Construction of a recombinant baculovirus expressing the major capsid protein (VP6) of bovine rotavirus

(Received: 05.01.2007; Accepted: 18.01.2007)

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ABSTRACT

The present study reports the expression of VP6, the major inner capsid protein of bovine rotavirus Nebraska calf diarrhea virus (NCDV) strain in a baculovirus expression system. The full-length DNA copies of RNA segment 6 (coding for VP6 protein) of NCDV were inserted into a baculovirus expression vector. A recombinant baculovirus carrying the VP6 gene was constructed through homologous recombination between the baculovirus recombinant plasmid carrying the VP6 gene and Autographa californica nuclear polyhedrosis virus (AcNPV) under the control of the polyhedrin promotor. Infection of Spodoptera frugiperda (Sf9) cells with the recombinant baculovirus expressing VP6 protein revealed a high-level of expression when tested by immunoflurescence and solid phase ELISA tests using BRV-specific polyclonal antibodies. The VP6 expressed protein was detected in Coomassie blue stained SDS-PAGE and produced a detectable band in Western blot assay. The high degree of reactivity with BRV-specific polyclonal antibodies confirmed that the antigenic determinants of the expressed protein were unaltered. The use of the in vitro expressed VP6 protein in the field diagnosis and vaccine development to control rotavirus infection is of considerable interest.

Key words: *Rotavirus, VP6 capsid protein, recombinant baculovirus.*

INTRODUCTION

Rotaviruses constitute a genus of the family Reoviridae. The virus contains 11 genome segments of double-stranded RNA in a 70 nm, double-shelled, icosahedral capsid. Rotaviruses are common causes of sever gastroenteritis in neonates of animals and human (Estes, 2001).

Rotavirus is composed of three layers of structural capsid proteins. The core comprises of three viral proteins VP1, VP2 and VP3. The inner capsid consists of the most abundant protein, VP6, to which a majority of the group-specific antibodies is directed (Ericson *et al*., 1982). The outer capsid layer contains 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 serogroups (A-G) determined by the antigenic properties of VP6. Group A, the most common, is further divided into serotypes determined by VP4 (P type) and VP7 (G type) (Estes and Cohen, 1989). In bovine population, there are at least ten G (G1, G2, G3, G5, G6, G7, G8, G10, G11 and G15) and six P (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; Lucchelli *et al*., 1994 and Hussein *et al*., 1996).

VP6 protein is the major structural protein in the virus particles. It represents 80% of the protein in single-shelled particles and 51-60% of the total viral proteins (Mattion *et al*., 1994). The VP6 protein is an important target antigen of Th cells, as it carries an epitope that stimulates proliferation of cross-reactive helper T cells of $L3T4^+$ phenotype and has also a serotype cross-reactive CTL epitope (Banos *et al*., 1997). The Th cells specific for VP6 can provide cognate help to B cell specific for neutralizing epitopes on the VP7 and or VP4 molecules. Thus, the VP6 seems to be a good candidate to be used as vaccine or included in vaccine formulation (Esquivel *et al*., 2000).

The baculovirus system has been successfully used for production of different proteins, including rotavirus proteins (Estes *et al*., 1987; Labbe *et al*., 1991; Sabara *et al*., 1991; Crawford *et al*., 1994; and Kim *et al*., 2002a). The expressed proteins are made in high yields and possess native conformation when compared immunologically and biochemically to native viral proteins. The aim of the present study is to construct a recombinant baculovirus expressing the VP6 protein to be used in further studies.

MATERIALS AND METHODS

Cells and Virus

MA-104 cell line, derived from African green monkey kidney, was grown and 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% fetal calf serum (FCS) [Sigma]. The NCDV strain of bovine rotavirus (serotype G6) were propagated in MA-104 cells in the presence of 5 µg / ml trypsin [DEFCO] and harvested by freezing and thawing of the infected cells three times as previously described by Saif *et al*. (1988).

Sf9 cells (Invertogen) were maintained in complete TNM-FM medium [GIBCO] including 10% fetal bovine serum gamma irradiated [GIBCO] in the presence of 1% pluronic F-68 solution (10%) [Sigma].The Sf9 cells were used for baculovirus infection until they were passage up to 30 times and then they were replaced with new cells at low passage level.

