Egytptian Virol. 5 (1): 239-259 (2008)

Synthesis of the Bovine Rotavirus Major Neutralization Antigen (VP7) Using A Baculovirus Expression System

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

The present study reports the successful production of the major neutralization antigen (VP7) of bovine rotavirus Nebraska calf diarrhea virus (NCDV) strain in insect cells. The full-length DNA copies of RNA segment 9 (coding for VP7 protein) of NCDV was inserted into a baculovirus transfer vector under the control of the polyhedrin promotor. A recombinant baculovirus carrying the VP7 gene was constructed through homologous recombination between the baculovirus transfer vector carrying the VP7 gene and *Autographa californica* Nuclear Polyhedrosis Virus (AcNPV). Infection of *Spodoptera frugiperda* (Sf9) cells with Baculovirus recombinants expressing VP7 protein revealed high reactivity with hyperimmune antiserum to BRV when tested by immunoflurescence assay. Using solid phase ELISA, the VP7 expressed protein was detected intracellulary and extracellulary at 48 and 96 hours post-inoculation using polyclonal antibodies against BRV, respectively. The VP7 expressed protein was not detected in Coomassie blue stained SDS-PAGE but produced a detectable band in Western blot assay. The reactivity of the VP7 expressed protein with BRV-specific hyperimmune antiserum confirmed that the antigenic determinants of the expressed protein were unaltered. The recombinant VP7 expressed protein can provide an effective tool for development of new diagnostic measures and novel vaccine candidate for improved diagnosis and control rotavirus infection in young calves.

Key words: BRV, VP7, Baculovirus expression system, Sf-9 cells.

Department of Virology, Faculty of Veterinary Medicine, Cairo University.

(Received March 2008)
(Accepted May 2008)

INTRODUCTION

Rotavirus is a nonenveloped segmented (11 segments), double-stranded RNA (dsRNA) virus belonging to family Reoviridae. Rotaviruses are the single most important etiological agent of diarrheal disease in infants and young animals throughout the world (Estes, 2001).The rotavirus virion consists of a three concentric protein shells, or layers. The innermost layer, or core, is composed of the VP2 protein, the middle layer is composed of the VP6 protein, and the outer layer is composed of the major surface glycoprotein, VP7 (glycoprotein, G) and haemagglutinin spike, VP4 (Protease sensitive, P); both enclose neutralization antigens (Estes and Cohen, 1989).

Rotaviruses are classified into seven serogroup (A-G) determined by the antigenic properties of VP6. Group A , the major cause of diarrhea, is future divided into serotypes and genotypes determined by VP4 (P type) and VP7 (G type). Nowadays, 15G-serotypes/genotypes and 14 P-serotypes including 3 subtypes/26P-genotypes has been identified in human and animals species (**Mattion et al., 1994; Rao et al., 2000; Hoshino et al., 2002; Rahman et al., 2005 and Martella et al., 2005**).In bovine population, there are at least 10G (G1, G2, G3, G5, G6, G7, G8, G10, G11 and G15) and 6P (P6[1], P7[5], P8[11], P?[14], P?[17] and P?[21]) types have been reported (**Matsuda et al., 1990; Hussein et al., 1993; Rao et al., 2000; Fukai et al., 2004 and Alfieri et al., 2004**) with G6, G10, P7[5] and P8[11] are the most predominant types (**Snodgrass et al., 1990; Parwani et al., 1993; Suzuki et al., 1993; Hussein et al., 1996; Chang et al., 1996; and Alfieri et al., 2004**).

VP7 is the second most abundant rotavirus capsid protein, which has a molecular weight of 38,000 Dalton. It constitutes 30% of the virion protein and located on the outer capsid, forming the smooth external surface of the outer shell (**Mattion et al., 1994**). Biochemical analysis revealed that VP7 protein is a glycoprotein that contains only N-linked high-mannose oligosccharide residues that are processed by trimming (**Kabcenell and Atkinson, 1985**). VP7 is the major neutralizing

protein of rotaviruses that help for identification of rotavirus serotypes **(Estes, 2001)**. Also, it is a major target for rotavirus-specific cytotoxic T lymphocytes (CTLs) **(Dharakul et al., 1991 and Offit et al., 1994)**.

Recently, baculovirus expressed rotavirus proteins were used in the form of subunit vaccine to enhance the homotypic and heterotypic protection to rotavirus infection **(Crawford et al., 1999; Jiang et al., 1999 and Iosef et al., 2002)**. The maternal vaccination of cows with virus like particles (VLPs) significantly increased the neutralizing and ELISA antibodies in serum, milk and colostrum. The results suggested that the VLPs vaccines might constitute a new approach for BRV vaccination strategies **(Fernandez et al., 1998 and Kim et al., 2002b)**.

The aim of the present study is expression of VP7 rotavirus structural protein in baculovirus expression vector system (BEVS) to be use in the development of a safe, low cost and effective rotavirus subunit vaccine for control of diarrheal disease syndrome in the newly born calves also as antigen for preparation of diagnostic kits, monospecific hyperimmune and monoclonal antibodies.

MATERIALS AND METHODS

Cells and Virus: Bovine Rotavirus strain NCDV (serotype 6) was propagated in Rhesus monkey kidney (MA104) cells. Trypsin [DEFCO] was used at a final concentration of 10 µg / ml for 1 hour at 37°C to activat virus infectivity. After adsorption of virus, the inoculated cells were maintained in Minimal essential medium (MEM) with Earl's salts [Sigma] supplemented with 2mM L-glutanine, nonessential amino acids, 100 U/ml penicillin, 100 µg / ml streptomycin and 5 µg / ml trypsin as previously described (Saif et al., 1988). Sf9 cells obtained from Invertogen were grown in complete TNM-FM medium [GIBCO] supplemented with 10% fetal bovine serum gamma irradiated [GIBCO], 1% pluronic F-68 solution (10%) [Sigma].

cDNA synthesis and Cloning: The rotavirus dsRNA were extracted using Trizol reagent. The VP7 gene of NCDV was RT-PCR amplified as described previously (Hussein et al., 1996) with some modification. Briefly, 5µl of extracted dsRNA was mixed with

