

## Cloning and Expression of Simian Rotavirus Spike Protein (VP4) in Insect Cells by Baculovirus Expression System

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Received 22 January 2008; revised 7 July 2008; accepted 13 July 2008

### ABSTRACT

**Background:** VP4 protein is as spikes on rotavirus outer capsid shell which is responsible for virus attachment to the host. VP4 induces production of neutralizing antibodies which could be used for serotyping of different isolates. **Methods:** Simian rotavirus SA11 gene 4 cDNA was cloned into a cloning plasmid pDONR<sup>TM</sup> by recombination reaction using clonase II enzyme mix. The resulting clone was called *VP4-entry clone*. In the second recombination reaction, cloned gene was inserted into the linear DNA of the Baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV) downstream of the strong polyhedrin promoter. The recombinant AcNPV-VP4 DNA was transfected by lipofection into the insect cell line, *Spodoptera frugiperda* (Sf9) cells. Expression of VP4 in the Sf9 cells was confirmed by the immunofluorescence test using rabbit polyclonal anti-rotavirus and anti-rabbit FTIC-conjugated antibodies by Western immunoblotting technique. The antigenicity of the expressed protein was determined by immunizing rabbits and testing the sera by Western-blotting and neutralization method. **Results:** The cloned VP4 gene was obtained and expressed in baculovirus system. The specificity of the expressed protein was confirmed by its reactivity with anti-rotavirus antibody. Antibody produced against the expressed protein showed neutralizing activity for rotavirus indicating that the protein was biologically active and could induce natural antibody response. **Conclusion:** the expressed protein from rotavirus VP4 gene has a potential for development of rotavirus vaccine. *Iran. Biomed. J. 13 (1): 9-18, 2009*

**Keywords:** Rotavirus, VP4, Cloning, Expression, Baculovirus

### INTRODUCTION

Rotavirus is one of the main cause of severe diarrhea in infants and young children in both developed and developing countries, accounting for 30% to 50% of the illnesses [1]. Because of the significant burden of rotavirus disease among children and animals, considerable efforts have been devoted towards the development of vaccines for control and prevention of the disease [2]. Nevertheless, host susceptibility and immunity to severe rotavirus-induced diarrhea are not fully understood [2, 3].

The outer shell of rotavirus is composed of proteins VP7 and VP4 in molar ratio of 13 to 1. VP7 is a glycoprotein (38 kDa) which specifies the virus

G serotypes. The other protein, VP4, is present as a series of 60 dimeric spikes, 10-12 nm in length, with a knob-like structure at the distal end, which projects outward from the VP7 shell [1, 2, 4]. Simian rotavirus (SA11) spike, VP4 protein has a molecular mass of 88 kDa, constitutes 1.5% of the virus protein, and is encoded by genomic RNA segment 4 and determines the virus P [for protease-sensitive protein] serotype. VP4 has been implicated in several important functions, including attachment to cellular receptors, cell penetration, hemagglutination (HA), virulence, and neutralization [1, 5]. In presence of trypsin, VP4 is cleaved to VP5\* (60 kDa, C-terminal region) and VP8\* (28 kDa, N-terminal region), two virion-associated fragments, resulting in the conversion of non-infectious

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rotavirus to an infectious form [1, 6]. Trypsin-activated virus enters cells rapidly by direct cell membrane penetration [1, 7, 8]. Proteolytic cleavage stabilizes the spike ensemble, confers icosahedral ordering on the VP4, and strongly enhances rotavirus infectivity [9]. Inner capsid is composed of VP6, which makes up approximately 50% of the VP4 and is the major antigenic determinant of group specificity. Core is composed of VP1, VP2 and VP3 that surrounds 11 dsRNA segments inside the core [1].

Neutralization assays can measure reactivity of antibody with the two outer capsid neutralizing antigens VP4 and VP7 independently [1, 10]. Monoclonal antibodies to VP4 (VP8\* or VP5\*) can inhibit virus attachment to cells and neutralize the virus *in vitro* [1, 11, 12]. Also, antibodies directed toward the VP4 or VP7 proteins inhibit HA, and passively protect mice against rotavirus challenge *in vivo* [6, 13, 14]. Studies with animal and human rotavirus have identified eight neutralization epitopes on VP4, with five of them located on the VP8\* region and three sites on VP5\* [1, 15, 16].

The individual contributions of antibodies to VP4 and VP7 to protective immunity have not been fully determined [3, 6]. The importance of the immune response to VP4 was emphasized by Ward *et al.* [17, 18], who have determined that 80% of the serum neutralizing antibodies detected following rotavirus infection of adult volunteers are directed toward the VP4 protein.