Gene Cloning

The clarified supernatant containing the NCDV was collected and rotavirus dsRNA were extracted using Trizol reagent. The VP6 gene of NCDV was RT-PCR amplified as described previously (Hussein *et al*., 1996) with some modification. Briefly, 5 µl of extracted dsRNA was mixed with 3.5 µl of dimethylsulfoxide in a microcentrifuge tube, denaturated by heating at 95ºC for 5 min and immediately cooled on ice. The denaturated RNA was RT-PCR amplified using 10 µm of each forward and reverse primers specific to full length VP6 gene. The primers sequences for RT-PCR are as follows: VP6 forward primer, 5′-GGCTTTTAAACGAAGTCTTCAACATGG-3′ (nucleotide 1- 27) and VP6 reverse primer 5′-GGTCACATCCTCTCACTACGC-3′ (complementary to nucleotide 1336- 1356). Reverse-IT TM one-step RT-PCR Kit [AB gene] containing 2x Reddy Mix Master mix buffer optimized for RT-PCR was used. A dye and precipitant to facilitate gel loading, dNTP mix (final conc.: 0.2 mM each), thermoprime puls DNA polymerase (Final Conc.: 1.25 µ/50 ul) and $MgCl_2$ (final conc.: 1.5 mM) and reverse transcriptase blend (RT) (Patented) (50 µ/µl) including RNase inhibitors are the components of the kit. First-strand cDNA was synthesized by incubating the RT-PCR mixture for 30 min at 47°C then at 94°C for 2 min for inactivation of RT enzyme and initial denaturation. 35 amplification cycles were conducted, each cycle contain 3 steps of a 94°C

for 45 sec (denaturation), 55°C for 45 sec (annealing) and 72°C for 1.5 min (extension) followed by a final extension cycle for 5 min at 72°C. The VP6 full-length RT-PCR products were analyzed on 1.25% agarose gel containing 0.5 µg/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 carried out as recommended by kit's manufactures.

Analysis of positive clones

After transformation into *Escherichia coli* (top 10 strain), plasmids in recombinant ampicillin-resistant colonies were screened by colony PCR for inserts in the correct transcription orientation. The colony PCR was performed as previously described by Sambrook *et al*. (1989) with some modifications. Briefly, 5 separate colonies were selected and cultured overnight in LB broth containing 50-100 µg/ml ampicillin. The cultured bacteria were harvested by centrifugation. The bacterial pellet was resuspended in 100 µl TE buffer, and then boiled at 100ºC for 10 min to lyse the cells. The bacterial lysates were clarified by centrifugation at 10,000 rpm/5min. The supernatant was PCR amplified using 10 µM of each forward VP6 gene primer and V5C-term reverse primer [5´-ACCGAGGAGAGGGTTAGGGAT-3´] of the cloning vector in PCR reaction containing 2x Reddy Mix Master mix buffer optimized for PCR, which included a dye and precipitant to facilitate gel loading, dNTP mix (0.2 mM each), thermoprime puls DNA polymerase (1.25 µ/50 µl) and $MgCl_2$ (1.5 mM) [AB gene]. The PCR cycling profile consisted of one cycle at 94ºC for 2 min for initial denaturation, and then 30 amplification cycles, with each cycle consists of 94ºC for 60 sec (denaturation), 55ºC for 60 sec (annealing) and 72ºC for 2 min (extension) followed by a final extension cycle of 5 min at 72ºC. The PCR products were analyzed on 1.25% agarose gel containing 0.5 µg/ml ethidium bromide.

Construction of baculovirus recombinants

The transfection procedures were conducted as previously described by Kitts, (1995) with some modifications. Briefly, the recombinant baculovirus transfer plasmids containing the VP6 gene of BRV were extracted using QIAPrep Miniprep Kit [QIAGEN]. The Sf9 cells ($1x10^6$ 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, 20 µl of Cellfectin reagent and 1ml of Grace's insect cell culture medium (modified 1X)[GIBCO]. The mixtures were incubated at room temperature for 15 min. 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 hr of incubation at room temperature on a side-to-side rocking platform adjusted at 2 side-to-side motion/minutes, then 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 recombinant baculoviruses

Recombinants, in which the polyhedrin gene had been replaced by polyhedrin-VP6 transfer vector by homologous recombination, were selected by plaque purification. Purification of a recombinant plaque is carried out by infecting cells with dilutions of P-1 stocks and isolating focal points of infection (plaques) from agarose overlay. 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 500 µl 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 by Day *et al*. (1995). Briefly, Sf9 cells ($2.5X10^5$ cells) were plated in 24 well tissue culture plate then 250 µl from each harvested blue plaques were inoculated in one well. Three days later, 500 µl 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 purified DNA was PCR amplified using 10 µM of each forward and reverse primers specific to baculovirus. The primers for PCR were as follows: baculovirus forward primer, 5′- TTTACTGTTTTCGTAA-CAGTTTTG-3′ (binds from -44 nt 4049 to -21 nt 4072 in front the start of the polyhedrin gene) and baculovirus reverse primer, 5′- CAAC-AACGCACAGAATCTAGC-3′ (binds at +794 nt 4886 to +774 nt 4866 3′ to the polyhedrin gene). The PCR reaction was conducted in 2x Reddy Mix Master mix buffer [AB gene] as mentioned above. The PCR cycling profile consisted of one cycle of 94ºC for 2 min for initial denaturation, 30 amplification cycles were conducted, each cycle consists of 94ºC for 1 min (denaturation), 55ºC for 2 min (annealing) and 72ºC for 3 min (extension) followed by a final extension cycle of 7 min at 72ºC. The PCR products were analyzed on 1% agarose gel containing 0.5 µg/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 and known multiplicity of infection (MOI). To prepare P-2 viral stocks, SF9 cells ($2X10^6$ cells/5ml complete TNM-FH medium) were seeded in 25 cm^2 flasks, then 50 µl 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 were 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 post-inoculation; respectively as previously descried by Ausubel *et al*. (1994).