3.5µl of dimethylsulfoxide in a microcentrifuge tube, denaturated by heating at 95°C for 5 minutes and immediately cooled on ice. The denaturated RNA was RT-PCR amplified using 10 µm of each forward and reverse primer specific to VP7 full length in Reverse-IT TM one-step RT-PCR Kit [AB gene]. First-strand cDNA synthesis was synthesized by incubating the RT-PCR mixture for 30 minutes at 47°C then at 94°C for 2 minutes for inactivation of RT enzyme and initial denaturation. 35 amplification cycles were conducted, with each cycle contain 3 steps of 94°C for 45 second (denaturation), 55°C for 45 second (annealing) and 72°C for 1.5 minutes (extension) followed by a final extension cycle of 5 minutes at 72°C. The primers for RT-PCR were as follows: VP7 forward primer, 5′-GCGGTTAGCTCCTTTTAA-TGTATGG-3′ (nucleotide 32- 56) and VP7 reverse primer 5′-GGTCACATCATACAACTCTAATCTAACATG-3′ (complementary to nucleotide 1033-1062). The VP7 full-length RT-PCR products were analyzed on 1.25% agarose gel containing 0.5 ug/ml ethidium bromide. The RT-PCR products were cloned into Blue Bac 4.5/V5-His Topo ® TA Expression kit [Invirtogen]. All cloning steps were recommended by kit's manufactures.

Selection of positive clones: Plasmids in recombinant ampicillin-resistant Escherichia coli bacterial colonies were screened by colony PCR for inserts in the correct transcription orientation. The colony PCR was performed as previously described (Sambrook et al., 1989) with some modification. Briefly, 5 separate colonies were selected and cultured overnight in LB broth containing 50-100µg/ml ampicillin. The cultured bacteria were harvested by centrifugation then the bacterial pellet was resuspended in 100µl TE buffer and boiled at 100°C for 10 minutes to lyse the cells. The bacterial lysates were clarified by centrifugation at 10,000rpm/5minutes. The supernatant was PCR amplified using 10 µm of each forward VP7 gene primer and V5C-term reverse primer [5′- ACCGAGGAGAGG-GTTAGGGAT-3′] of the cloning vector in 2X Reddy mix PCR Master mix [AB gene]. The PCR cycling profile consisted of one cycle at 94°C for 2 minutes for initial denaturation. 30 amplification cycles were conducted, with each cycle of 94°C

for 60 second (denaturation), 55°C for 60 second (annealing) and 72°C for 2 minutes (extension) followed by a final extension cycle of 5 minutes at 72°C. The PCR products were analyzed on 1.25% agarose gel containing 0.5 ug/ml ethidium bromide.

Construction of VP7 recombinant baculovirus: The transfection procedures were conducted as previously described (Kitts, 1995) with some modification. Briefly, the recombinant baculovirus transfer plasmid carrying the VP7 gene of BRV were extracted using QIAPrep Miniprep Kit [QIAGEN]. The Sf9 cells (10^6 cells) were plated on 35-mm tissue culture dishes. The Bac-N-Blue linearized baculovirus DNA [Invitrogen] was mixed with 20µl of recombinant transfer plasmids, 20µl of Cellfectin reagent and 1ml of Grace's insect cell culture medium (modified 1X)[GIBCO]. The mixtures were incubated at room temperature for 15 minutes. During incubation, the plated Sf9 monolayer was washed with Grace's medium (modified 1X) without supplements or FBS. The transfection mixtures were added to SF9 cells and after 4 hours of incubation at room temperature on a side-to-side rocking platform, 1ml TNM-FH complete medium (10% FBS) was added. The culture fluid was harvested 3 days later and stored for plaque purification (P-1 stocks).

Selection of VP7 recombinant baculoviruses: Recombinants, in which the polyhedrin gene had been replaced by polyhedrin-VP7 transfer vector by homologous recombination, were selected by plaque purification. The recombinant plaques were selected by formation of blue plaques in the presence of chromogenic substance (X-gal) due to presence of lacZ gene in the baculovirus transfer vector. The obtained blue plaques were harvested, added in 500µl complete TNM-FH medium and used for preparation of P-1 virus stock. The propagation of P-1 virus stocks was conducted as previously described (Day et al., 1995). Briefly, Sf9 cells ($2.5X10^5$ cells) were plated in 24 well tissue culture plat then 250µl from each harvested blue plaques were inoculated in one well. 3 days later, 500µl of the infected cells were harvested for PCR analysis of recombinant baculoviruses. The DNA from recombinant baculoviruses was purified by QIAamp DNA blood Mini kit [QIAGEN]. The extracted DNA was

PCR amplified as previously described (Day et al., 1995). The purified DNA was PCR amplified using 10 µm of each baculovirus forward primer [5′-TTTACTGTTTTCGTAACAGTTT TG-3′ (binds from -44 nt 4049 to -21 nt 4072 in front the start of the polyhedrin gene)]and baculovirus reverse primer [5′-CAACAACGCACAGAATCTAGC -3′ (binds at +794 nt 4886 to +774 nt 4866 3′ to the polyhedrin gene)]. The PCR reaction was conducted using 2X Reddy Mix PCR Master mix [AB gene]. The PCR cycling profile consisted of one cycle at 94°C for 2 minutes for initial denaturation. 30 amplification cycles were conducted, with each cycle of 94°C for 1 minutes (denaturation), 55°C for 2 minutes (annealing) and 72°C for 3 minutes (extension) followed by a final extension cycle of 7 minutes at 72°C. The PCR products were analyzed on 1% agarose gel containing 0.5 ug/ml ethidium bromide.

Preparation and titration of high-titer viral stocks (P-2 viral stocks): The *in vitro* expression studies were performed using a high, known multiplicity of infection (MOI). To prepare P-2 viral stocks, Sf9 cells ($2X10^6$ cells/5ml complete TNM-FH medium) were seeded in $25cm^2$ flasks then 50µl of the P-1 stocks were inoculated to the cells and incubated for 7 days. The prepared virus was harvested by one cycle of freezing and thawing. The P-2 viral stocks was titrated using plaque assay in which, the produced plaques were observed through staining of infecting cells by trypan blue and neutral red stains at day 5 and 7 postinoculation, respectively as previously descried (Ausubel et al., 1994).