A lack of readily available typing serum or monoclonal antibodies to different VP4 types, however, has hampered classification of VP4 (or P) serotypes [1, 10]. The availability of purified VP4 protein would allow the production of serotype-specific polyclonal sera against spike proteins belonging to different P types, which would be useful for typing of human rotaviruses [2, 10, 19].

Baculovirus expression system has several advantages over *E. coli* system such as: high yields of expression, solubility, correctly folding, oligomerization and functional activity of recombinant proteins [20].

Since there is no safe vaccine available in Iran for prevention of rotavirus gastroenteritis; attempt was made to clone VP4 gene of major neutralizing induced antibody of the virus into the baculovirus expression system to obtain good quality of pure protein which, could be used for future vaccine development.

## MATERIALS AND METHODS

**Cell and virus.** African green monkey kidney epithelial cell line (BSC-1 cells) were grown as a monolayer in DMEM (Himedia, India) supplemented with 10% Fetal Bovine Serum (FBS), 2 mM L-glutamine, and 100 U penicillin and 100 µg streptomycin per ml at 37°C. Simian rotavirus SA11 was treated with 5 µg/ml trypsin at room temperature for 60 min to activate the virus. Virus was propagated in serum free medium supplemented with 0.5 µg/ml trypsin. When infected cells showed 80% rotavirus cytopathic effects (CPE), cultured flasks were harvested by freeze-thawing three times and clarified by low speed centrifugation. The virus was concentrated by ultracentrifugation on 40% sucrose cushion, at 100,000 × g for 2 h (CENTRIKON T-1180, Rotor: TST 28.38/17, R = 20 cm). Viral RNA was extracted from the virus pellet using RNAfast RNA extraction reagent. Extracted RNA was electrophoresed on 10% polyacrylamide or on 1% agarose and bands were identified by staining with ethidium bromide.

**Primer design.** Oligonucleotide primers specific for genome segment no. 4, were designed according to rotavirus SA11 sequence data (accession number: D16345) [21]. To generate *att* (attachment sites) flanked VP4 PCR product suitable for use as substrate in Gateway<sup>®</sup> BP recombination reaction with donor vector (pDONR<sup>TM</sup>221) (Invitrogen, USA), incorporation of *attB* sites into PCR product was necessary [22, 23]. In forward primer, four G residue at the 5' end were followed by a 25 bp *attB1* site and by gene-specific sequences (nucleotide no. 10 to 29). For reverse primer, four G residues at the 5' end followed by a 25-bp *attB2* site and by gene-specific sequences (nucleotide no. 2316 to 2338) were designed. Stop codon was removed from VP4 gene in the reverse primer for in frame C-terminal 6 × His epitope tagging to our recombinant gene in the BaculoDirect<sup>TM</sup> Linear DNA (Invitrogen, USA). Primers were 51 and 52 nucleotides long with following sequence. *AttB1* and *attB2* sites are bolded.

VP4-F1: 5'GGGG-ACAAGTTTGTACAAAAAAGCAGGCT-**ATATGGCTTCGCTCATTATAG**-3'

VP4-R11: 5'GGGG-ACCACTTTGTACAAGAAAGCTGGGT-**ACAACCTGCATTGCATAATCAG**-3'

**cDNA synthesis.** Rotavirus dsRNA was used as template to synthesize cDNA copies from the both viral RNA strands. Reverse transcription was carried

out using a mixture of 10  $\mu$ l dsRNA template, and 1  $\mu$ l of 100  $\mu$ M of each of the primers. The reaction tubes were heated at 95°C for 5 min and quickly cooled at 4°C, then the reverse transcription mixture consisting of 5 $\times$  incubation buffer, 1  $\mu$ l of 2.5 mM dNTP mix (TAKARA, Japan), 20 U/ $\mu$ l of RNase inhibitor (Fermentas, Germany) and 40 U of *M-MuLVirus reverse transcriptase* (Roche Diagnostic, Germany) were added in total volume of 20  $\mu$ l. The tubes were incubated in a thermocycler at 37°C for 60 minutes.

**PCR amplification.** PCR reaction was carried out in total volume of 50 $\mu$ l containing: 5  $\mu$ l of cDNA synthesis mixture, 5  $\mu$ l of 10 $\times$  pfu PCR buffer with MgSO<sub>4</sub>, 1  $\mu$ l of 20  $\mu$ M of primers, 4  $\mu$ l of 10 mM dNTP mix, and 2.5 U of *pfu DNA polymerase* (Fermentas, Germany). Thermal cycler was programmed with an initial denaturation at 95°C for 5 min followed by 30 cycles: 95°C for 30sec, 60°C for 30sec, 72°C for 2 min; and final extension time of 10 min at 72°C and final hold at 4°C. PCR product (called *VP4-attB-PCR* product) was electrophoresed in 1% agarose gel, bands were identified primarily by restriction enzyme NdeI (Fermentas, Germany) digestion then purified by 30% polyethylene glycol 8000 (Fluka, Germany) precipitation for removing primers and primer-dimers.