Expression of VP6 protein

The preparation and harvest of expressed protein were performed according to the standard protocol described by Ausubel *et al*. (1994) with some modifications. Briefly, Sf9 cells ($3X10^6$ cells/5ml TNM-FH medium containing 2% FBS) were seeded in 25 cm^2 flasks then inoculated by recombinant baculovirus carrying the VP6 gene with a MOI of 10. The cells and medium were harvested at 96 hr post-infection, and the medium was clarified by centrifugation at 2000 rpm/10 min at 4ºC. The supernatant was collected, and then the cells in pellets were washed by PBS and centrifuged again at 2000 rpm/10 min at 4ºC. The cells were lysed by addition of 300 µl lysis buffer (1% triton X100 [BDH] in PBS containing 1 µg/ml pepstatin A acid protease inhibitor [APPLICHEM]) and incubation on ice for 45 min, followed by three cycles of freezing and thawing. The lysed cells were centrifuged at 4000 rpm/10 min at 4ºC. The cell lysates were harvested and the cell debris was resuspended in 300 µl PBS containing pepstain A (cell debris). Baculovirus wild type and cell control were included as a control in the characterization studies.

Characterization of expressed baculovirus recombinant VP6 protein

Fluorescent antibody technique (FAT)

Indirect FAT was performed as previously described by Ausubel *et al*. (1994) with some modifications. Briefly, VP6 baculovirus recombinant and wild type baculovirus were infected Sf9 cells grown in cell culture staining chamber (CCSCs) containing cover slips with MOI of 10. Two days post-infection, the cells were washed with PBS and fixed with 80% cold acetone for 20

min at 4ºC. The cells were then incubated with rabbit rotavirus hyperimmune antisera diluted 1/20 in PBS for 1 hr 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 hr at room temperature, followed by three times of washing and examined under fluorescent microscope for detection of the fluorescent reaction.

Enzyme linked immunosorbent assay (ELISA)

Solid-phase ELISA was carried out using the different preparations of recombinant VP6, wild type baculoviruses and cell control (Supernatant, cell lysate and cell debris) as antigen for coating of 96-well microtiter plate [FALCON] as previously described by Mackow *et al*. (1989) with some modifications. 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 hr at 37ºC then blocked with 10% nonfat dry milk in PBS overnight at 4ºC. The plate was washed once with 0.05% Tween in PBS and incubated for 2 hr at 37ºC 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 hr at 37ºC.Three cycles of washing were employed and the reaction was developed with the addition of H_2O_2/ABTS for 20 min. Further color development was stopped by SDS (0.5%), then the plate was read at 405 nm wavelength.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot assays

The cell debris of recombinant VP6, wild type baculoviruses and cell control were solubilized in Laemmli buffer and separated on polyacrylamide gels as previously described by 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 4ºC, washed three times with 0.05% Tween in PBS and incubated for 1 hr 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 hr 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 VP6-baculovirus recombinants

The main objective of the present study was to express the major inner capsid VP6 protein of bovine rotavirus using insect cells infected with a recombinant baculovirus and evaluate if the expressed VP6 protein retains the antigenic and functional characteristics of the VP6 protein. The study was initiated by amplification and cloning of the full-length DNA copies of RNA segment 6 (coding for VP6 protein) into baculovirus shuttle plasmid. Fig. (1) shows the amplified PCR specific band of VP6 gene (1356 bp).

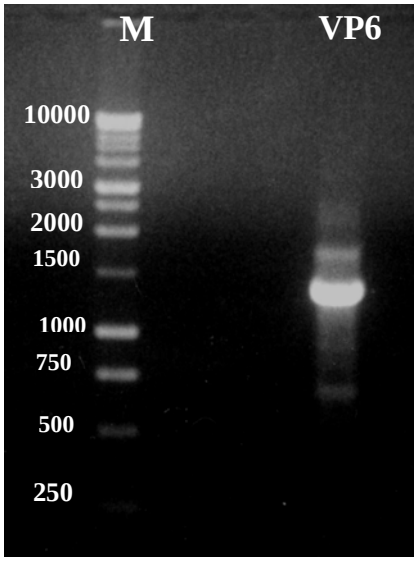

Fig. (1): RT-PCR band of VP6 gene (1356bp) along with 1 kbp ladder.