Expression of bovine rotavirus VP7 protein: The preparation and harvestation of expressed protein were performed according to standard protocol (Ausubel et al., 1994) with some modification. Briefly, two sets of Sf9 cells ($3X10^6$ cells/5ml TNM-FH medium containing 2% FBS) were seeded in $25cm^2$ flasks then inoculated by recombinant baculovirus carrying the VP7 gene with a MOI of 10. The cells and medium of one set were harvested at 48 and the other at 96 hours post-infection, and the medium was clarified by centrifugation at 2000rpm/10 minutes at 4°C. The supernatant was collected, and then the pellets were washed by PBS and centrifuged again at 2000rpm/10 minutes at 4°C. The cells were lysed by addition of

300μl lysis buffer (1% triton X100 [BDH] in PBS containing 1μg/ml pepstatin A acid protease inhibitor [APPLICHEM]) and incubated on ice for 45 minutes followed by three cycles of freezing and thawing. The lysed cells were centrifuged at 4000rpm/10 minutes at 4°C. The cell lysates were harvested and the cell debris was resuspended in 300μl PBS containing pepstain A (cell debris). Baculovirus wild type and cell control were included as control in the characterization studies.

Characterization of bovine rotavirus VP7 protein expressed by recombinant baculovirus:

Fluorescent antibody technique (FAT): Indirect FAT was performed as previously described (Ausubel et al., 1994) with some modification. Briefly, VP7 baculovirus recombinant and wild type baculovirus were infected Sf9 cells with MOI of 10. Two days post-infection, the cells were washed with PBS and fixed with 80% cold acetone for 20 minutes at 4°C. The cells were then incubated with rabbit rotavirus hyperimmune antisera diluted 1/20 in PBS for 1 hour at room temperature and washed three times with PBS. FITC conjugated anti-rabbit IgG [Sigma] diluted 1/20 was added to the cells and incubated for 1 hour at room temperature followed by three times of washing and examined under fluorescent microscopy for detection of the fluorescent reaction.

Enzyme linked immunosorbent assay (ELISA): Solid-phase ELISA was carried out using the different preparations of recombinant VP7, wild type baculoviruses and cell control (Supernatant, cell lysate and cell debris) that harvested at 48 and 96 hours post inoculation as antigen for coating of 96-well microtiter plat [FALCON] as described by (Machow et al., 1989) with some modification. The cell lysates and debris were diluted 1/10 with coating buffer (Carbonate-Bicarbonate buffer, pH 9.6) while the supernatant was used undiluted. The plate was coated for 1 hour at 37°C then blocked with 10% nonfat dry milk in PBS overnight at 4°C. The plate was washed 1 time with 0.05% Tween in PBS and incubated for 2 hours at 37°C with bovine rotavirus specific polyclonal antibodies diluted 1/50. After washing three times affinity purified antibody peroxidase labeled goat anti-bovine IgG (H+L) [KPL] diluted 1/1000 was added and incubated for 1 hour at 37°C. Three cycles of washing were employed and the reaction was developed with

addition of H_2O_2/ABTS for 20 minutes. Further color development was stopped by SDS (0.5%) then the plate was read at 405nm wavelength.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot assays: The cell debris of recombinant VP7 , wild type baculoviruses and cell control, which harvested at 48 and 96 hours post inoculation were solubilized in Laemmli buffer and separated on polyacrylamide gels as previously described (Laemmli, 1970). The gels were stained with Coomassie blue stain (R-250) [ICN] as described by (Zacharius et al., 1969). For Western blotting assay, the separated proteins in SDS-PAGE were electroblotted onto the nitrocellulose membranes in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) (Towbin et al., 1979). The membranes were blocked with 10% nonfat dry milk in PBS overnight at 4°C, washed three times with 0.05% Tween in PBS and incubated for 1 hour at room temperature with rabbit rotavirus hyperimmune antisera diluted 1/75 . After three times washing, alkaline phosphatase conjugated goat anti-rabbit IgG whole molecule [Sigma] diluted 1/30,000 was added and incubated for 1 hour at room temperature. The blots were washed again and developed with alkaline phosphatase substrate solution (BCIP [Sigma] and NBT[BDH]) until the bands became visible.

RESULTS

Construction of VP7-baculovirus recombinants: The objectives of this study were express the VP7 protein of bovine rotavirus in insect cells infected with recombinant baculovirus then determine whether the expressed VP7 protein retains the antigenic functional characteristics of the bovine rotavirus VP7 protein. Therefore, the present study was initiated by amplification and cloning of the full-length DNA copies of RNA segment 9 (coding for VP7 protein) into baculovirus plasmid vector.

Figure 1 shows the amplified PCR specific band of VP7 gene (1031 bp).

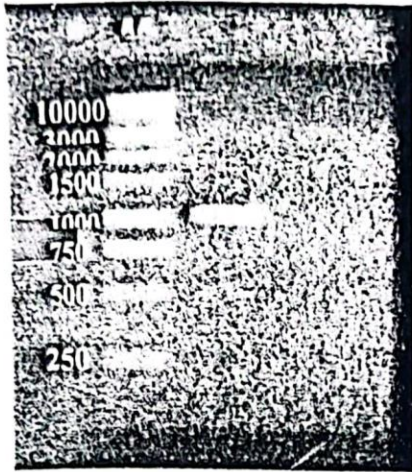

Fig. (1): RT-PCR specific band of VP7 gene (1031) along with 1Kbp leader.

The amplified VP7 gene was successfully cloned in the pBlueBac 4.5/V5-His-TOPO®TA baculovirus shuttle vector and transformed in the chemically competent E.coli cells. Plasmids carrying VP7 gene of BRV were screened for correct orientation for translation using standard PCR assay utilizing the forward primer of VP7 gene and the reverse primer of the baculovirus transfer vector. VP7 gene was successfully cloned in the correct orientation and yielded positive specific band of corrected size (1085 bp) [1031 (VP7) + 54 (vector)]. Figure (2) demonstrates the positive with correct orientation colonies out of the five tested colonies in the colony PCR assay.