**Cloning.** The VP4 gene flanked by *attB1* and *attB2* sites was transferred from *VP4-attB-PCR* product to an attP-containing vector (pDONR<sup>TM</sup>221) by BP recombination reaction, that creates an entry clone [22]. The following components were added to a 1.5-ml tube: 7  $\mu$ l *VP4-attB-PCR* product (50 fmol), 1  $\mu$ l pDONR221 vector (50 fmol) and 2  $\mu$ l BP clonase<sup>TM</sup> II enzyme mix (*lambda* phage and *E. coli* recombination factors and enzymes) (Invitrogen, USA). The reaction mix was incubated overnight at room temperature then, 1  $\mu$ l of 2  $\mu$ g/ $\mu$ l proteinase K solution (Invitrogen, USA) was added and incubated at 37°C for further 10 min [22]. The classical CaCl<sub>2</sub> procedure was used to prepare chemically competent cells [24]. Transformation of competent *E. coli* cells (One Shot<sup>®</sup> OmniMAX<sup>TM</sup> 2-T1) (Invitrogen, USA) was performed as Mandel & Higa methods [24]. Colonies were cultured in LB broth, containing 50  $\mu$ g/ml kanamycin. Plasmid was extracted and screened for the desired clone by EcoRI and Bgl II (Fermentas, Germany) restriction enzymes. The resulting clone was named *VP4-entry clone* and

sequenced in an automated ABI 3130XL genetic analyzer using universal M13F and M13R primers. The results were analyzed by BLAST software.

**Recombinant baculovirus construction.** The construction and characterization of the recombinant baculovirus DNA containing recombinant VP4 gene were done according to manufacturer's instructions [23]. Briefly, the recombination reaction was performed to transfer the *attL1* and *attL2* flanked VP4 gene from *VP4-entry-clone* into the attR containing BaculoDirect<sup>TM</sup> Linear DNA by LR Clonase<sup>TM</sup> II enzyme mix (*lambda* phage and *E. coli* recombination factors and enzymes) [22, 23]. The resulting recombinant baculovirus DNA was transferred into *Spodoptera frugiperda* (Sf9) cells by lipofection.

**Sf9 cell culture.** Sf9 cells were grown as monolayer in Grace's Insect Medium (Gibco, Canada) with supplements (lactalbumin hydrolysate, L-glutamine, and yeast extract) and 10% FBS. The cells were seeded 8  $\times$  10<sup>5</sup> per well in 2 ml of complete Grace's Insect Medium in a six-well plate and incubated at 28°C for one hour [25].

**Transfecting Sf9 cells.** Transfecting the LR recombination reaction into Sf9 cells was as follow: transfection mixture A: 5  $\mu$ l LR recombination reaction, and 100  $\mu$ l Grace's Insect Medium, transfection mixture B: 6  $\mu$ l Cellfectin reagent (Invitrogen, USA) and 100  $\mu$ l of Grace's Insect Medium. The mixtures A and B were combined and incubated at room temperature for 45 min. Then, 800  $\mu$ l of Grace's insect medium was added to the mixture. The entire transfection mixture was added to the cells and incubated at 28°C for 5 h. The transfection mixture was then removed and 2 ml of complete growth medium containing 100  $\mu$ M ganciclovir was added to each well. For generating non-recombinant baculovirus as negative control, transfected Sf9 cells by BaculoDirect<sup>TM</sup> Linear DNA alone and Sf9 cells cultured in the absence of ganciclovir were used. At late stage of infection (4-5 days post infection), the P1 virus was harvested from the cell culture medium by centrifuging at 1000  $\times$ g for 5 min. The supernatant was transferred to a 15-ml fresh tube as P1 baculovirus stock [23]. The P1 viral stocks that determined by plaque assay.

**Analyzing recombinant baculovirus DNA for VP4 gene.** To verify the presence of VP4 gene in baculovirus, viral DNA was extracted from the

supernatant of infected Sf9 cells using 20% PEG 8000 (Fluka, Germany) in 1 M NaCl, 0.1% Triton X-100, proteinase K, followed by phenol:chloroform: isoamyl alcohol extraction (25:25:1). DNA was precipitated by sodium acetate, ethanol, and centrifugation. The sediment was dissolved in 10  $\mu$ l sterile water and used in PCR [23].

