutes in a dark place.

RESULTS

Generation of baculovirus shuttle vector carrying N gene:

The complete N gene sequence was amplified in a single-step RT-PCR using two specific primers located 44 nucleotides upstream and 29 nucleotides downstream the gene sequence. The amplified fragment was extracted from agarose gel and cloned in the baculovirus shuttle vector pBlueBac 4.5/V5-His-TOPO®. The cloning process was very robust and resulted in appearance of a multitude of bacterial colonies following transformation of the cloned plasmids in competent TOP10 *E. coli* cells and propagation of the transformed bacteria on a selective semi-solid medium containing ampicillin. Analysis of five selected colonies with a modified method of colony PCR using the forward N gene primer and a reverse primer of the plasmid vector enabled identification of two colonies that harbor the N gene sequence in a correct orientation, a prerequisite for generation of recombinant baculoviruses expressing immunologically and biologically active protein(Figure1)

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Construction of baculovirus recombinants:

Baculovirus shuttle vector DNA was extracted from one of the PCR-identified bacterial colonies and co-transfected with *Ac*MNPV linearized DNA into Sf9 cells. Recombinant baculoviruses generated from the homologous recombination between both DNA molecules become circular, replicative and have the ability to infect insect cells. Signs of infection usually appear in the form of cellular enlargement, nuclear distention, growth cessation and detachment of the cell monolayer. A complete lysis of cells appears at the end stages of infection (after 72 hours). Purification of the recombinant baculoviruses was achieved by plaque assay using X-gal as a chromogenic substrate. Ten recombinant blue plaques were

identified, harvested and propagated in Sf-9 cells for generation of a satisfactory viral stock (Figure 2). One pure plaque uncontamined with the wild-type baculovirus was recognized by PCR analysis of two representative viral stocks using a primer pair flanking the polyhedrin region of baculovirus genome (Figure 3). This clone was further propagated for generation of high-titer viral stock, titrated and utilized in gene expression studies.

Expression analysis of recombinant N protein:

For examining the expression potential of the constructed baculovirus recombinant carrying N gene in insect cells and for studying the identity and antigenicity of the expressed protein, several serological techniques were carried out including immuno-fluorescence (Figure 4), ELISA and Western blot (Figure 5). All the performed tests indicated high reactivity of the expressed protein with the BCV-specific polyclonal antibodies particularly in the cytoplasmic extracts of infected cells. Results of Western blot assay also revealed that the recombinant N-protein was expressed in substantial quantities within insect cells, a matter that could be helpful in utilizing such protein for further field applications