The baculovirus shuttle vector containing correct orientation of the VP7 and Bac-N-Blue™DNA were co-transfected into Sf9 cells. Homologous recombination of the shuttle vector carrying the VP7 gene and baculovirus DNA resulted in the insertion of VP7 gene under the control of the polyhedrin promoter. Recombinant viral plaques (blue) were screened for the absence of occlusion bodies formed by the polyhedrin protein using specific baculovirus primers that amplify fragments of [435 bp from vector + size of insert gene (VP7 "1031 bp"] in recombinant virus and 839 bp in case of baculovirus wild type. Figure (3) shows the macroscopic and microscopic features of the produced recombinant blue plaques in comparison with non-infected Sf9 cell culture. Figure (4) demonstrates the PCR reaction of check recombination of three representative VP7 plaques; two plaques showing the specific bands (1466 bp) of recombinant baculovirus carrying the VP7 gene and one plaque showing the specific band (839 bp) of wild-type baculovirus.

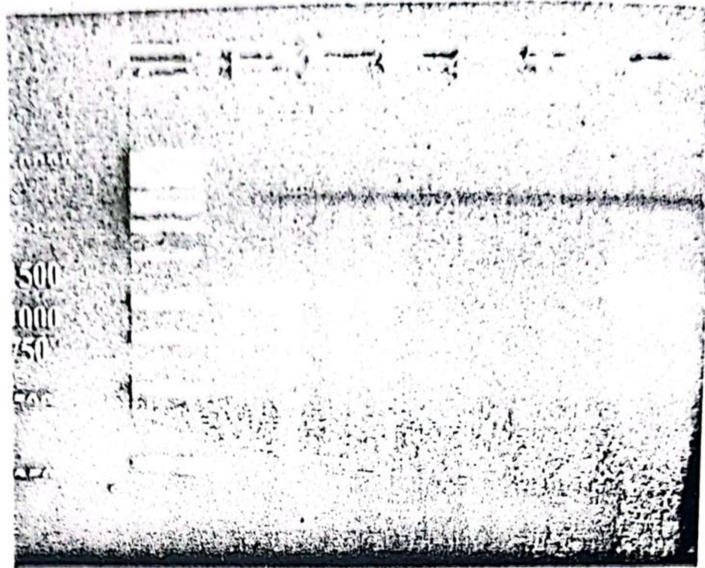

Fig. (2): The PCR specific bands of check cloning orientation of VP7 gene along with 1 Kbp leader

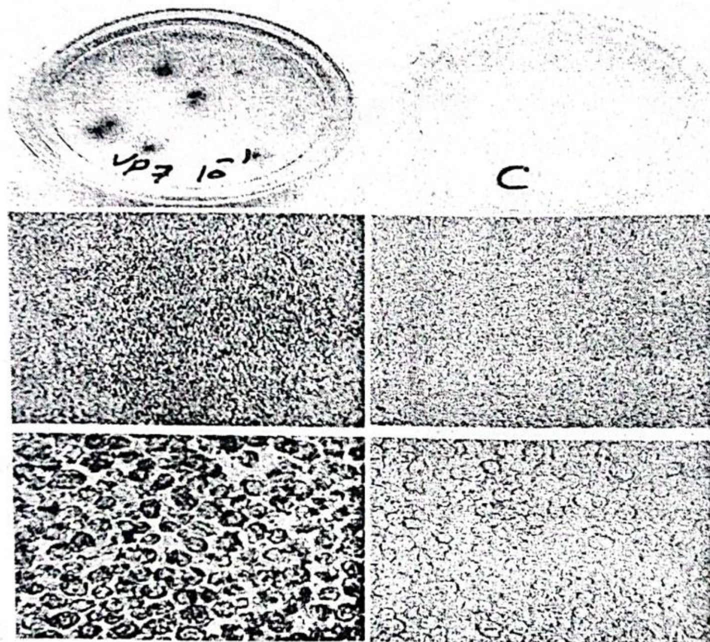

Fig. (3): Macroscopical and microscopical features of blue plaques for recombinant baculovirus carrying the VP7 gene in SF9 cells in comparison with control cells.

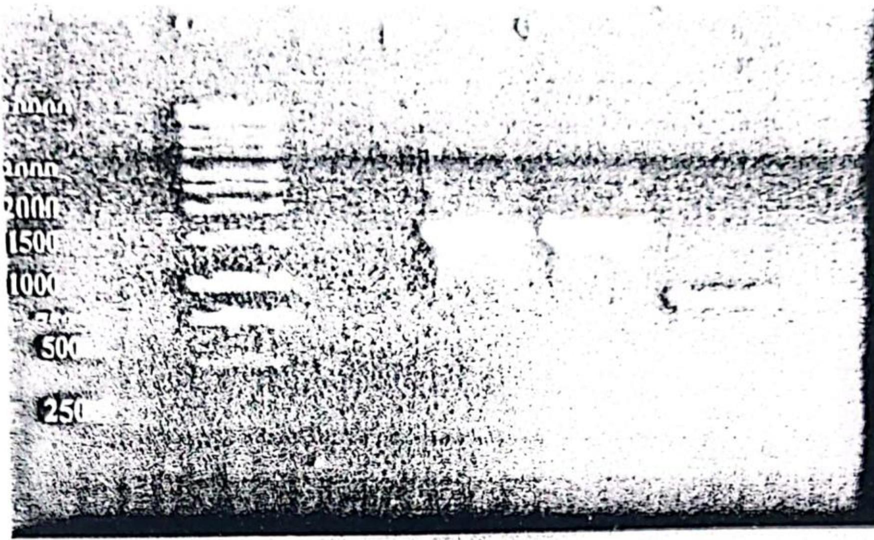

Fig. (4): The PCR specific bands of check recombination of VP7 gene along with 1 Kbp leader