**Immunofluorescence tests.** Infected and uninfected Sf9 cells were washed twice in PBS then, placed on a slide and fixed in acetone at  $-20^{\circ}\text{C}$  for 10 min. Expression of recombinant VP4 protein was verified by indirect immunofluorescence test using rabbit polyclonal anti-rotavirus antibody, and anti-rabbit FITC-conjugate antibody.

**SDS-PAGE.** Protein electrophoresis was carried out by the method of Laemmli [26], using 10% separating and 5% stacking gels. Briefly, samples of Sf9 cells infected by nuclear polyhedrosis virus *Autographa californica* nuclear polyhedrosis virus (AcNPV)-VP4 expressing VP4, AcNPV and uninfected cells and cells supernatant were lysed in SDS-PAGE sample buffer and boiled at  $95^{\circ}\text{C}$  for 5 min. Prepared samples subjected to electrophoresis and gels were stained with Coomassie blue.

**Western immuno-blotting.** Briefly, SDS-PAGE resolved protein bands were transferred to nitrocellulose or polyvinylidene fluoride (PVDF) membranes using liquid transfer system. Membranes were soaked in 5% skim milk, to block the non-specific sites and were incubated with 1/500 dilution of rabbit anti-rotavirus antibody at room temperature for 1.5 h. After washing, the bound antibody was detected by 1/1000 dilution of polyclonal HRP-conjugated goat anti-rabbit antibody (KOMA, Korea) at room temperature for 1.5 h. Color development was observed by addition of 4-Chloro-1-naphtol in methanol as substrate [27]. Because the VP4 was a protein with C-terminal 6 $\times$ His tag, mouse monoclonal anti-6 $\times$ His tag and anti-mouse HRP-conjugated antibodies (Roche, Germany) were used in Western-blotting.

**Time points for maximal expression.** To optimize time points for maximal expression of VP4 by recombinant baculovirus, Sf9 cells in a six-well plate were infected at a multiplicity of infection about: 5 pfu/cell and were assayed by SDS-PAGE for recombinant VP4 protein expression at different days post infection. The gels were stained by Coomassie brilliant blue and were scanned by a densitometry scanner (Pharmacia LKB

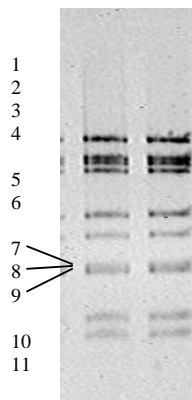
Ultrascan XL, USA).

**Immunization of rabbits.** Rabbits were immunized by injecting 0.5 ml of cell extracts containing 250  $\mu$ g of VP4 protein mixed with equal volumes of complete Frond's adjuvant. Rabbits were injected 3 times at weekly intervals and were bled 10 days later. Pre-immunized serum was taken as control and potency of serum was tested against VP4 by dot-blot and neutralization experiments. Partially, purified SA11 rotavirus was blotted on PVDF membrane. Non-specific sites were blocked by incubation for 2 h by 5% skim milk prepared in western washing buffer. Anti-rotavirus SA11 antiserum was used as positive control and pre-immune serum was used as negative control. Unbound antibodies were washed three times in washing buffer. Bound antibody was detected by incubation in a 1:1000 dilution of HRP-conjugated goat anti-rabbit antibody diluted in washing buffer. Following additional washing steps, the membranes were developed by transferring to 0.06% 4-chloro-1-naphtol prepared in washing buffer-methanol (4:1; vol/vol) containing 5  $\mu$ M  $\text{H}_2\text{O}_2$ .

**Neutralization test.** Neutralizing effect of antiserum produced in rabbits against recombinant VP4 was determined on BSC-1 cells in a microplate. Serial dilutions of heat-inactivated antisera were incubated with 100 TCID<sub>50</sub> of SA11 rotavirus at  $37^{\circ}\text{C}$  for 1 h. Then, inoculated into BSC-1 cells in microplate and incubated for 1 h. DMEM (without serum) was added to the wells and incubated at  $37^{\circ}\text{C}$ . Final dilution of antiserum, that could prevent CPE development, was considered as neutralizing titer. Pre-immune serum was used as negative control.