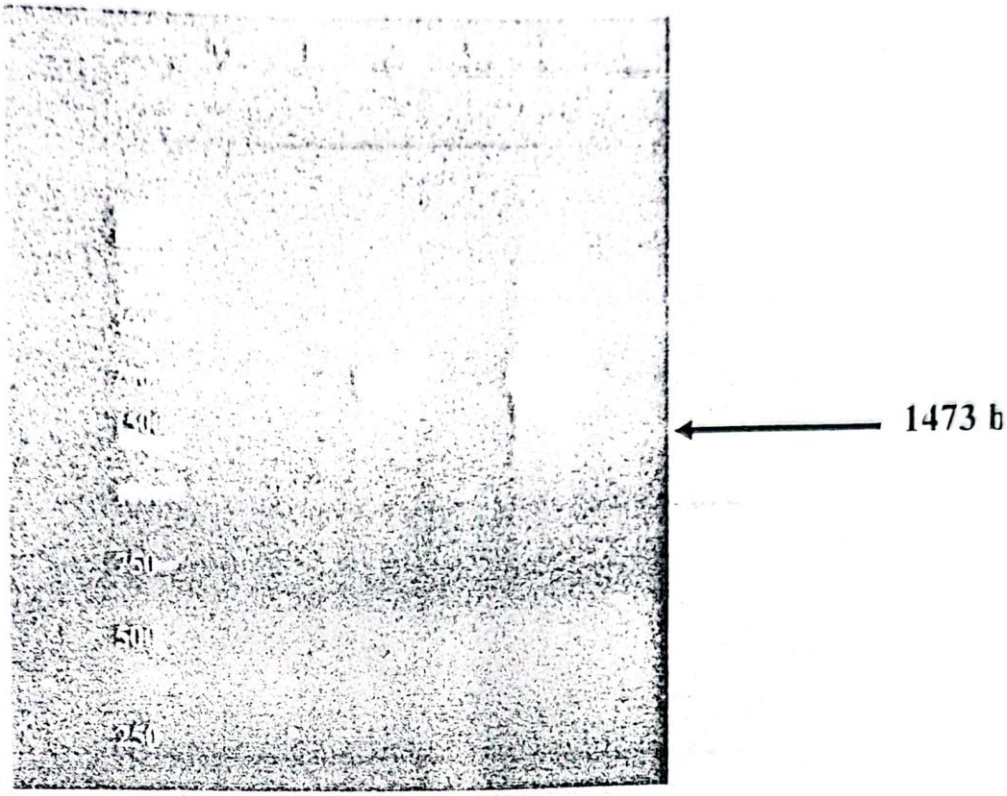


Figure (1): PCR products of the tested bacterial colonies in ethidium bromide-stained agarose gel electrophoresis. A distinct specific band of 1473 bp indicates the presence of cloned N gene sequence in the recombinant plasmid in correct orientation. M represents 1 kbp DNA ladder.

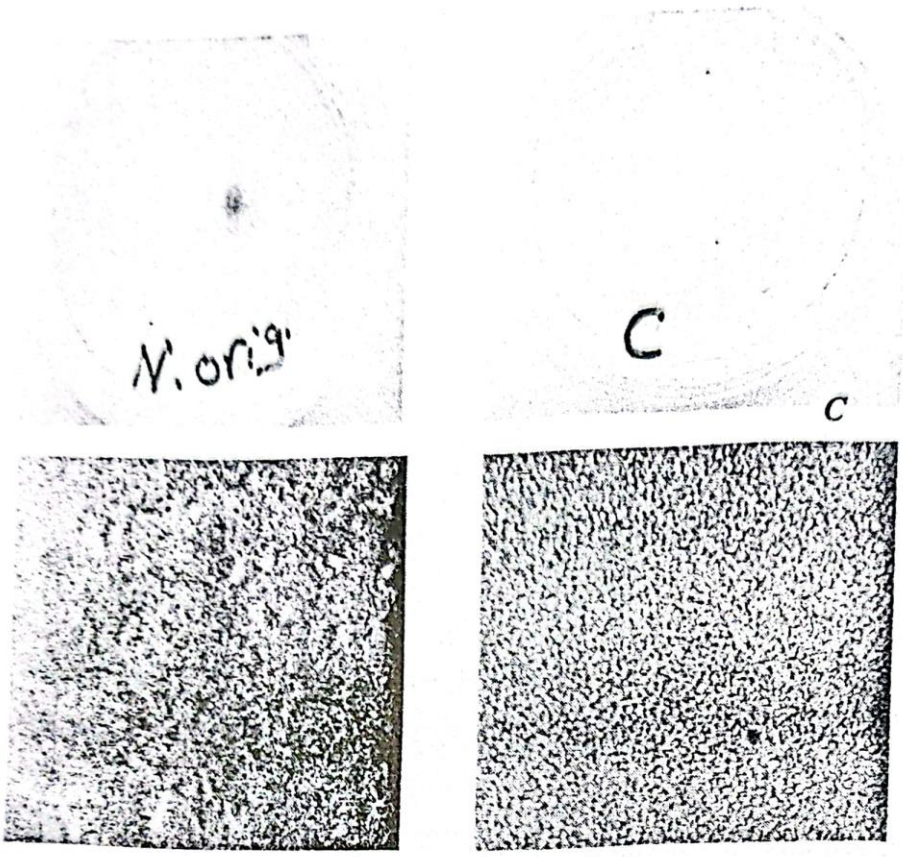


Figure (2): Deep blue rounded plaques appeared in Sf-9 cells infected with recombinant baculovirus carrying N gene (A,C) compared with control non-infected cells (B,D).

