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EXPRESSION OF NONSTRUCTURAL GLYCOPROTEIN NSP4 OF SA11 SIMIAN ROTAVIRUS IN *ESCHERICHIA COLI* AND PRODUCTION OF ANTIBODY AGAINST IT

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ABSTRACT

Rotavirus nonstructural glycoprotein NSP4 can induce diarrhea in newborn mice. It has been suggested that NSP4 may be a key determinant for rotavirus pathogenesis and a target for vaccine development. In order to study the biological and morphological role of NSP4 a large amount of the purified protein and antibody against it are required.

Simian rotavirus SA11 was propagated in BSC1 cell, purified on cesium chloride gradients, and its genomic RNA was extracted. A cDNA from RNA segment 10 was synthesized and amplified by RT-PCR. cDNA fragment was cloned into plasmid vectors. The recombinant plasmid was characterized by restriction enzyme and sequencing. Construction of expression plasmid containing nsp4 gene was performed and expression of NSP4 was demonstrated by SDS-PAGE, Western blot, ELISA and IF using polyclonal antibody against NSP4 from SA11 infected BSC1 cells. A polyclonal antiserum against purified recombinant NSP4 was raised in rabbit; which was reacted with NSP4 in BSC1 cells infected with SA11 rotavirus.

These results indicated successful expression of the full-length NSP4 in *E.coli* to produce antibody against it and to study its biological activities.

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Keywords: Expression, NSP4, Rotavirus, *E.coli.*

INTRODUCTION

Rotaviruses are major pathogens causing life-threatening dehydrating gastroenteritis in children and young animals.[1-3]

Rotaviruses are members of the Reoviridae family and contain 11 double-stranded RNA segments inside a100 nm triple-layered protein capsid.[4,5] The genome codes for six structural and six nonstructural proteins.[1,4] The nonstructural protein NSP4, which is encoded by gene segment 10 for most strains, is a glycoprotein an-

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choring the endoplasmic reticulum membrane.[6]

It has been demonstrated that NSP4 serves as an intracellular receptor for double-layered rotavirus particles and interacts with viral capsid proteins during viral morphogenesis.[7,8,9] Both et al. (1983) cloned SA11 rotavirus gene 10 segment into pBR322 and determined its nucleotide and encoded protein sequences,[10,11-12] Taylor et al. (1996) and Horie et al. (1999) expressed NSP4 as fusion with glutathione-S-transferase (GST) in *E. coli*.[13,14]

Au et al. (1989) reported expression of NSP4 in spodoptera frugierda cell using a recombinant baculovirus.[7] Expression of NSP4 in insect cells triggers the release of calcium from the ER.[8]

NSP4 has been proposed as the first viral enterotoxin capable of inducing diarrhea in young mice.[5,14] NSP4 protein consists of 175 amino acids, and its toxic effect has been attributed to the carboxy-terminal half of the protein.[9,12] The 114-135 peptide ($NSP4_{114-135}$) was reported to be capable of inducing a calcium-mediated increase in chloride secretion by intestinal epithelial cells as well as diarrhea in suckling but not adult mice.[15]

Antibodies against NSP4 might diminish the frequency and severity of diarrhea.[16] Increasing evidence indicates that this enterotoxin activates a signal transduction pathway that increases the intracellular calcium level by mobilizing calcium from the ER and ultimately resulting in chloride secretion.[17-19] Despite extensive studies of different animal models, rotavirus pathogenesis is still not completely understood.[20]

Since the mechanisms by which rotavirus and NSP4 cause diarrhea are not completely understood, investigating possible, alternative mechanisms might prove useful.[21,22] To study the biological and morphological effects of NSP4, purified protein and antibody against it would be necessary.

In this study an attempt was made to clone and express the NSP4 protein to use it for a better understanding of its role in inducing gastroenteritis.