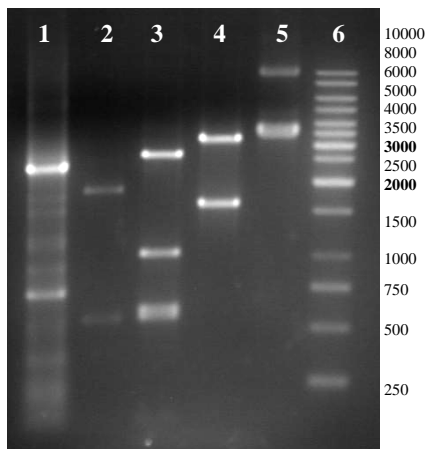
## RESULTS

The study was initiated by culturing SA11 rotavirus and extracting its dsRNA segments from partially purified virus (Fig. 1). The cDNA of VP4 was synthesized and amplified by RT-PCR from viral RNA. The gene specific sequences of forward and reverse primers were designed such as, 5' and 3' consensus sequences were not included in cDNA, but added *attB1* and *attB2* sites to 5' ends of forward and reverse primers respectively. Thus, attB-flanked VP4 PCR product had a total length of 2386 nucleotides (Fig. 2, lane 1).



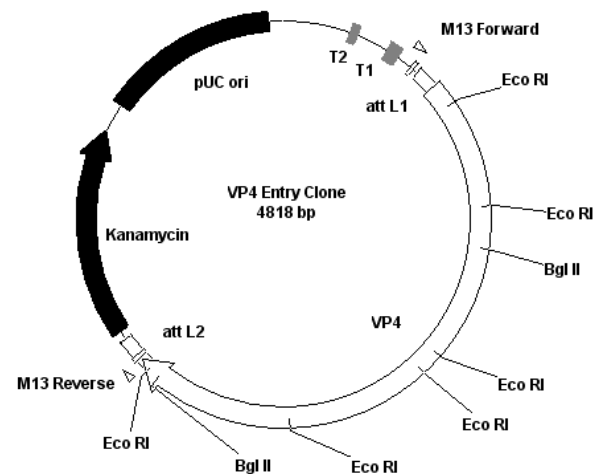
**Fig. 1.** Electropherogram of simian rotavirus RNA segments. one percent agarose gel showing 11 segments of dsRNAs extracted from partially purified, SA11 rotavirus pellet and stained with ethidium bromide.

**Cloning.** A complete sequence of SA11 rotavirus VP4 ORF was cloned into baculovirus entry vector (pDONR™ 221) by recombination reaction and resulting clone (*VP4-entry clone*) (Fig. 3) was confirmed by restriction digestion enzymes (Fig. 2). Sequencing of the product showed that it had about 100% homology with SA11 rotavirus genome segment 4. Sequencing result confirms highly conserved nucleotide sequence of VP4 gene after long and continuous passage of SA11 rotavirus in our laboratory.



**Fig. 2.** One percent of agarose gel electrophoresis of DNA fragments. Lane 1, RT-PCR product (VP4-attB-PCR product), 2386 bp; lane 2, VP4-attB-PCR product digested by restriction enzyme, Nde I; because this product has one site for this enzyme at nucleotide no 559, therefore two segments: 1827 bp, and 559 bp resulted; lane 3, VP4-entry clone digested by EcoRI, five DNA fragments resulted, segments no 3 and 4 are close together and segment 84 bp appeared very faint; lane 4, VP4-entry clone digested by Bgl II, because this enzyme have two RE sites, two pieces of 1608 and 3210 bp fragments were resulted; lane 5, VP4-entry clone undigested, lane 6, 1 kb molecular weight DNA markers.

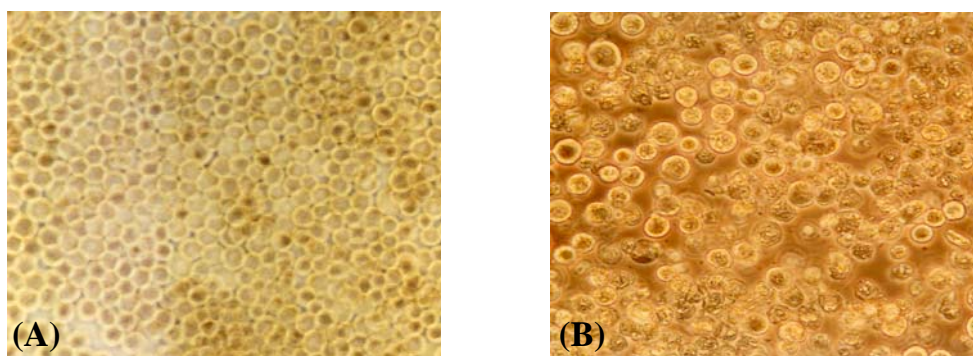
**Recombinant baculovirus construction.** VP4 gene was transferred from *VP4-entry clone* into the Gateway® cassette of baculovirus™ linear DNA under the control of the polyhedrin promoter by LR recombination reaction. Thus, HSV1 tk and lacZ genes were deleted from BaculoDirect™ Linear DNA as by-products. The resulting recombinant baculovirus DNA (AcNPV-VP4) was transferred into Sf9 cells by lipofection. In presence of ganciclovir, the non-recombinant baculovirus DNA (AcNPV) or by-products were omitted from the transfected cells [23]. Figure 4 shows CPE of baculovirus in comparison to uninfected Sf9 cell.