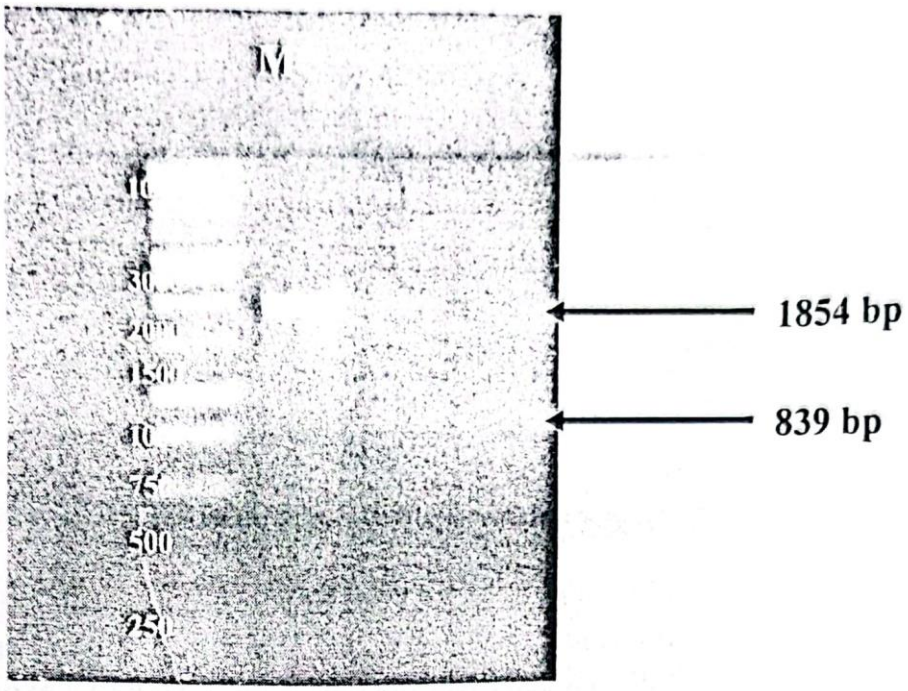
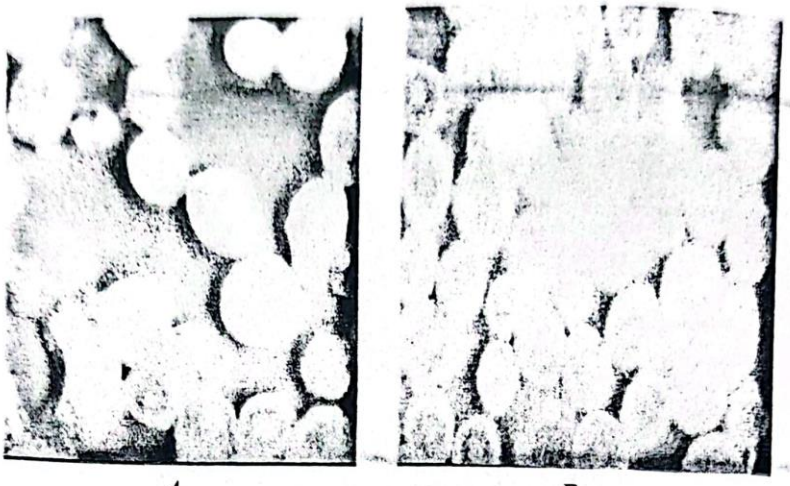


Figure (3): A specific band of 1854 bp indicates the presence of N gene sequence in the recombinant baculoviruses. Appearance of another band at a size of 839 bp indicates presence of wild-type baculovirus along with the recombinant one. M represents 1 kbp DNA ladder.