MATERIAL AND METHODS

Propagation of cells

Monkey kidney cell line, BSC1, was grown in Dulbecco's Modified Eagle's medium (DMEM) (GIBCO BRL) supplemented with 10% fetal calf serum (FCS, GIBCO BRL) at 37°C in a 5% CO_2 controlled atmosphere. The cells were infected with simian rotavirus SA11 in presence of 5 µg/mL trypsin and titrated by TCID50 method.[23]

Purification of SA11 rotavirus

Virus particles from infected BSC1 cells were layered over 40% sucrose cushion and centrifuged at 22000 rpm for 2.5 hrs in a Kontron type TST28.38 rotor followed by ultracentrifugation on a linear gradient of 1.2 g/mL and 1.4 g/mL CsCl at 25000 rpm for 3 hrs in a Kontron type TST 55.5 rotor.[24]

Extraction of viral RNA

Genomic RNA was extracted with phenol-chloroform from purified virions.[25] Extracted RNA was electrophoresed on polyacrylamid gel according to the Laemmli method, and the gel was stained by silver nitrate.[26]

Reverse transcription-polymerase chain reaction (RT-PCR)

Extracted RNA was used as template to synthesize cDNA in RT-PCR mixture that included 2µL of 10X reaction buffer, 2µL of 50Mm $MgCl_2$, 2µL of 10mM deoxynucleoside triphosphates, and 0.5µL of each 38 µM nsp4 gene-specific 3'-terminal primer (End G10: 5'GGTCACATTAAGACCATTCC 3') and the 5'-terminal primer (Beg G10: 5' GGCTTTTAAAAGTTCTGTTC 3').[27,28] The reaction mixture was heated for 15 min at 65°C, cooled on ice for 5 min, and then 0.8 µL avian myeloblastosis virus (AMV) reverse transcriptase (20 unit) was added to the mixture and reverse transcription was carried out for 1h at 42°C.

Cloning of nsp4 gene

The cDNA of the SA11 gene 10 was amplified by PCR with the same 3' and 5' terminal primers for 30 cycles of denaturation for 1 min at 95°C, annealing for 30 sec at 56°C, and extension for 1 min at 72°C. Cloning vector pBluescript-KS (+) and the expression vector pET-26a (+) (Novagen) was used for cloning and expression of recombinant protein. For maintenance of the plasmid, ampicillin (100µg/mL) and kanamycin (30µg/mL) was added to the culture medium.

DNA isolation, manipulation and transformation were carried out as described by Sambrook et al.[29]

The plasmid pBS-KS (+) DNA was digested with EcoRV and the amplified DNA was ligated into it. The recombinant plasmid was transformed into *E.coli* TG1 strain using $CaCl_2$ treatment procedure. Competent cells and plasmid DNA were mixed and incubated on ice for 30 min. The reaction mixture was heated at 42°C for 1.5min, then transferred on ice for 3 min and then cultured on LB-Agar containing Xgal and IPTG.

White bacterial colonies were screened for the presence of gene 10 DNA by PCR using specific primers. Cloning of the *nsp*4 gene was confirmed by restriction enzymes analysis and sequencing of the selected clones. DNA sequencing was performed by the dideoxynucleotide chain termination method using automated DNA sequencer Applied Biosystem.

Expression of the NSP4 protein

The *nsp*4 gene DNA was excised from the cloning

vector as a BamHI fragment and cloned into the *Bam*HI site of pET-26a (+), downstream of the pel B signal sequence as in frame fusion under the control of T7/lac promoter-operator.

The recombinant plasmid was transformed into *E.coli* BL21 (DE3) and was verified by PCR and restriction enzyme digestion. Overnight cultures of *E.coli* strain BL21 (DE3), harboring either the pET-26a (+) or the pET-26a (+) carring *nsp*4, were diluted 1:20 into fresh Luria-Bertain (L.B.) medium containing kanamycin (30 μg/mL) and grown to an OD 600 of 0.5-0.7. Gene expression was induced by addition of isopropylthio-β-D-galactoside (IPTG) to 1mM final concentration and six hours further incubation.

SDS-PAGE and Western blot analysis of NSP4 protein

Expression of the NSP4 was monitored by SDS-PAGE and Western blot analysis.Whole cell lysate and the periplasmic fluid of *E.coli* expressing NSP4 were mixed with sample buffer and boiled for 5 min. Samples were electrophoresed on 13.5 % SDS-polyacrylamide gel and stained with coomassie brilliant blue G-250.

For Western blot, electrophoresed proteins were transferred to a nitrocellulose membrane in a transfer buffer (25mM Tris, 192 mM glycine, and 20% methanol) at 86 mA overnight and probed with a polyclonal antiserum prepared against SA11 infected cell lysate and recombinant NSP4. The NSP4 protein-antibody complexes were treated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin and visualized using a solution of 4-chloro-1-naphthol with hydrogen peroxidase as enzyme substrate.