**Fig. 3.** Map of VP4-entry clone.

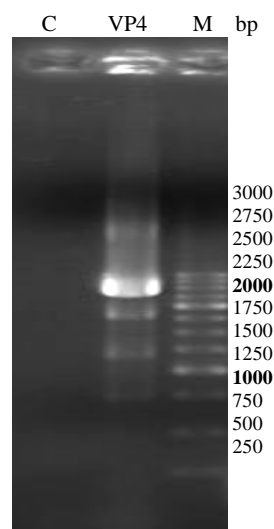
New construct (AcNPV-VP4) was verified by PCR after viral DNA extraction using specific primers for VP4 gene. Figure 5 shows PCR product of VP4 gene cloned into the baculovirus. Gene 4 insert contains an intact translation start site and lacks the stop codon, but has in frame C-terminal V5 epitope and 6×His tag.

**Expression of VP4 protein.** Recombinant baculovirus was screened for VP4 expression in the cytoplasm of infected Sf9 cells by indirect immunofluorescent technique using SA11 rotavirus specific antibody (Fig. 6). As shown in Figure 6, the cytoplasm of Sf9 cells shows staining of the entire cytoplasm. Expression of VP4 protein was increasingly evident in day 3 days post infection by SDS-PAGE (Fig. 7). The protein band, shown by Coomassie blue staining, was confirmed by immuno-blotting using polyclonal antibody to SA11 rotavirus (Fig. 8). It appeared that VP4 to be

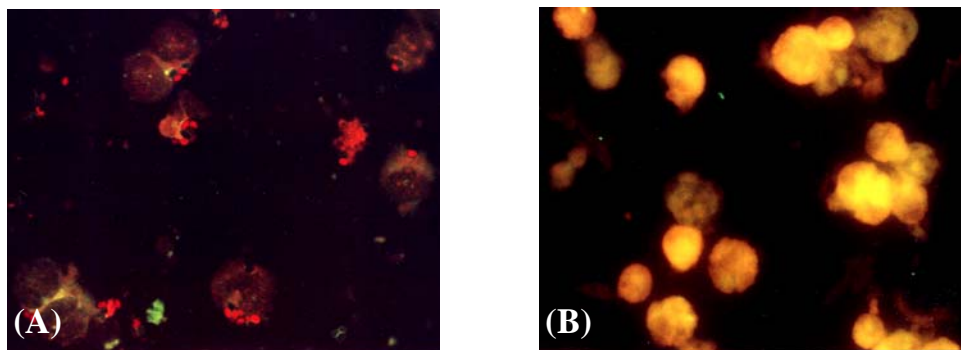


**Fig. 4.** Comparison of uninfected and infected Sf9 cell monolayers. Sf9 cells uninfected (A) and CPE of infected Sf9 cells with recombinant VP4 baculovirus (B).

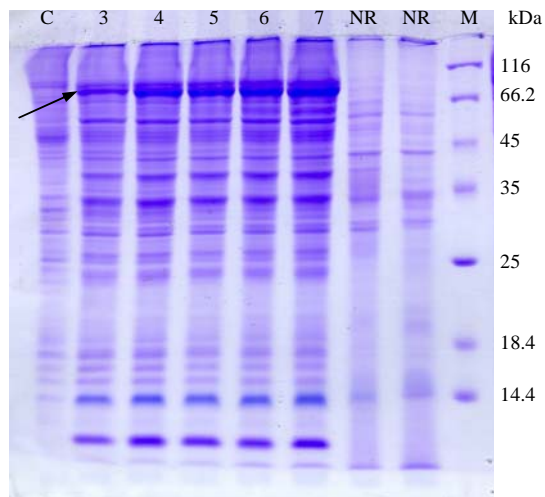
localized within the cells, because VP4 protein was not determined extracellularly by SDS-PAGE (data not shown). A densitometry scan of Coomassie blue stained SDS-PAGE showed that VP4 found in cells increased with the time. Accumulation of VP4 increased to 9.21% of total protein of infected Sf9 cells in day six and then decreased 8.39% in day seven (Fig. 7). Considering the size of VP4 (88 kDa) and in frame V5 epitope and  $6 \times$  His tag (4 kDa) in baculovirus DNA, in sum recombinant VP4 protein would be 92 kDa. Yields of VP4 were greatest when cells were grown in complete Grace's Insect Media containing 10% FBS. Figure 8 shows Immuno-reactivity of SA11 VP4 expressed in the cytoplasm of Sf9 cells. Western immuno-blotting of recombinant VP4 protein was expressed in Sf9 cells by baculovirus using mouse anti-6 $\times$ His tag and anti-mouse HRP-conjugated antibodies also showed a 92-kDa protein (data not shown).