A *B*

Figure (4): Distinct intra-cytoplasmic and peripheral fluorescent granules are shown in the majority of infected cells with recombinant baculovirus expressing the N gene under fluorescent microscope (A), in comparison with the control wild type infected cells (B). Power 40 X

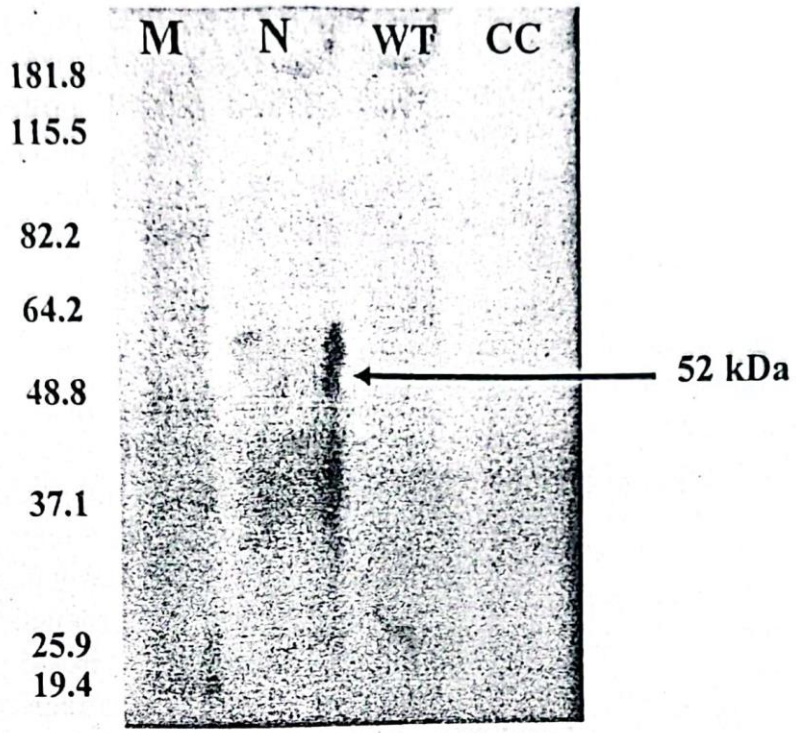


Figure (5): Western blot analysis of the expressed N protein. A specific band of 52 kDa appears in Sf-9 cell extracts after 96 hours of infection with the recombinant baculoviruses carrying N gene sequence. This band is completely absent in similar extracts prepared from non-infected (CC) and wild-type baculovirus-infected (WT) cells. M represents a prestained molecular weight protein marker.

DISCUSSION

Coronaviruses, like virtually all single-stranded RNA viruses, contain a nucleocapsid (N) protein in close structural association with their genomes **(Regenmortel et al., 2000)**. In addition to its fundamental importance for protecting the viral RNA from adverse environmental conditions, N protein has a crucial impact in the viral multiplication cycle and pathogenesis **(Zhou et al., 1996 and Cavangh, 1997).** In addition, N protein of BCV bears several B and T cell antigenic determinants that play a distinctive role in protection against field infections **(Derget and Babuik, 1987; Boots et al., 1990 and Hussain et al., 1991)**.

In contrast to spike (S) and haemagglutinin-esterase (HE) proteins of BCV that were expressed in both insect and mammalian host systems **(Parker et al., 1990; Pulford et al., 1990; Vennema et al., 1990; and Yoo et al., 1990; 1992 and Reddy et al., 2000)**, the N protein did not receive any attention in such direction. This may be referred to the absence of any neutralizing epitope on its surface as well as the completed picture of N protein structure and function did not elevate the opportunity for in vitro expression of such protein for advanced fundamental studies as the case in S and HE proteins. In the current report, we describe for the first time, according to our available data, the generation of baculovirus recombinants that express BCV N protein in insect cells. This recombinant protein can be utilized in field applications for diagnosis of BCV infections and vaccination of susceptible animals.