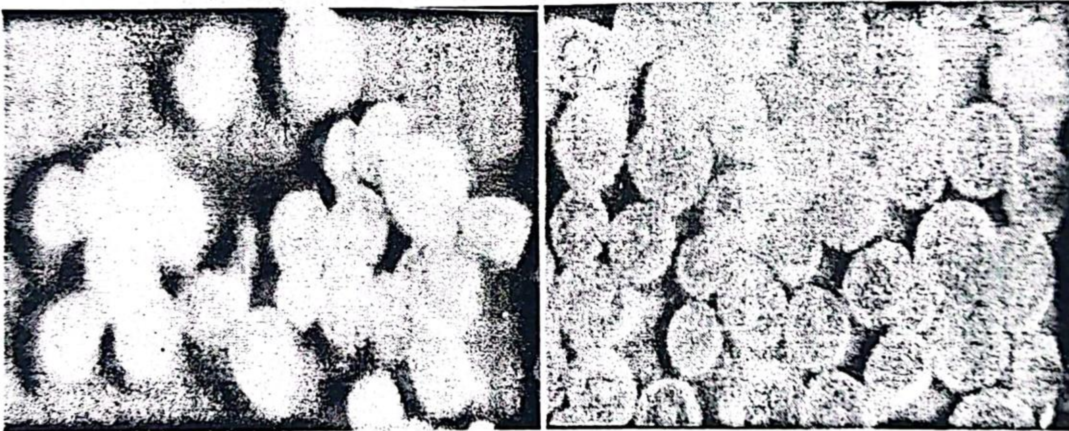

Fig. (5): Green fluorescent reaction of the VP7 expressed protein in comparison with control Sf9 cells.

The selected, purified VP7-recombinant baculovirus was titrated using plaque assay and the obtained plaques were observed through staining of agarose overlay by both trypan blue and neutral red stains. The results of plaque titration revealed that the VP7 recombinant baculovirus titer was $2X10^8$ PFU/ml.

Characterization of VP7 baculovirus expressed protein: Recombinant titrated baculovirus carrying the VP7 gene of BRV was evaluted for VP7 expression by several techniques as FAT, Solid phase ELISA, Coomassie blue staining of SDS-PAGE and Western blot. The VP7 recombinant baculovirus produced high fluorescent intensity when reacted with rotavirus polyclonal antibodies in comparison with the baculovirus wild type and control Sf9 insect cells. Figure (5) demonstrates the intensity of the intranuclear fluorescent reaction of the recombinant baculovirus expressing VP7 protein of BRV in insect cells in comparison with control non-infected Sf9 cells.

n an ELISA, the expressed baculovirus VP7 protein in addition to wild type baculovirus and Sf9 cell controls were harvested at 48 and 96 hour post-inoculation and the different preparation (cell debris, cell lysate and supernatant) were bound to mictotiter plat then identified with bovine hyperimmune anti-rotavirus serum. The VP7 expressed protein harvested at 48 hours was recognized with high intensity in cell debris than the cell lysate while the supernatant shows low reactivity whereas, the VP7 expressed protein harvested at 96 hours was recognized with high intensity in the supernatant when compared with the baculovirus wild-type and Sf9 cell controls . Table 1 demonstrates the optical densities of the baculovirus VP7 expressed protein in comparison with baculovirus wild-type and Sf9 cell controls.

In Western blot assay, the recombinant baculovirus expressing VP7 protein produced a detectable band of approximately 38KDa, which represent the molecular weight of the native rotavirus VP7 protein at 48 hours. Figure (6) demonstrates the Western blot assay of Sf9 cells infected with the recombinant baculovirus expressing VP7 protein and baculovirus wild-type and control Sf9 cells.

Table (1): The optical densities of different preparations of VP7 expressed protein in solid phase ELISA in comparison with baculovirus wild-type and control cells.

Time of harvestation	Protein	Cell debris	Cell lysate	Supernatant
48 hours	VP7	0.670	0.493	0.320
	Wild type	0.195	0.189	0.282
	Cell control	0.205	0.175	0.254
96 hours	VP7	0.417	0.275	0.948
	Wild type	0.387	0.205	0.210
	Cell contol	0.356	0.195	0.225

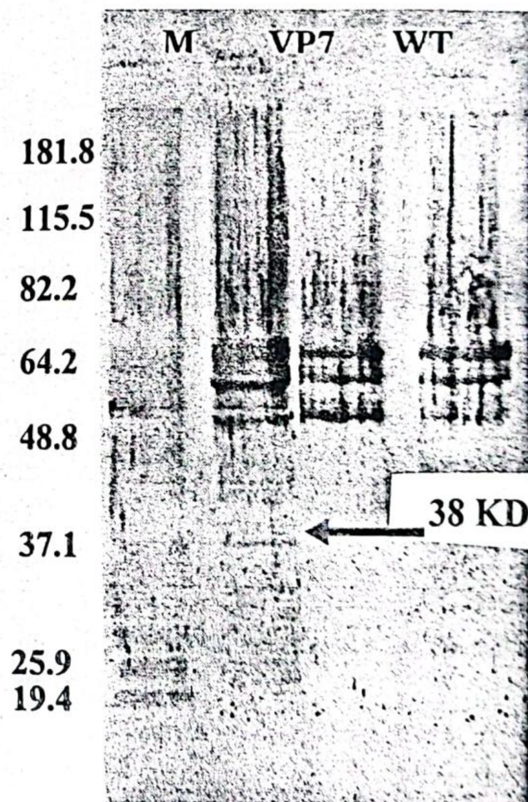

Fig. (6): Immunoreactivity of VP7 expressed by a recombinant baculovirus. Sf9 cell debris were solubilized and after electrophoresis, polypeptides were transferred to nirocellulose and reacted with BRV hyperimmune serum. Bound antibodies were visualized by immunoalkaline phosphatase-coupled reaction (BCIP-NBT). Sf9 cells infected with VP7 recombinant baculovirus shows a specific band of VP7 protein at 38 KDa.