**Fig. 5.** PCR screening of recombinant baculovirus. One percent of agarose gel electrophoresis showing PCR product of VP4 gene (2386 bp) cloned into the baculovirus, using VP4-F1 and VP-R11 primers and baculovirus extracted DNA as template. C, non-recombinant baculovirus DNA as negative control; M, molecular weight DNA markers.



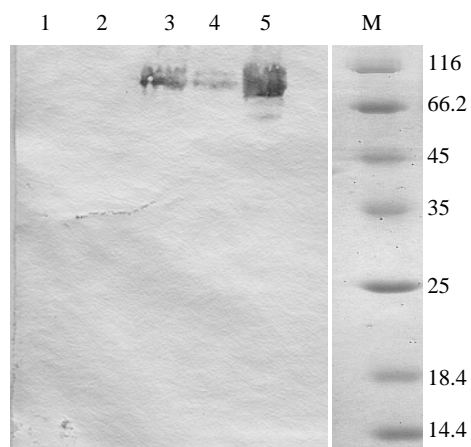
**Fig. 6.** Expression of rotavirus VP4 protein by recombinant baculovirus in Sf9 cells detected by indirect immunofluorescence. Uninfected Sf9 cells (A) and Sf9 cells infected by recombinant baculovirus expressing VP4 (B) after staining with rabbit anti-rotavirus SA11 polyclonal and anti-rabbit FITC-conjugated antibodies. Cells were examined and photographed under UV microscope at a magnification of 400 $\times$ .



**Fig. 7.** Kinetics of VP4 synthesis in Sf9 cells by Coomassie blue staining of 10% SDS-PAGE. The proteins synthesized in un-infected Sf9 as negative control (C), non-recombinant (NR) or recombinant VP4 gene baculovirus at indicated times (3-7 days post infection). VP4 is 92kDa protein (arrow). Molecular weight protein markers (M).

#### **Immune response against recombinant VP4.**

Soluble fraction of AcNPV-VP4 infected Sf9 cells were injected into rabbits and the resulting antibody titer to recombinant VP4 were measured by reactivity to SA11 rotavirus particles in a dot- and Western-blot assay. Rabbit antiserum after final step of inoculation could recognize rotavirus in dot-blot



**Fig. 8.** Immuno-reactivity of SA11 VP4 expressed in Sf9 cells. Western immuno-blotting carried out by using rabbit anti-rotavirus serum and HRP-conjugated goat anti-rabbit antibodies. recombinant VP4 (92 kDa) expressed in Sf9 cells by baculovirus in various passages (lane 3-5); uninfected Sf9 cell proteins as negative control (lane 1), and Sf9 cells infected by non-recombinant baculovirus proteins (lane 2) resolved by 10% SDS-PAGE and transferred on nitrocellulose membrane and molecular weight protein markers are shown at right, kDa (M).

in titer of 400 and native VP4 protein band (88 kDa) in Western- immune blotting test. In contrast pre-immune serum could not recognize any rotavirus proteins (data not shown). This result indicates that induced antibody functions specifically. We tried to measure neutralizing antibodies produced against recombinant VP4 protein. Antiserum against baculovirus-infected cells containing recombinant VP4 reduced rotavirus infectivity with a neutralizing titer of approximately 320. By contrast pre-immune serum from inoculated rabbits failed to elicit a neutralizing response.

## **DISCUSSION**

A recombinant baculovirus containing a full-length VP4 ORF, that expressed VP4 protein, was constructed. The expressed polypeptide maintained viral antigenic sites in the absence of other rotavirus outer or inner capsid components. This indicates that the expressed VP4 protein is antigenically highly similar to VP4 on the rotavirus outer capsid [6, 10, 28]. Expression and antigenicity of VP4 protein were detected by immunofluorescence, Coomassie blue staining of SDS-PAGE, and Western immunoblot analysis. However, it has been shown that in group A strains of rotavirus, VP4 protein is antigenically, functionally, and immunologically intact when expressed in a recombinant baculovirus. Also, the immunization with the expressed VP4 provides passive protection to a rotavirus challenge in an animal model [6, 14, 29]. The expressed VP4 protein in this study could be similarly used to develop assays for viral functions, neutralization and protection.