The amplified VP6 gene was successfully cloned in the pBlueBac 4.5/V5-His-TOPO®TA baculovirus transfer plasmid and transformed in the chemically competent *E. coli* cells. Plasmids carrying VP6 gene of BRV were extracted from transformed *E. coli* and screened for correct orientation for translation using standard PCR assay, utilizing the forward primer of VP6 gene and the reverse primer of the baculovirus transfer vector. VP6 gene was successfully cloned in the correct orientation and yielded a positive specific band of corrected size (1410 bp) [1356 bp from the VP6 + 54 bp from the vector]. Fig. (2) demonstrates the positive colony with correct orientation out of five tested colonies in the colony PCR assay.

The plasmid containing the correct orientation of the VP6 and Bac-N-Blue™DNA were co-transfected into SF9 cells. Homologous recombination of the plasmid and baculovirus DNA resulted in the insertion of VP6 gene under the control of the polyhedrin promoter. Recombinant viral plaques (blue) were screened for the absence of onclusion bodies formed by the polyhedrin protein using specific baculovirus primers that amplify fragments of 1791 bp [435 bp from vector + 1356 bp of the VP6] in recombinant virus and 839 bp in case of baculovirus wild type. Fig. (3) demonstrates the PCR specific bands of the two representative VP6 plaques. Fig. (4) shows the transfected SF9 cells and blue plaques in comparison with SF9 control cells.

The purified VP6-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 VP6 recombinant baculovirus titer was of 10^9 PFU/ml.

Expression and characterization of the VP6 protein

VP6-recombinant baculovirus that had been titrated was screened for VP6 expression by FAT (Fig. 5), Solid phase ELISA (Table 1), Coomassie blue staining of SDS-PAGE and Western blot assay (Fig. 6 A and B). The VP6-recombinant baculovirus was found to produce a high fluorescent intensity in comparison with the wild-type baculovirus and control non-infected cells.

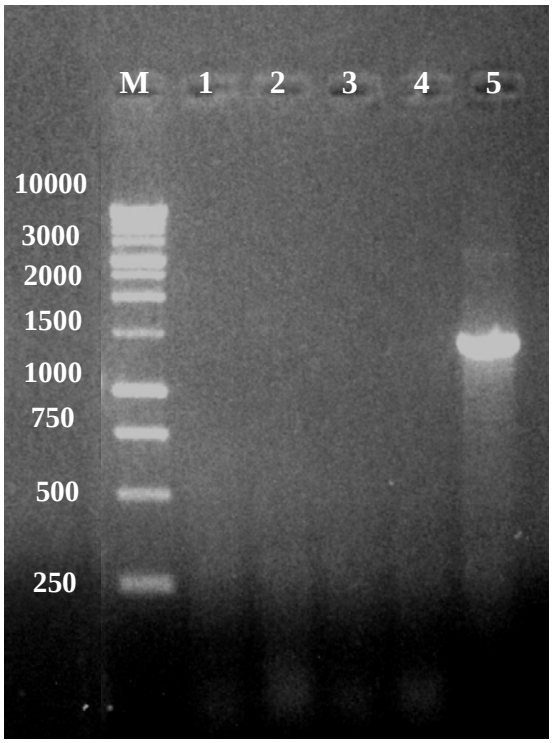

Fig. (2): The PCR specific bands of check orientation for cloned VP6 gene (lane 5) along with the 1 Kbp ladder (lane M).

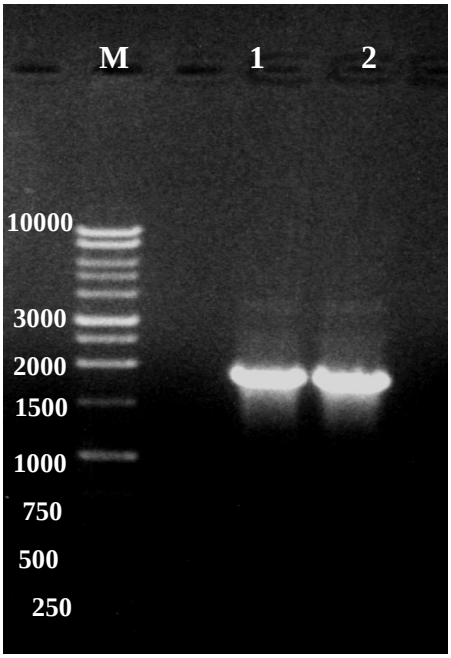

Fig. (3): The PCR specific bands of 2 representative VP6 recombinant baculovirus plaques (lanes 1 and 2) along with 1 Kbp ladder (lane M).