DISCUSSION:

Rotaviruses are the most common etiological agents of severe acute gastroenteritis in young children and animals worldwide. Group A rotavirus is a major cause of diarrhea in calves occurring most frequently in 1-3 week-old calves. The recognition that the rotavirus are the major cause of life-threatening diarrheal disease and of significant morbidity in the young has focused efforts on disease prevention and control against these viruses (Estes, 2001).

The development of safe effective rotavirus vaccines is an international priority, and significant efforts have focused on the conventional vaccines as modified live attenuated and inactivated adjuvanted rotavirus vaccines **(Rousic *et al.*, 2000)**. Under field conditions, the efficiency of these vaccines in cattle varied and many researchers are not satisfied with the efficiency of these vaccines in enhancing the rotavirus antibody titers and protecting calves against rotavirus infection **(Myers and Snodgrass, 1982 and Saif *et al.*, 1983)**.

An alternative to conventional rotavirus vaccines is virus-like particles (VLPs) of different rotavirus proteins composition that self-assemble in insect cells coinfected with recombinant baculovirus expressing the roravirus structural proteins. Rotavirus double-layered particles (DLPs) 2/6-VLPs are made by coinfecting insect cells with recombinant baculovirus expressing VP2 and VP6, whereas co-expression of VP2, VP6 and VP7, with or without VP4, results in the protection of triple-layered particles (TLPs) 2/6/7-VLPs or 2/4/6/7-VLPs, respectively **(Labbe et al., 1991; Crawford et al., 1994 and Kim et al., 2002a)**.Such VLPs have been shown to induce antibody responses and protection from rotavirus challenge, notably in rabbits after parentral immunization **(Crawford et al., 1999)**, in mice treated orally and intranasally **(Bertolotti-Ciarlet et al., 2003)**, cows **(Fernandez et al., 1998 and Kim et al., 2002b)** and pigs **(Iosef et al., 2002)**.

Therefore, the current study was directed for expression and characterization of rotavirus VP7 protein in insect cells to be furthure use in both diagnostic tests and vaccines production. The first step for expression of any proteins is

cloning the gene of interst in suitable baculovirus transfer vector. Therefore, the RT-PCR product of VP7 gene of BRV was cloned in pBlueBac4.5/V5-His-TOPO® TA baculovirus transfer vector because this vector utilizes the polyhedrin promotor from AcNPV for high-level expression **(Crawford and Miller, 1988)** and contains the 5′ portion of the LacZ gene and 3′ portion of ORF1629 in which recombination occurs with similar sequences in Bac-N-Blue™ DNA of baculovirus to generate recombinant virus and forms blue plaques when 5-bromo-4-chloro-3-indolyl-D-galactosidase (X-gal) is present in the agarose overlay to facilitate the selection of recombinant viruses **(Vialard et al., 1990)**.

The correct orientation for translation of cloned VP7 genes in baculovirus shuttle vector and insertion of the cloned VP7 gene in the DNA of recombinant baculovirus was identified by PCR because it allows a quick and safe method to determine the presence of the interest gene in a putative recombinant virus and confirms the isolation of pure, recombinant plaques. Colony PCR was performed using the forward primer of VP7 gene with the V5C-term reverse of the vector to identify the correct orientation of the cloned gene. Using these primers, the correct orientation of the cloned VP7 gene yields a fragment of 1031 bp which is the full length of VP7 plus 54 from vector with a total of 1085bp **(Sambrook *et al.*, 1989)**. Also, the check recombination was performed using the baculovirus forward and reverse primers, which flank the polyhedrin region and are compatible with all polyhedrin promotor-based baculovirus transfer vectors **(Webb *et al.*, 1991)**. These primers are able to identify recombinant viruses as the wild type baculovirus DNA produced a fragment of 839 bp, while the recombinant virus produced a fragment of 435 bp plus 1031 bp the size of VP7 gene.

Identification and characterization of baculovirus VP7 expressed protein were performed by different techniques as FAT, ELISA, SDS-PAGE and Western blot. Although the VP7 expressed protein was detected in SF9 cells by FAT using rotavirus specific antiserum it was not detected in polyacrylamide gel stained with Coomassie blue stain. The reasons of such result may be due low level of expression that may be due to the codon usage, which dose not reflects the tRNA population available in the infected insect cells **(Hastings and Emerson, 1983)** and/or masking of the VP7

expressed protein with other cellular protein that have the same molecular weight of VP7 protein. This suggestion was confirmed by Western blot and ELISA assays. In Western blot assay the VP7 expressed protein produced a detectable band of approximately 38KDa, which represent the molecular weight of VP7 protein at 48 hours postinfection this band was detected in cell lysates by rotavirus specific antiserum. Furthermore, the ELISA results were confirmed the results of Western blot as the VP7 expressed protein was detected in cell lysates fraction as early as 48 hours postinfection and detected in the supernatant fraction as late as 96 hours postinfection, suggesting the possibility that it was being secreted from cells. These results are agreed with the results obtained by (Sabara et al., 1991). Reactivity of the VP7 baculovirus expressed protein with rotavirus hyperimmune antisera confirmed the expression of VP7 protein in its native conformation and its native immunoreactive determinants.

The present study has validated the baculovirus expression vector system (BEVS) as a cheap alternative for production of recombinant rotavirus proteins. The high level of expression of VP7 protein by the recombinant baculovirus provides sufficient material for functional analysis of the expressed proteins as well as for production of virus like particles (VLPs) by co-infection of insect cells with recombinant baculovirus containing the VP6 gene **(El-Sabagh et al., 2007)** to be use fartherly for preparation of subunit vaccine. Furthermore, the establishment of the BEVS offers the opportunity to express the rotavirus genes from the reference and local serotypes to prepare chimeric particles containing serotypically different proteins in order to study the role these proteins in the protective response and provide the opportunity to prepare a subunit vaccine containing the predominant locally serotype to control the rotavirus infection in young calves in our country.

ACKNOWLEDGMENT

This work was supported by the National Academy of Science and Technology as a research project (No. 34) under title, production of a combined genetically engineered bovine rotavirus/coronavirus subunit vaccine.

