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Expression and Immunoreactivity of a Human Group A Rotavirus Vp4

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Abstract: Rotavirus capsid protein Vp4 plays an important role in the virus adhering and entering the cells. In this study, a Vp4 gene cloned from a rotavirus strain TB-Chen was highly expressed in *E.coli* BL21 (DE3). The results of the Western blot showed that the protein possesses specific immuno-reactivities and can be specifically recognized by guinea pig antibodies against rotavirus strain SA11 or Wa. Some Vp4 dimers were formed during renaturation. These data obtained from this study provide a strong basis for further study on the structure and function of the Vp4.

Key words: Recombinant rotavirus VP4; Prokaryotic expression; Immunoreactivity.

Rotavirus (RV), belonging to the family of *Reoviridae*, is the leading cause of severe viral gastroenteritis in infants and children worldwide. In developing countries RV infections cause approximately 600,000 children deaths per year(15). In addition, rotaviruses also infect mammals and poultry. Therefore, RV not only threaten human health but also impact the development of the stockbreeding(14). RV are non-enveloped icosahedral viruses and their genome contains 11 segments of double-stranded RNA coding six structural proteins (Vp1, Vp2, Vp3, Vp4, Vp6, Vp7) and five nonstructural proteins (NSP1, NSP2, NSp3, NSp4, NSP5) (7). The Vp4 is the outermost spike protein of RV and accounts for 1.5% of the total viral proteins. The gene of Vp4 is made up of 2359 base pairs, coding a protein of 775 amino acids, with a molecular weight ~ 88 kDa. The Vp4 protrudes from the outer capsid glycosylated protein Vp7 and has many functions such as cell binding, entry, hemagglutination and neutralization. The Vp4 can be cleaved into Vp5* and Vp8* by the trypsin-like proteases, and virus infectivity is enhanced by this cleavage (6). From the above discussion, it is clear there are several theoretical and practical benefits to further study of this protein. This study reports the results of the gene cloning, recombi- nant expression and immunity characteristics of Vp4 of a human group A RV strain TB-Chen, which was isolated from a clinical patient in two years with acute gastroenteritis.

MATERIALS AND METHODS

Experiment materials

The *E.coli* strains DH5 α and BL21(DE3) were used for cloning and protein expression respectively. The RV strain TB-Chen was isolated in our lab from a clinical patient two years old with acute gastroenteritis and the full sequence of the Vp4 was cloned (the Vp4 gene sequence refers to Genebank, Accession Number:

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AY787644). The pBS (pBluescrip k/s) vector was used for cloning and amplification of the Vp4 gene. The plasmid pETL, which was modified from the pET-3a in our lab, was used for protein expression. DNA restriction enzyme, Taq DNA polymerase, and T4 DNA ligase are all purchased from TaKaRa company (Dalian). Guinea pig antibodies against strain SA11 and Wa were prepared in our lab. Goat anti guinea pig IgG labeled with horseradish peroxidase and 3.3-Diaminobenzidine were purchased from Sigma company.

PCR of the Vp4 gene

TB-Chen strain RV Vp4 was cloned and constructed onto the pBS vector (pBS-Vp4). The PCR reaction mixture solution contained 30ng template DNA of pBS-Vp4, 50 pmol/L dNTPs, 10 μL PCR buffer, 5U EX Taq DNA polymerase, 20 pmol/L upstream primer (5'-CATCATATGGCTTCGCTCATTTATAGACA-3'), 20 pmol/L downstream primer (5'-TTGTAAAACGACGGCCAG-3'). The upstream primer contains an *Nde* I restriction site (underlined) and ATG was the initiation codon of the Vp4 gene. The downstream primer was from the downstream sequence of the poly-link region of the pBS vector DNA. The end of PCR amplification product of the Vp4 gene contained *Sac* I (GAGCTC) and *Bam*H I (GGATCC) sites in the pBS. Total volume of PCR reaction mixture solution was 100μL. The conditions of PCR reactions were: heat for 3 mins at 94℃ with one cycle; denaturalize at 94℃ for 45 seconds, renature at 56℃ for 45 seconds, extending at the 72℃ for 1.5 min, with 30 cycles. Five microliters of the PCR product were detected in 1.2% agarose gel electrophyresis.