Enzyme-Linked Immunosorbent Assay

ELISA test was developed to detect the NSP4 protein in bacterial lysates. Bacterial cultures were grown and induced with IPTG. The cells were then suspended in lysis buffer and sonicated (3 times, 3sec). Polystyrene 96-well plates were coated with 100μL of bacterial lysate at a predetermined concentration in coating buffer and incubated overnight at 4°C; free protein binding sites were blocked by incubation with 200μL of 1% BSA in TBST (Tris-Buffer-Saline-Tween 20) for 1h at 37°C.

The primary antibody (1:200 dilution of the rabbit-antiserum) against rotavirus infected cell lysate-which was previously absorbed with uninfected cell lysate- was added for 1h at 37°C. After 3 times of washing with buffer, the secondary antibody (1:2000 dilution of horseradish peroxidase conjugated goat anti-rabbit Ab obtained from DAKO) was added and incubated for 1h at 37°C. After 5 times of washing with buffer, tetramethylbenzidine(TMB), H_2O_2 was added as substrate, the reaction was stopped with 1.25 M H_2SO_4 and the absorbance was measured at 450 nm.

Simian rotavirus-infected BSC1 cells were recovered at 8 hours post-infection (hpi) by scraping, washed twice in phosphate buffered saline (PBS) and lysed in lysis buffer (10 mM Tris containing 2% sodium salt of deoxycholic acid pH 7.4). Proteins in the cell lysates was tested by ELISA using rabbit antiserum against purified recombinant NSP4 protein.

Production of antibody against NSP4

A polyclonal antiserum against SA11 infected BSC1 cell lysate was raised in New Zealand white rabbit.[7,30] The reactivity of the polyclonal antibody with viral protein including NSP4 in SA11 infected cells was confirmed by the immunofluorescent test. The whole cell lysate of the bacteria was separated by preparative SDS-PAGE. The area containing recombinant NSP4 was excised from the gel and then eluted into 0.05 M Tris-HCl buffer.[31] Protein concentration was determined according to the method of Lowry.[32] A polyclonal antiserum against the purified recombinant NSP4 was prepared in N.Z.white rabbits.[31] The first inoculum of 100 μg protein was emulsified in Freund's complete adjuvant (Gibco); all subsequent inoculations were prepared in Freund's incomplete adjuvant. Rabbits were injected subcutaneously into 6-8 sites on the animal's back (0.1-0.2 mL/site). Boosting doses of emulsified antigen were given every 4 weeks.

Immunofluorescence (IF) staining

Confluent monolayers of BSC1 cell were grown on coverslip in 24-well tissue culture plates, infected with rotavirus and incubated at 37°C in a 5% CO_2 atmosphere. The culture medium was removed and coverslips were dried at room temperature. Cells were fixed in cold acetone for 10 min. After 3 times washing with PBS (0.01 M, pH 7.2), antiserum against recombinant NSP4 (1:20 dilution) was added to the coverslips in humid chamber and then were incubated at 37°C for 45 min. After 3 times washing with PBS, secondary antibody, FITC conjugated antibody to rabbit IgG (1:50 dilution) (DAKO) was added and incubated in a humid chamber at 37°C for 45 min. The stained cells were examined by a fluorescent microscope.

RESULTS

Production of antibody against infected cell lysate with rotavirus

The BSC1 cell line was infected with SA11 rotavirus. At 7 to 8 hrs post-infection the culture medium was removed and cells were rinsed with ice-cold PBS and pelleted at 2500 rpm for 10 min at 4°C. The cells were treated with 1mM $MgCl_2$ and were kept on ice. Swollen

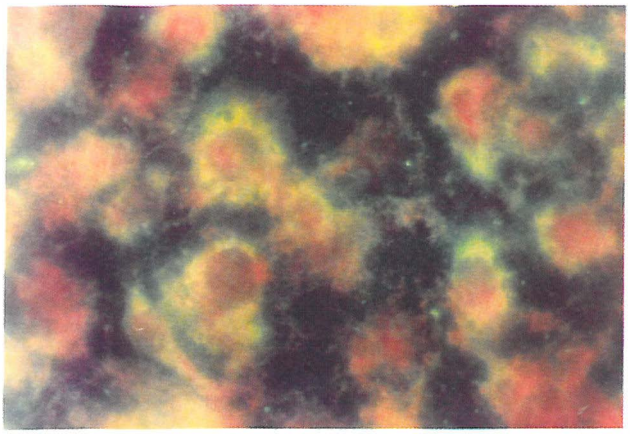

Fig. 1. IF staining of SA11 infected cells using antiserum against infected cell lysate with rotavirus, which was previously absorbed with uninfected cell lysate.