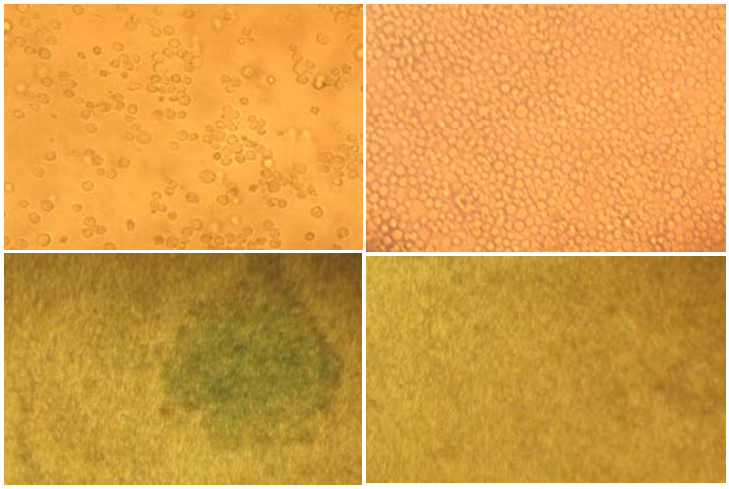

Fig. (4): The characteristic cytopathic changes of transfected SF9 cells and obtained blue plaques (Left upper and lower), in comparison with control cells (Right upper and lower).

Testing the different preparations (cell debris, cell lysate and supernatant) harvested 96 hr post-inoculation of cells by recombinant and wild-type baculoviruses in addition to the preparation from the control non-infected cells in an ELISA, revealed a high intensity reaction in case of the preparation from recombinant baculovirus, confirming the presence of the expressed VP6 protein in the cell debris and cell lysate. However, The cell debris preparation revealed a high reactivity than the cell lysate, whereas the supernatant harvest was low in reactivity (Table 1).

The use of SDS-PAGE stained with Coomasie blue confirmed that the Sf9 cells infected with VP6-recombinant baculovirus expressed a protein of approximately 44 KDa, which represents the same molecular weight of the rotavirus VP6 protein. The SF9 cells infected with wild-type baculovirus expressed a protein of approximately 30 KDa that represents the molecular weight of polyhedrin protein. The results showed no detectable band at 30 KDa in the VP6-recombinant baculovirus. The presence of the expressed VP6 protein was confirmed by Western blot assay when reacted with rabbit hyperimmune antisera to bovine rotavirus (Fig. 6 A and B).

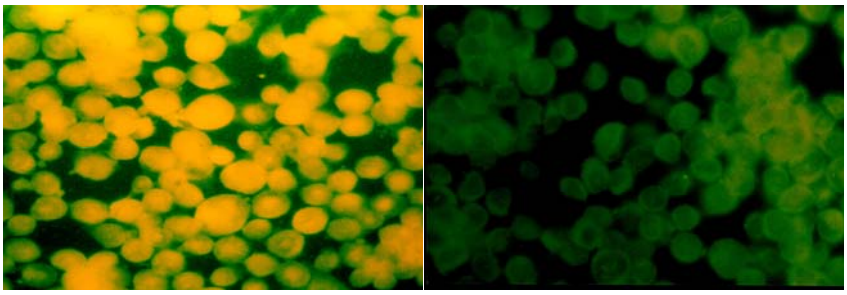

Fig. (5): Green fluorescent reaction of VP6 expressed protein (left) in comparison with wild-type baculovirus infected Sf9 cells (right).

Table (1): The optical densities of the baculovirus-expressed VP6 protein in comparison with wild-type baculovirus and control non-infected cells produced from ELISA test using bovine rotavirus polyclonal antibodies.

Proteins	Optical densities		
	Cell debris	Cell lysate	Supernatant
VP6	1.047	0.490	0.329
Wild type	0.387	0.205	0.210
Cell control	0.356	0.195	0.225