A wide variety of expression systems have been developed and established for the production of antigens. Since coronavirus proteins undergo several posttranslational modifications such as glycosylation and phosphorylation, the eukaryotic expression systems have to be used to obtain recombinant proteins that closely resemble the authentic viral proteins **(Fernandez and Hoeffler, 1999)**. Among the most robust eukaryotic systems for gene expression is the baculovirus-vectored system. This system allows production of high levels of biologically active recombinant proteins in insect cells using safe and economic procedures **(O'Reilly et al., 1992)**. Despite the presence of certain differences in the glycoprotein processing pathways of insects and higher eukaryotes,

which may lead to production of less complex and trimmed glycoproteins in insect cells **(Bienkowska-Szewczyk and Szewczyk, 1999)**, no similar events were recorded for the phospho-proteins. Therefore, baculovirus expression system could be considered the best choice for obtaining a functional phospho-protein like BCV N protein *in vitro*. Bac-N-Blue™, Invitrogen is one the most widely acceptable baculovirus expression systems utilized successfully in various laboratories worldwide. This system involves Bsu36I linearization of genetically-engineerined wild-type baculovirus DNA and removal of sequences essential for virus replication **(Kitts and Possee, 1993 and Zhao et al., 2003).** The deleted sequences are regained by the Bac-N-Blue DNA with insertion of the heterogonous gene of interest by a process of homologous recombination between a baculovirus shuttle vector and the Bac-N-Blue DNA in insect cells. Such system provides high recombination efficiency that exceeds 90%, low background of non-recombinant viruses and easy identification of recombinant viruses through development of blue plaques **(Fernandez and Hoeffler, 1999)**.

Analysis of N protein expression in insect cells infected with the recombinant baculovirus carrying the entire N gene under control of the polyhedron promoter was carried out using several serological tests like ELISA, immune-fluorescence and Western blot. All the utilized techniques proved the efficiency of insect system in production of high quantities of a recombinant protein that is antigenically identical to the authentic BCV N protein. Different variables including: the multiplicity of infection needed for inoculating the recombinant virus; the time and site for protein harvestation and finally the degree and efficiency of the post-translational modifications of the expressed protein were tested (data not shown). These optimization procedures allowed complete understanding for the expression kinetics of the recombinant N protein in Sf-9 cells and permitted establishment of a standard method for collection of the highest possible protein level to be exploited in further production of diagnostic utilities and subunit vaccines, in which the economic aspect is highly considerable.

Similar to the nucleocapsid protein of many RNA viruses, N protein of coronaviruses is considered the

most prominent and conserved viral antigen (**Daginakatte et al., 1999**). The N molecule is typically divided into three relatively conserved structural domains that are separated with two variable spacer regions (**Parker and Masters, 1990**). Mabs developed against N protein were highly reactive with the different vaccine and wild-type BCV strains in Western blot (**Hussain et al., 1991**). Although the use of baculovirus expressed N protein of different animal and human viruses, including vesicular stomatitis (**Ahmad et al., 1993**), measles (**Hummel et al., 1992**), hepatitis C (**Chiba et al., 1991**) and rinder pest (**Ismail et al., 1994**) was high successful in recognition of specific antibodies and development of diagnostic kits, the potential for using the expressed N protein of BCV as a diagnostic tool needed to be examine.

On the other hand, the recombinant N protein of BCV is a vulnerable product for use in formulation of subunit vaccines for protection of calves against the fatal neonatal diarrhea. Moreover, N protein carries multiple epitopes that stimulate a protective T-cell response (**Boots et al., 1990**) and a series of non-neutralizing B-cell epitopes that are responsible for some protection in vivo (**Hussain et al., 1991**). The most efficient induction of protection against coronavirus infections has been achieved with a combination of S and N proteins (**Regenmortel et al., 2000**). Utilizing the recombinant S protein subunits previously expressed in the baculovirus system (**Amer et al., 2007**), along with the expressed N protein and a proper highly efficient adjuvant can offers an alternative vaccine candidate for immunization of cows and buffaloes at late stages of pregnancy to provide their offspring with adequate levels of protective immune response against BCV field infections.

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