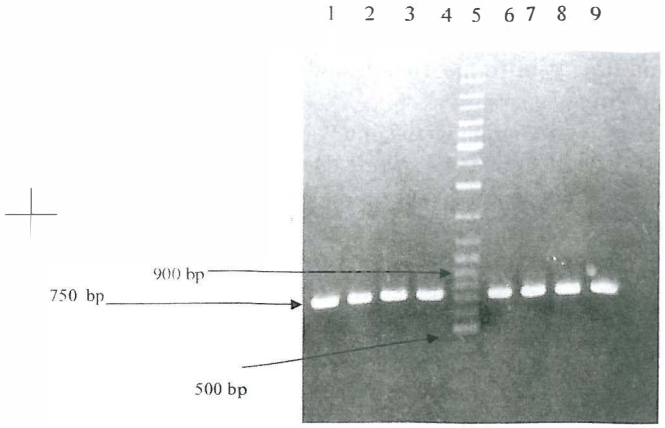

Fig. 2. Agarose gel electrophoresis of RT- PCR product of SA11 rotavirus gene 10 cDNA. Lanes 1-4 and 6-9, PCR product with 750 base pair length generated using gene sepecific primers of SA11 gene 10 cDNA. Lane 5, DNA size marker (100 bp ladder Fermentas).

cells were homogenized with a Dounce type A pestle. The nuclei and unbroken cells were pelleted at 2500 rpm for 10 min. The resulting pellet was suspended in sterile PBS. A polyclonal antibody was raised in rabbit and then absorbed with uninfected cell lysate. IF test was done on infected cells at 8 h post-infection using antiserum against infected cell lysate. Fluorescent stained cells indicated presence of antibody to NSP4 protein (Fig.1).

Cloning of nsp4 gene

Total RNA was extracted from SA11 virus particles and cDNA strand was synthesized and amplified by RT-PCR using *nsp*4 gene specific 3`, 5` terminal primers; Figure 2 shows RT- PCR products of approximately 750 bp fragment containing nsp4 gene.

The PCR product was ligated to Eco RV site of pBS-KS (+) to produce plasmid pYS48 and transferred into *E. coli*. Resulting clones were screened by PCR using specific primers, and restriction enzyme digestion. The recombinant plasmid was digested by Xbal endonuclease which confirmed the construction of 3711 bp recombinant plasmid pYS48 containing the complete gene 10 compared with pBS-KS(+) with 2961bp length (Figure 3). The cloned DNA was subjected to sequence analysis. Analysis of the nucleotide sequence by blast program showed that the entire amino acid-coding sequence of gene 10 was present in the recombinant plasmid.

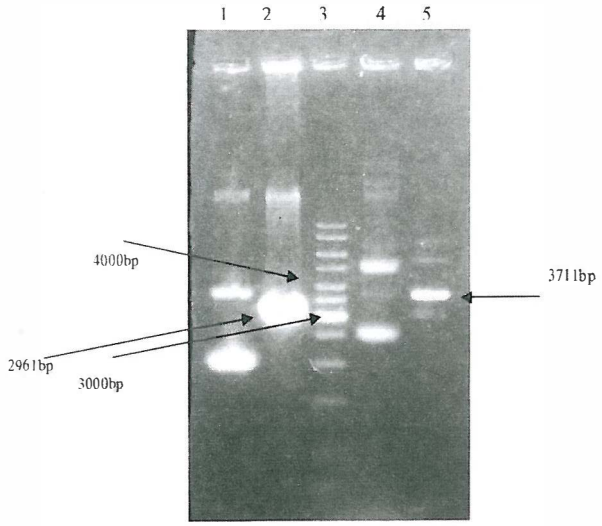

Fig. 3. Agarose gel analysis of restriction enzyme digestion of recombinant vector containing nsp4 gene. Lane 1, p BS-KS (+) plasmid uncut. Lane 2, Xbal digestion of pBS-KS (+) with 2961 base pair length. Lane 4, pYS48 recombinant plasmid uncut. Lane 5, Xbal digestion of pYS48 recombinant plasmid with 3711 base pair. Lane 3 100 bp DNA size marker.