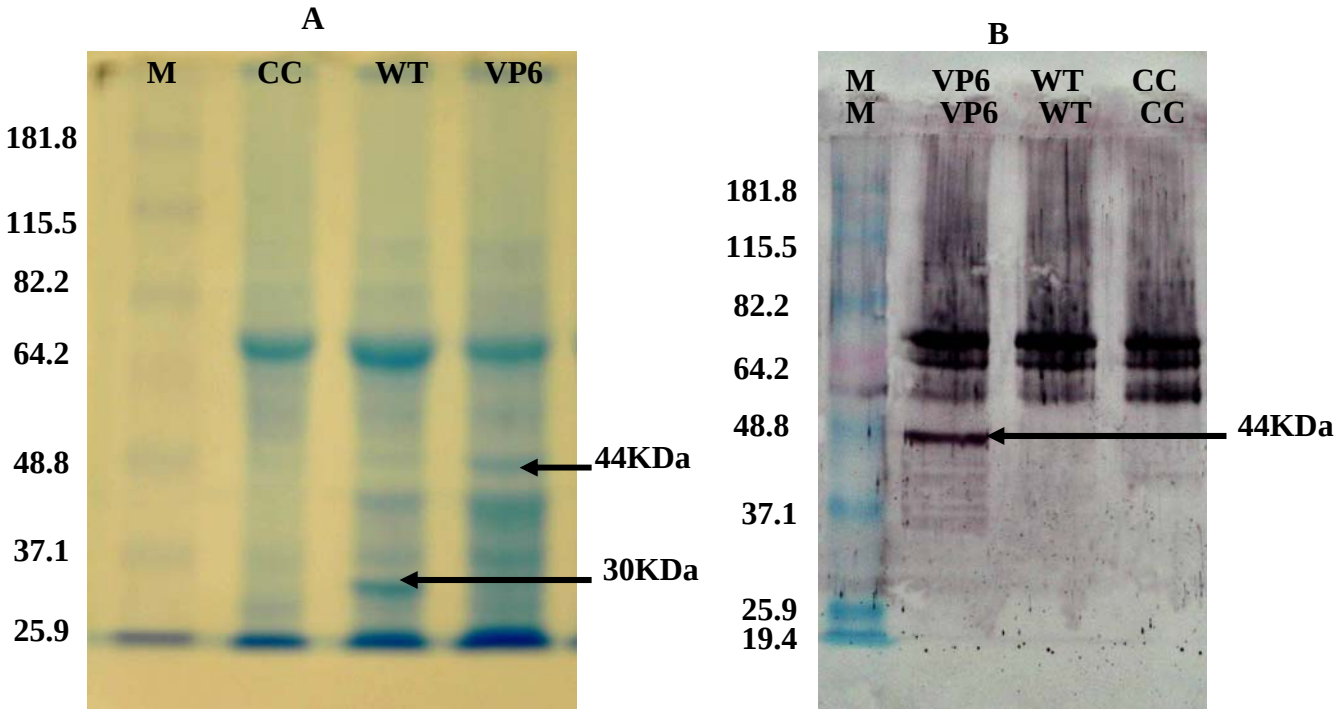

Fig. (6): A. Expression of VP6 protein of BRV by a recombinant baculovirus. Lysate of Sf9 cells were solubilized and assayed directly by SDS-PAGE and then stained with Coomasie blue. Sf9 cells infected with wild type shows a protein of 30KDa represent the polyhedrin protein. Sf9 cells infected with VP6 recombinant baculovirus show a protein of approximately 44 KDa represent the rotavirus VP6 protein. B. Immunoreactivity of VP6 expressed by a recombinant baculovirus. Sf9 lysates were solubilized and after electrophoresis, polypeptides were transferred to nitrocellulose and reacted with BRV hyperimmune serum. Bound antibodies were visualized by the immuno-alkaline phosphatase-coupled reaction enhanced (BCIP-NBT). Sf9 cells infected with VP6 recombinant baculovirus shows a specific band of VP6 protein at 44KDa.

DISCUSSION

Rotavirus is a major cause of diarrhea in young animals and children worldwide (Estes, 2001). The economic impact of rotavirus infections in animal husbandry and human society has led to extensive studies of rotavirus and the development of rotavirus vaccines. The production of viral proteins in high yields from baculovirus expression system offers new ways to study viral protein function and to develop diagnostic tests and vaccines.

Two vaccination strategies have been developed to control rotavirus infection in young calves. First, the induction of active immunity in young calves by using modified live attenuated or inactivated rotavirus vaccines. However, this strategy was failed under field condition (Saif and Jackwood, 1990). Second, the induction of passive immunization to prevent BRV infection in young calves through immunization of pregnant cows with live attenuated or inactivated adjuvanted rotavirus vaccines to increase the colostrum antibodies (Rousic *et al*., 2000). Under field conditions, the efficiency of the commercial maternal 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).