REFERENCES

Alfieri, A.F.; Alfieri, A.A.; Barreirous, M.AS.; Leite, J.P. and Richtzenhain, L.J. (2004). G and P genotypes of group A rotavirus strains circulating in calves in Brazil, 1996-1999. Vet. Microbiol. 99: 167-173.

Ausubel, F.M.; Brent, R.; Kingston, R.E.; Moore, D.D.; Seidman, J.G.; Smith, J.A. and Struhl, K. (1994). Current Protocols in Molecular Biology (New York: Greene Publishing Associates and Wiley-Interscience).

Bertolotti-Ciarlet, A.; Ciarlet, M.; Crawford, S.E,; Conner, M.E. and Estes, M.K. (2003). Immunogenicity and protective efficacy of rotavirus 2/6-virus-like particles produced by a dual baculovirus expression vector and administered intramuscularly, intranasally, or orally to mice. Vaccine 21:3885-3900.

Chang, K.O.; Parwani, A.V. and Saif. L.J. (1996). The characterization of VP7 (G type) and VP4 (P type) genes of bovine group A rotaviruses from field samples using RT-PCR and RFLP analysis. Arch. Virol. 141:1727-1739.

Crawford, A.M. and Miller, L.K. (1988). Characterization of an early gene accelerating expression of late genes of the baculovirus autographa californica nuclear polyhedrosis virus. J. Viro. 62: 2773-2781.

Crawford, S.E.; Estes, M.K.; Ciarlet, M.; Barone, C.; O'Neal, C.M.; Cohen, J. and Conner, M.E. (1999). Heterotypic protection and induction of a broad heterotypic neutralization response by rotavirus-like particles. J. Virol. 68:5945-5952.

Crawford, S.E.; Labbe, M.; Cohen, J.; Burroughs, M.H.; Zhou, Y.J. and Estes, M.K. (1994). Characterization of virus-like particles produced by expression of the rotavirus capsid proteins in the insect cells. J. Virol. 68:5945-5952.

Day, A.; wright, T.; Sewall, A.; Price-Laface, M.; Srivastava, N. and Finlayson, M. (1995). Rapid procedures for isolation and PCR analysis of recombinant baculovirus. Methods Mol. Biol. 39. Baculovirus expression protocols. Ed. Richardson, C.D. Human Press, 143-159.

Dharakul, T.; Labbe, M.; Cohen, J.; Bellamy, R.A.; Street, L.E.; Mackow, E.R.; Flore, L.; Rott, L. and Greenberg, H.B. (1991). Immunization with baculovirus expressed recombinant rotavirus

proteins VP1, VP4, VP6 and VP7 induces CD8+ T lymphocytes that mediate clearance of chronic rotavirus infection in SCID mice. J. Virol. 65:5928-5932.

El-Sabagh, I.M.; Hussein, H.A.; Amer, H.M.; El-Sanousi, A.A.; Reda, I.M. and Shalaby, M.A. (2007). Construction of a recombinant baculovirus expressing the major capsid protein (VP6) of bovine rotavirus. Arab J. Biotech., 10 (2): 369-384.

Estes, M.K. (2001). Rotaviruses and their replication. Chapter 54: Field's Virology 1747-1785.

Estes, M.K. and Cohen, J. (1989). Rotavirus gene structure and function. Microbiol. Rev. 53:410-449.

Fernandez, F.M.; Conner, M.E.; Hodgins, D.C.; Parwani, A.V.; Nielsen, P.R.; Crawford, S.E.; Estes, M.K. and Saif, L.J. (1998). Passive immunity to bovine rotavirus in newborn calves fed colostrum supplements from cows immunized with recombinant SA11 rotavirus core-like particle (CLP) or virus-like particle (VLP) vaccines. Vaccine 16: 507-516.

Fukai, K.; Onoda, H.; Iton, T.; Sato, M.; Miura, Y. and Sakai, T. (2004). Genetic and serological characterization of novel serotype G8 bovine group A rotavirus strains isolated in Japan. J. Vet. Med. Sci. 66(11): 1413-1416.

Hastings, K.E.M. and Emerson, C.P.JR. (1983). Codon usage in muscle genes and liver genes. J. Mol. Evolution 19: 214-218.

Hoshino, Y.; Jones, R.W. and Kapikian, A.Z. (2002). Characterization of neutralization specificities of outer capsid spike protein VP4 of selected murine, lapine and human rotavirus strains. Virology299:64-71.

Hussein, A.H.; Frost, E.; Talbot, B.; Shalaby, M.; Cornaglia, E. and El-Azhary, Y. (1996). Comparison of polymerase chain reaction and monoclonal antibodies for G-typing of group A bovine rotavirus directly from fecal material. Vet. Microbiology. 51:11-17.

Hussein, A.H.; Parwani, A.V.; Rosen, B.I. Lucchelli, A. and Saif, L.J. (1993). Detection of rotavirus serotypes G1, G2, G3 and G11 in feces of diarrheic calves by polymerase chain reaction-derived cDNA probes. J. Clin. Microbiol. 31:2491-2496.

Iosef, C.; Van Nguyen, T.; Jeong, K.; Bengtsson, K.; Morein, B. and Kim, Y. (2002). Systemic and intestinal antibody secreting cell responses and protection in gnotobiotic pigs immunized orally with attenuated Wa human

rotavirus and Wa 2/6-rotavirus-like-particles associated with immunostimulating complexes. Vaccine 20:1741-1753.

Jiang, B.; Estes, M.K.; Barone, C.; Barniak, V.; O'Neal, C.M.; Ottaiano, A.; Madore, H.P. and Conner, M.E. (1999). Heterotypic protection from rotavirus infection in mice vaccinated with virus-like particles. Vaccine 17:1005-1013.

Kabcenell, A.K. and Atkinson, P.H. (1985). Processing of the rough endoplasmic reticulum membrane glycoproteins of rotavirus SA11. J. Cell. Biol. 100:1270-1280.

Kim, Y.; Chang, K.O.; Kim, W.K. and Saif, L.J. (2002a). Production of hybrid double-or triple-layered virus-like particles of group A and C rotaviruses using a baculovirus expression system. Virology 302:1-8.