Expression of NSP4 in *Escherichia coli*

SDS-PAGE and Western blotting

The cDNA encoding NSP4 from pYS48 was cloned into expression vector pET-26a (+) resulting in pYS50, allowing expression of NSP4 from T7/Lac promoter/ operator and targeting the recombinant protein to the periplasm of *E. coli,* BL21 (DE3) strain using the pelB signal sequence (Fig. 4).

The recombinant NSP4 was expressed by induction with IPTG as shown by SDS-PAGE (Figure 5a) and Western blot analysis (Fig. 5b). SDS-PAGE analysis of

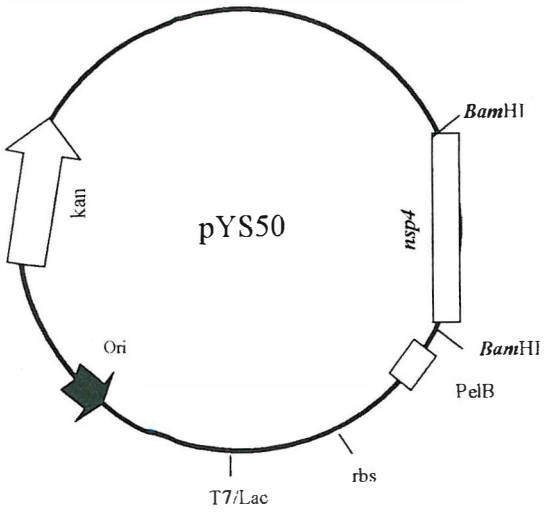

Fig. 4. Schematic diagram of pYS50 carrying nsp4 gene in Bam HI site of pET-26a (+) under the control of T7/lac promoter-operator.

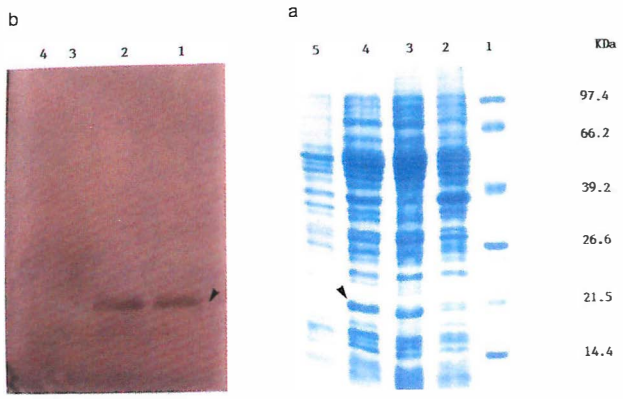

Fig. 5a. SDS- PAGE analysis of expressed gene product. Lane 1, protein molecular weight marker (Top to down 97.4, 66.2, 39.2, 26.6, 21.5, 14.4 KD). Lane 2, induced cells containing plasmid pET-26a (+) (negative control). Lane 3, 4, induced cells containing pYS50. Lane 5, uninduced cells containing pYS50.

Fig. 5b. Western blot analysis of expressed gene product. Lane 1, 2, induced cells containing pYS50. Lane 3, uninduced cells containing pYS50. Lane 4, induced cells containing pET-26a (+) (negative control).

induced cells containing pYS50 showed a band corresponding to 20KD (Fig. 5a, lanes 3,4 arrow), in comparison with induced cells containing pET-26a(+) (negative control), uninduced cells containing pYS50 (Fig. 5a, lanes5,2,1, respectively) and molecular weight marker.

For Western blotting, the resolved proteins were electroblotted onto a nitrocellulose membrane and reacted with rabbit polyclonal antiserum against lysate of infected BSC1 cell with SA11 rotavirus. The induced cells containing pYS50 showed bands (Fig. 5b, 1,2 arrow) corresponding to 20 KDa protein in SDS-PAGE analysis, compared to uninduced and induced cells containing pET-26a(+) (negative control).

Enzyme-Linked Immunosorbent Assay

Bacterial lysate was examined by ELISA for confirmation of NSP4 expression. Cells containing pYS50 and pET-26a (+) were grown and induced with IPTG.The cells were suspended in lysis buffer and sonicated. Supernatant of bacterial lysate was coated in ELISA microplates. ELISA was done using a rabbit polyclonal antiserum against lysate of infected cells with rotavirus; which was previously absorbed with lysate of uninfected cells. The result (Fig. 6) confirmed the expression of NSP4 in bacteria containing plasmid pYS50 and the results obtained from SDS-PAGE and Western blotting.