Recently, a new strategy based on the use of non-replicating rotavirus subunit vaccine, such as virus-like particles (VLPs) of different protein composition that self-assemble in insect cells co-infected with recombinant baculovirus expressing the rotavirus structural proteins has been described. The VLPs are stable noninfectious particles that are morphologically and antigenically similar to the native virus (Labbe *et al*., 1991; Crawford *et al*., 1994; O'Neal *et al*., 1998; Fernandez *et al*., 1998; and Yuan *et al*., 2000). Co-expression of insect cells with VP2 and VP6 recombinant baculoviruses results in the production of double-layered particles (DLPs) 2/6-VLPs, whereas, co-expression of VP2, VP6 and VP7 with or without VP4 results in the production 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). The co-expression of VP6 and VP7 without VP2 results in production of double-shelled VLPs 6/7 (Sabara *et al*., 1991). Recently, rotavirus 2/6 VLPs was produced by cloning the VP2 and VP6 into a single baculovirus transfer vector (Bertolotti-Ciarlet *et al*., 2003). The immunological studies showed that the VLPs inoculated into pregnant animals induced high antibody responses in mammary secretions (Fernandez *et al*., 1998 and Sheoron *et al*., 2000) and the colostrum or milk from vaccinated animals protected calves from diarrhea (Fernandez *et al*., 1998 and Kim et al., 2002b). The VP6 is a target of both G serotype-specific and G cross-reactive cytotoxic T lymphocytes (CTLs) in mice (Offit *et al*., 1991). The Th cells specific for VP6 can provide cognate help to B cell specific for neutralizing epitopes on the VP7 and/or VP4 molecules. Thus, the VP6 is seem to be a good candidate to be used as a vaccine or included in vaccine formulation (Esquivel *et al*., 2000).

In the present study, the RT-PCR product of VP6 was cloned in pBlueBac4.5/V5-His-TOPO® TA expression kit. The pBlueBac4.5/V5-His-TOPO® is a baculovirus transfer vector used for expression of recombinant proteins in insect cell lines. It utilizes the polyhedrin promotor from AcNPV for high-level expression of the cloned gene (Crawford and Miller, 1988). In addition, this transfer vector contains the 5′ portion of the LacZ gene and 3′ portion of ORF1629. Recombination occurs between these sequences and the *LacZ* and ORF1629

sequences in Bac-N-Blue™ DNA 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, which facilitate the selection of recombinant viruses (Vialard *et al*., 1990).

Screening of colonies for identification of the correct orientation of cloned VP6 in baculovirus shuttle vector and plaques to check recombination was carried out by PCR, which represents and 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 VP6 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 VP6 gene yields a fragment of 1356 bp, which is the full length of VP6, plus 54 from vector with a total of 1410 bp (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 1356 bp the size of VP6 gene.

Reactivity of the VP6 baculovirus expressed protein with rotavirus hyperimmune antisera using different serological tests (FAT, ELISA and Western blot) confirmed the expression of VP6 protein in its native conformation and its native immunoreactive determinants, which are conserved. SF9 lysates infected with the VP6-recombinant baculovirus expressed a protein of approximately 44 KDa that had the same mobility in SDS-PAGE stained with Coomassie blue stain as authentic VP6 protein of native rotavirus (Cohen *et al*., 1984). The VP6 expressed protein was detected in the cell lysate and not appeared in significant amounts in the supernatant fraction, even when cell viability dropped below 100% at 96 hr post-infection. This suggests that the recombinant VP6 expressed protein is mainly cell-associated, which agreed with the early observation by Sabara et al. (1991).

Expression of the VP6 and other proteins of other rotavirus strains, particularly non-cultivable strains are particularly beneficial. The possible uses of expressed VP6 to improve current diagnostic tests for rotaviruses are of considerable interest. Future efforts are needed to evaluate the use of expressed VP6 as a subunit vaccine, using animal models that can measure passive and active protection against the virus challenge. Baculovirus expressed VP6 protein could also a useful component of future subunit vaccines containing VP2, VP4 and VP7 proteins of rotaviruses

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ACKNOWLEDGMENTS

This work was supported by the National Academy of Science and Technology as a research project (No. 34) under the title, production of a combined genetically engineered bovine rotavirus/coronavirus subunit vaccine. Professors M.A. Shalaby and H.A. Hussein are the principle and co-principle investigators of the project, respectively.

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).

Banos, D.M.; Lopez, S.; Arias, C.F. and Esquivel, F.R. (1997). Identification of a T-helper cell epitope on the rotavirus VP6 protein. J. Virol., 71:419-426.

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.

Cohen, J.; Lefevre, F.; Estes, M.K. and Bremont, M. (1984). Cloning of bovine rotavirus (RF strain): nucleotide sequence of the gene coding for the major capsid protein. Virol., 138:178- 182.

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. Virol., 62: 2773-2781.

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.

Ericson, B.L.; Graham, D.Y.; Mason, B.B. and Estes, M.K. (1982). Identification, synthesis and modifications of simian rotavirus SA11 polypeptides in infected cells. J. Virol., 42:825-839.