Kim, Y.; Nielsen, P.R.; Hodgins, D.; Chang, K.O. and Saif, L.J. (2002b). Lactogenic antibody responses in cows vaccinated with recombinant bovine rotavirus-like particles (VLPs) of two serotypes or inactivated bovine rotavirus vaccines. Vaccine15: 1248-1258.

Kitts, P.A. (1995). Production of recombinant baculoviruses using linearized viral DNA. Methods Mol. Biol. 39. Baculovirus expression protocols ed. Richardson, C.D. Human Press, 129-142.

Labbe, M.; Charpilienne, A.; Crawford, S.E.; Estes, M.K. and Cohen, J. (1991). Expression of rotavirus VP2 produces empty core-like particles. J. Virol. 65:2946-2952.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, London 227: 680-685.

Mackow, E.R.; Barnett, J.W.; Chan, H. and Greenberg, H.B. (1989). The rhesus rotavirus outer capsid protein VP4 functions as a hemagglutinin and is antigenically conserved when expressed by a baculovirus recombinant. J. Virol. 63:1661-1668.

Martella, V.; Ciarlet, M.; Banyai, K.; Lorusso, E.; Cavalli, A.; Corrente, M.; Elia, G.; Arista, S.; Camero, M.; Desario, C.; Decaro, N.; Lavazza, A. and Buonavoglia, C. (2005). Identification of a novel Vp4 genotype carried by a serotype G5 porcine rotavirus strain. Virology, in press.

Matsuda, Y.; Nakagomi, O. and Offit, P.A. (1990). Presence of three P types (VP4 serotypes) and two G types (VP7 serotypes) among bovine

rotavirus strains. Arch. Virol. 115:199-207.

Mattion, N.M.; Cohen, J. and Estes, M.K. (1994). The rotavirus proteins. In: Kapikian A, ed. viral infections of the gastrointestinal tract. New York: Marcel Dekker, 169-249.

Myers, L.L. and Snodgrass, D.R. (1982). Colostral and milk antibody titers in cows vaccinated with a modified live rotavirus-coronavirus vaccine. JAVMA 181: 486-488.

Offit, P.A.; Coupar, B.E.H.; Svodoba, Y.M.; Jenkins, R.J.; McCrae, M.A.; Abraham, A., Hill, N.L.; Boyle, D.B.; Andrew, M.E. and Both, G.W. (1994). Induction of rotavirus specific cytotoxic T lymphocytes by vaccinia virus recombinants expressing individual rotavirus genes. Virology 198:10-16.

Parwani, A. V.; Hussein, A. H. and Rosen, B. I. (1993). Characterization of field strains of group A bovine rotaviruses by using polymerase chain reaction-generated G and P type-specific cDNA probes. J. Clin. Microbiol. 31:2010-2015.

Rahman, M.; Matthijnssens, J.; Nahar, S.; Podder, G.; Sack, D. A.; Azin, T. and Van Ranst, M. (2005). Characterization of a novel P[25], G11 group A rotavirus. J. Clin. Microbiol. 43:3208-3212.

Rao, C.D.; Gowda, K.; and Yugandar Reddy, B.S. (2000). Sequence analysis of VP4 and VP7 genes of nontypeable strains identifies a new pair of outer capsid proteins representing novel P and G genotypes in bovine rotaviruses. Virology. 276:104-113.

Rousic, S. Le; Klein, N.; Houghton, S. and Charleston, B. (2000). Use of colostrum from rotavirus-immunized cows as a single feed to prevent rotavirus-induced diarrhea in calves. Vet. Record 147 (6) 160-161.

Sabara, M.; Parker, M.; Aha, P.; Cosco, C.; Gibbons, E.; Parsons, S. and Babiuk, L.A. (1991). Assembly of double-shelled rotavirus like particles by simultaneous expression of recombinant VP6 and VP7 proteins. J. Virol. 65:6994-6997.

Saif, L.J.; Redman, D.R.; Smith, K.L. and Theil, K.W. (1983). Passive immunity to bovine rotavirus in newborn calves fed colostrum supplements from immunized or nonimmunized cows. Infect. Immun. 421:1118-1131.

Saif, L.J.; Rosen, B.; Kang, S. and Miller, K. (1988). Cell culture propagation of rotaviruses. J.

Tissue Culture Meth. 11(3): 147-154.

Sambrook, J.; Fritsch, E.F. and Maniatis, T. (1989). Molecular cloning: A laboratory Manual, 2nd ed. Cold Spring Harbor laboratory, Cold Spring Harbor, NY.

Snodgrass, D.R.; Ojeh, C.K.; Campbell, I. and Herring, A.J. (1990). Rotavirus serotypes 6 and 10 predominate in cattle. J. Clin. Microbiol. 28:504-507.

Suzuki, Y.; Sanekara, T.; Sato, M.; Tajima, K.; Matsuda, Y. and Nakagomi, O. (1993). Relative frequencies of G (VP7) and P (VP4) serotypes determined by polymerase chain reaction assays among Japanese bovine rotaviruses isolated in cell culture. J. Clin. Microbiol. 31:3046-3049.

Towbin, H.; Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA. 76:4350-4354.

Vialard, J.; Lalumiere, M.; Vernet, T.; Briedis, D.; Alkhatib, G.; Henning, D.; Levin, D. and Richardson, C. (1990). Synthesis of the membrane fusion and haemagglutinin proteins of measles virus, using a novel baculovirus vector containing the β-galactosidase gene. J. Virol. 72: 1551-1557.

Webb, A. C.; Bradley, M. K.; Phelan, S. A.; Wu, J. Q. and Gehrke, L. (1991). Use of the polymerase chain reaction for screening and evaluation of recombinant baculovirus clones. Biotechniques, 11: 512-518.

Zacharius, R.M.; Zell, T.E.; Morrison, J.H. and Woodlock, J.J. (1969). Glycoprotein staining following electrophoresis on acrylamide gels. Analytical Biochemistry 30:148-152.