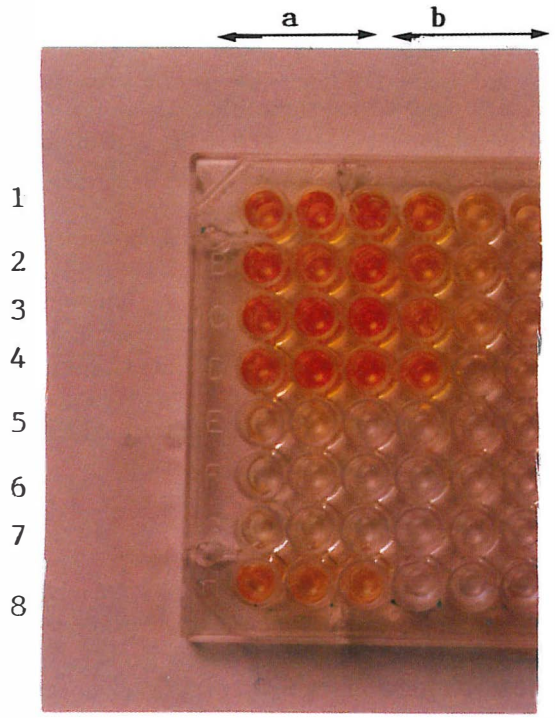

Fig. 6. ELISA of expressed gene product. Lane 1-4, induced cells containing pYS50. Lane 5, 6, uninduced cells containing pYS50. Lane 7, induced cells containing PET-26. Lane 8a, lysate of infected cell with rotavirus after 8 hrpi (positive control). Lane 8b, lysate of uninfected cell (negative control).

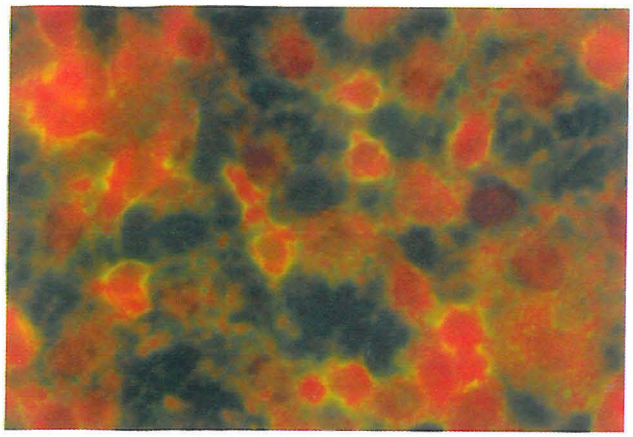

Fig. 7. IF staining of SA11 infected cells using antiserum against recombinant NSP4.

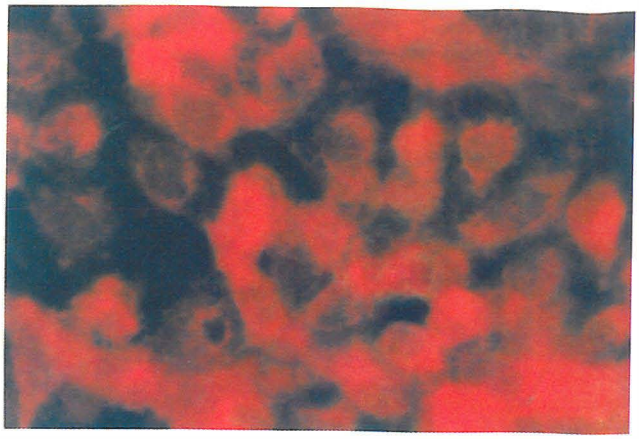

Fig. 8. IF staining of SA11 infected cells using rabbit unimmunized serum (as negative control).

Production of antibody against recombinant NSP4

The whole cell lysates of the bacteria were separated by electrophoresis. The recombinant NSP4 protein was purified from the gel and antiserum against it was produced in rabbit. Production of antibody against recombinant NSP4 was confirmed using ELISA and IF methods. SA11 rotavirus infected BSC1 cells were recovered at 8 hours post-infection (hpi) and lysed in lysis buffer. Proteins in the cell lysates was tested by ELISA using the rabbit polyclonal antiserum that raised against the purified recombinant NSP4 protein. This antibody reacted with lysate of infected cells with SA11 rotavirus but not with uninfected cells.