Construction of the expression plasmid

To prepare the insert segment, the PCR product of the Vp4 cDNA was digested by *Nde I* and *Sac I*. The Vp4 coding gene was ligated into pETL by T4 DNA ligase, and transformed into the competent DH5α cells. The amplified plasmid DNA was detected by restriction endonucleoside enzymes and sequenced by dideoxynucleoside termination reaction.

Expression of the Vp4 in *E.coli* BL21 (DE3)

The pETL-Vp4 final product was confirmed by restriction endonucleoside enzymes digestion and sequence containing the full Vp4 coding gene was transformed into competent *E.coli* BL21 (DE3). The transformed bacterial cells were incubated in LB liquid medium (containing 200 μg/mL of ampicillin), at 37℃. When absorbance value (OD_{600}) of the culture reached 0.8, bacterial cells were collected by centrifugation at 4 000g for 8 minutes at 4℃, suspended in sonication buffer (NaCl 200 mmol/L, Tris-HCl 50 mmol/L, pH 7.5, 5% Glycerol, 5% Triton X 100, EDTA 2 mmol/L, DTT 1 mmol/L) and lysed. The sonication solution was centrifuged at 12 000g, at 4℃ for 30 minutes. The supernatant was discarded, the pellet (inclusion bodies) dissolved in 8 mol/L urea, and stocked at 4℃.

SDS-PAGE and Western blot analysis

SDS-PAGE was performed on an 8%, 10%, 12% acrylamide gradient gel at constant voltage 120V. The proteins were visualized by Coomassie Blue R250 staining. The proteins separated by SDS-PAGE were transferred onto the nitrocellulose membrane. The membrane was blocked by incubation in TBS containing the 5% powdered skimmed milk at 4℃ overnight, then incubated with guinea pig anti-rotavirus Wa or SA11 strain antiserum (1:300) in the TBST containing 0.5% powdered skimmed milk at

room temperature for 2 h under shaking. The membrane was washed four times for 6 minutes. with 10mL TBST. The membrane was incubated with goat anti-guinea pig IgG labelled with horseradish peroxidase (1:1000) at room temperature for 2 h with shaking. After washing, the specific protein binding was detected by adding substrate solution containing DAB (1:1000) and H_2O_2 (1:1000).

Purification and renaturation of rVp4

Inclusion body proteins were separated by 10% SDS-PAGE, stained with the 150 mmol/L KCl, and the aim rVp4 band was cut from the gel. The gel band containing rVp4 was put into the dialysis tubing, electrophoresis buffer lacking SDS was added, and was run at 120V for 3 h at 4℃. The rVp4 solution was then taken out and put into fresh dialysis tubing and concentrated with PEG (polyethylene glycol, MW8000). The purified protein solution was quantified by the Brodford method.

The purified rVp4 protein was diluted with the phosphate buffered saline till the concentration reached 0.14 mg/mL, and then transferred into dialysis tubing and dialyzed in renaturation buffer (100 mmol/L NaH_2PO_4, 1 mmol/L EDTA, 10 mmol/L Tris-HCl, 1 mmol/L Oxydized Glutathione pH8.0, and different urea concentration, 6 mol/L, 4 mol/L, 2 mol/L, 1 mol/L). Each renaturation solution containing different urea concentrations was replaced five times and each time for 4 h at 4℃. The dialyzed protein solution was then centrifuged at 12 000g at 4℃ for 20 min. The supernatant was collected and detected by SDS-PAGE.