Analysis of the supernatant and pellet of infected Sf9 cells by SDS-PAGE revealed that the expressed VP4 protein remained cell associate and no detectible VP4 protein was found in the supernatant. This is compatible with the finding of other investigators [30]. A densitometry scan of a Coomassie blue stained SDS-PAGE gel indicated that 9.21% of total cellular protein was expressed as a 92-kDa VP4 protein on day 6<sup>th</sup> post infection when compared with a 5% expression level for the rhesus rotavirus VP4 protein reported by Mackow *et al.* [6]. Because of variable expression levels of foreign gene by recombinant AcNPV, the 9.21% expression level is remarkable.

It has been reported that VP2 and VP6 accumulated in both Sf9 and H5 cells were cultured in Excell medium in spinner flasks, from 3 to 5 days

post infection then, reached a plateau. However, the accumulation of VP7 in Sf9 cultures increased till 4 days post infection and decreased thereafter, whereas the maximal accumulation of VP7 in H5 cultures occurred at 4 or 5 days post infection and was sustained till 7 days [30]. Estes *et al.* [31] reported that SA11 inner capsid protein, cell associated VP6 was produced in infected Sf9 cells to the amount of about 20 to 150  $\mu\text{g}$  per  $10^6$  cells and the amount of VP6 found in the medium increased with time.

In a study, polyhydrin protein was expressed at a level of 15% of total cellular protein [6]. Because we did not have polyhydrin gene in wild type baculovirus control, VP4 expression level to native polyhydrin protein (29 kDa) could not be compared. Yields of VP4 were greatest when cells were grown in complete media containing 10% FBS. That is compatible with the result of VP6 expression in Sf9 cells by Estes *et al.* [31]. Several studies have shown that VP4 cross reacts among other rotaviruses in the same group [32-34]. The baculovirus expressed VP4 protein may be useful for studying immune responses to a single rotavirus outer capsid polypeptide [10, 35] which may be useful for production of virus like particles as a synthetic rotavirus vaccine [36]. The limited amount of VP4 in the native viral particles or produced during replication also makes it difficult to obtain enough VP4 for structural and functional analysis by virus propagation and purification from infected cell cultures [10].

To determine the immunogenicity of the baculovirus expressed VP4, rabbits were immunized with the VP4 lysate as described in Methods. The ability of rabbit antiserum to recognize VP4 in intact SA11 was investigated by dot-blotting and Western-blotting. The rabbit's sera showed a dot-blot titer of 400 against SA11 rotavirus. Pre-immune sera did not possess antibody. Sera from the immunized rabbits recognized VP4 in Western-blot using partially purified SA11 rotavirus, but not other SA11 proteins indicating that the produced antibody activity was specific. The result of neutralization test indicated that neutralizing epitopes of VP4 were retained. Monoclonal antibodies that recognize six discrete neutralization epitopes on rhesus rotavirus VP4, all bind to the baculovirus expressed VP4 protein in ELISA. These neutralization sites are involved in both heterotypic and homotypic neutralization and are located in the VP8\* and VP5\* portions of VP4 protein. Antibodies directed at regions on both VP5\* and VP8\* are capable of

mediating protective immunity [14, 37]. Expressed VP4 protein was also recognized by hyperimmune anti-rhesus rotavirus serum on Western-blot and neutralizing monoclonal antibodies under reduced and non-reduced conditions, suggesting that the VP4 neutralization epitopes are not conformationally determined and conserved on the expressed VP4 protein. Thus, the baculovirus-expressed VP4 protein may be useful for studying immune responses to a single rotavirus outer capsid polypeptide and as a synthetic rotavirus vaccine. Previous studies showed that neutralizing antibodies against VP4 protein were important for protection of animals from rotavirus diseases [14, 38, 39]. Immuno-dominant responses against natural rotavirus infections of adults are neutralizing antibodies [17].

To date the expression of the complete VP4 protein in *E. coli* has not been successful, though VP8\* and VP5\* have been expressed as fusion proteins in insoluble forms [2, 15, 40]. Kovacs-Nolan *et al.* [41] reported a system for the expression and purification of soluble VP8\*, as a fusion protein with glutathione S-transferase. The reasons for our low titer neutralizing antibody in comparison to the study reported could be due to the low amount of protein used in immunization.

In our study, it was found that using baculovirus expression system, the VP4 protein was not a fusion protein and the yield of expressed protein was satisfactory.

## ACKNOWLEDGMENTS

We would like to thank Iran University of Medical Sciences for their financial support and National Research Center of Genetic Engineering and Biotechnology for their technical assistance.

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