SA11 rotavirus infected BSC1 cells were stained with fluorescein conjugated anti-rabbit IgG antibody after treatment of cells with antibody against recombinant NSP4 and rabbit unimmunized serum (as negative control).The results showed foci of fluorescent dye in infected cell cytoplasm (Fig. 7) in comparison to rabbit unimmunized serum (Fig. 8) ; which confirmed the antibody against recombinant NSP4.

DISCUSSION

Expression of individual viral proteins by recombinant technology provides an opportunity to examine their properties and their role in the viral life cycle.

The association of Nsp4 with rotavirus pathogenesis was first suggested when SA11 NSP4, expressed and purified from insect cells, was shown to induce a dose dependent diarrhea in neonatal mice and rats after intraperitoneal or intraileal administration.[16,18] Both et al. (1983) cloned SA11 rotavirus gene 10 segment and determined its nucleotides with 751 base pair length.[10]

Nsp4 has been shown to function as an enterotoxin by stimulating chloride secretion through calcium-dependent signaling pathway.[18,19] Antibody against NSP4 might diminish the frequency and severity of rotavirus induced diarrhea. Thus, further study of NSP4 as a key pathogenic determinant of human rotavirus infection might justify it as a suitable target for vaccine development.

In this study, the product obtained from PCR on nsp4 cDNA oriented a band of 750 base pairs of the size marker, indicating amplification of nsp4 gene. The complete SA11 rotavirus gene 10 was cloned in pBluescript-KS vector. Cloning of the *nsp*4 gene was confirmed using restriction enzyme digestion and sequencing.

The protein product of gene 10 is a 20 KDa protein which is glycosylated to 28 KDa.[10]

The *nsp*4 gene was subcloned into pET-26a (+) expression vector. The expression of protein was studied by SDS-PAGE, Western blot analysis, ELISA and IF using a polyclonal antiserum against SA11-infected BSC1 cell. This antiserum reacted with whole cell lysate of *E.coli* strain BL21 containing the recombinant plasmid pYS50.

Ball *et al.* (1996) reported that intraperitoneal administration of 0.1 nmol of a full-length SA11 NSP4 derived from insect cells induced diarrhea in 60% (6/10) of 6-7 days old CD1 mice.[16] Zhang *et al.* (1998) have also reported that 56.5% of 6-7 days old CD1 mice developed diarrhea after intraperitoneal administration of 0.25 nmol of a full-length NSP4 of porcine (osu) rotavirus strain(1). Horie *et al.* (1999) reported that the GST-$NSP4_{96\text{-}175}$ peptide of murine (EW) rotavirus strain fusion protein could induce diarrhea in (8/14) CD1 neonatal mice.[14]

Thus the enterotoxigenic activity of the NSP4 is roughly proportional to the size of the NSP4 peptide. Since the small fragment of protein does not fold into its

native conformation, it is less likely that the NSP4 86-175 or NSP4 114-135 peptides fold into the native conformation of the full-length NSP4.[14] Hence we have attempted to express the full-length of the protein in *E.coli* and use it for better understanding of its pathogenesis in rotavirus infection.

It has been reported that expression of the rotavirus nonstructural glycoprotein NSP4 in *E.coli* leads to a decrease in the growth of the bacterium.[33] Therefore, to reduce its probable toxic effect, we expressed the full-length NSP4 in expression plasmid, carrying a pelB signal sequence. Signal sequence mediates the translocation of proteins across the bacterial plasma membrane into the periplasmic space.[34]

In our experiments the periplasmic fraction of the expressed NSP4 was much lower compared to the cytoplasmic fraction. Low expression of NSP4 in *E.coli* periplasm could be due to the unprocessing of the protein, since the signal sequence can anchor the protein within the membrane generating a transmembrane protein.

Polyclonal antiserum against recombinant protein, NSP4, was prepared in New Zealand rabbits and confirmed by ELISA and IF methods using infected cells with SA11.

Our results indicated that recombinant NSP4 expressed in *E.coli* would provide a useful source of antigen to produce antibody and to study the biological activity of NSP4.

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