Esquivel, F.R.; Lopez, S.; Guitierrez, X. and Arias, C. (2000). The internal rotavirus protein VP6 primes for an enhanced neutralizing antibody response. Arch. Virol., 145:813-825.

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.

Estes, M.K.; Crawford, S.E.; Penaranda, E.M.; Petrie, B.L.; Burns, J.W.; Chan, W-K.; Ericson, B.; Smith, G.E. and Summers, M.D. (1987). Synthesis and immunogenicity of the rotavirus major capsid antigen using a baculovirus expression system. J. Virol., 61:1488-1494.

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.

Hussein, H A.; 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, H A.; 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.

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. Vaccine,15: 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.

Lucchelli, A.; Kang, S.Y. and Jayasekera, M.K. (1994). A survey of G6 and G10 serotypes of group A bovine rotaviruses from diarrheic beef and dairy calves using monoclonal antibodies in ELIZA. J. Vet. Diagn. Invest., 6:175-181.

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.

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.

O'Neal, C.M.; Clements, J.D.; Estes, M.K. and Conner, M.E. (1998). Rotavirus 2/6 virus-like particles administered intranasally with cholera toxin, *Escherichia coli* heat-labile toxin (LT), and LT-R192G induced protection from rotavirus challenge. J. Virol., 72:3390-3393.

Offit, P.A.; Boyle, D.B.; Both, G.W.; Hill, N.L.; Svodoba, Y.M.; Cunningham, S.L.; Jenkins, R.J. and McCrae, M.A. (1991). Outer capsid glycoprotein VP7 is recognized by cross-reactive, rotavirus specific T lymphocytes. Virology, 184:563-568.

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.

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. and Jackwood, J.J. (1990). Enteric virus vaccines: theoretical considerations,

current status and future approaches. In viral diarrhea of man and animals. CRC Press, Boca Raton.

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.

Sheoron, A.S.; Karzeski, S.S.; Whalen, J.W.; Crisman, M.V.; Powell, D.G. and Timony, J.F. (2000). Prepartum equine rotavirus vaccination inducing strong specific IgG in mammary secretions. Vet. Rec., 146:672-673.

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.

Yuan, L.; Geyer, A.; Hodgins, D.C.; Fan, Z.; Qian, Y.; Chang, K.O.; Crawford, S.E.; Parreno, V.; Waed, L.A.; Estes, M.K.; Conner, M.E. and Saif, L.J. (2000). Intranasal administration of 2-6-rotavirus-like particles with mutant Escherichia coli heat-labile toxin (LT-R192G) induces antibody-secreting cell responses but not protective immunity in gnotobiotic pigs. J. Virol., 74:8843-8853.

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.

الملخص العربي

بناء فيروس مدمج من جنس الباكيلو للتعبير عن بروتين الغلاف الداخلى لفيروس الروتا البقرى

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في هذه الدراسة تم إنتاج و توصيف بروتين مدمج و مهندس وراثيا لفيروس الروتا في الأبقار باستخدام اسلوب التعبير في فيروسات الباكيلو الحشرية. و لتحقيق هذا الهدف تم استخلاص الحمض النووي الريبوزي من الخلايا المصابة بالفيروس، ثم استخدم تفاعل البلمرة المتسلسل المسبوق بتفاعل النسخ العكسى (RT-PCR) لإكثار جين الغلاف الداخلى المراد التعبير عنه ثم إدماجه في بلازميدات حاملة متخصصة للاستخدام مع فيروس الباكيلو. و لانتاج فيروسات مدمجة من جنس الباكيلو تحمل الجين المراد التعبير عنه تم حقن خلايا زرع نسيجي من أصل حشري بالبلازميدات الحاملة للجين مع فيروس الباكيلو غير المهجن في نفس الوقت و تجميع الفيروسات المتكاثرة، و فصل المدمج منها ثم إكثارها حتي تصل إلى الكم الملائم للتقييم. و للتعرف علي الفيروسات المدمجة التي تحمل الجين المقصود تم أستخدم تفاعل البلمرة المتسلسل مرة أخري، ثم استخدمت الفيروسات النقية في إنتاج البروتينات المدمجة بعد إكثارها و معايرتها. استخدمت أساليب متعددة لتوصيف البروتين المدمج الناتج مثل اختبار الفلورسنت المناعي غير المباشر و اختبار الإليزا و اختبار الفصل الكهربى للبروتينات، بالإضافة إلي اختبار اللاطعة الغربية (Western blot) و قد كان البروتين المنتج إيجابيا في جميع الإختبارات المستخدمة مما يثبت فعالية هذا الأسلوب في إنتاج بروتينات مدمجة و مهندسة وراثيا لا تختلف في صفاتها الأنتيجينية عن بروتينات الفيروس الأصلية.