Inoculation of guinea pigs with rVp4

Guinea pigs weighing about 150g were inoculated intramuscularly three times at a two week interval, each with 100μL rVp4 (containing 140μg of rVp4 protein). The rVp4 protein used for first inoculation was emulsified with equal volume of Freund's complete adjuvant, and the second and third inoculations were emulsified with equal volume of Freund's incomeplete adjuvant. Seven days after the last inoculation, the guinea pigs were bled by heart puncture. Inoculation of guinea pigs with Wa and SA11 virus (containing 1×10^6 $TCID_{50}$) were carried out as above.

Detection of neutralizing antibodies

RV strains Wa and SA11 were diluted to 200 $TCID_{50}$ in 0.1mL, and incubated with trypsin at 10μg/μL, at 37℃ for 1 hr. The trypsin treated virus solution was mixed with equal volume of guinea pig antiserum diluted at different dilutions and incubated at 37℃ for 1 h, then 100μL was added in each well onto MA104 cell monolayer in a 96-well culture plate. The cells were incubated at 37℃ and cytopathic effect (CPE) was observed 24 hrs after infection. Neutralization titers were defined as the dilution of antiserum that protected fifty percent of cells from virus infection.

RESULTS

PCR amplification of RV Vp4 coding gene

Using the pBS-Vp4 as template DNA, PCR was performed. The PCR product with 2450 bp was detected at 1.2% agarose gel electrophoresis (Fig. 1).

Digestion of the expression plasmid

PCR product of Vp4 gene was digested with *Nde* I and Sac I and inserted into the corres- ponding site in the pETL DNA. The recombinant expression plasmid pET-Vp4 which contain RV Vp4 full coding gene was selected and confirmed by digestion and sequencing (Fig. 2).

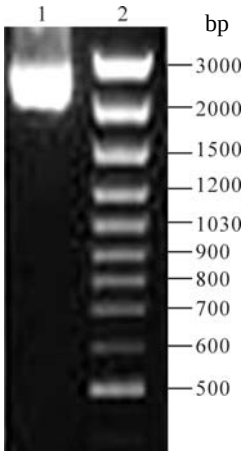

Fig.1. PCR of RV Vp4 coding gene. 1, PCR product of Vp4 gene cDNA. 2, DNA Marker.

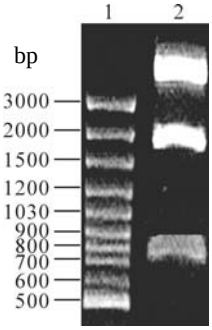

Fig. 2. Digestion of recombinant plasmid. 1, DNA Marker (bp); 2, Digestion products : one 1635bp (*Nde* I/*Spe* I) band and one 749bp (*Spe* I/*Bam*H I) band can be seen by digestion of pET-Vp4 with *Nde* I/*Spe* I/*Bam*H I .

SDS-PAGE analysis of rVP4 expressed

The 8%, 10%, 12% gradient SDS-PAGE was performed to detect the pET-Vp4 expression in the BL21(DE3) cells. The results of the SDS-PAGE showed that the pET-Vp4 was highly expressed in the BL21 (DE3) cells, and, as predicted, appeared as an ~87.6 kDa band on the gel (Fig. 3). The gel scanning analysis showed that the target protein accounts for about 17% of the total bacterial proteins.

Western blot of recombinant rVp4

The results of the Western blot indicated that the rVp4 could be specifically recognized by the guinea pig antiserum against SA11 or against Wa strain (Fig. 4 and Fig. 5).

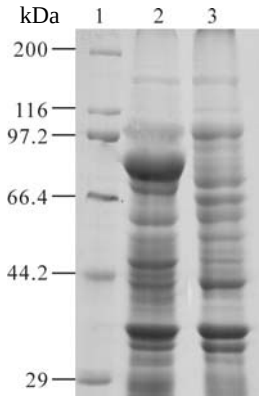

Fig.3. SDS-PAGE analysis of rVp4 expressed in BL21 (DE3) cells. 1, Protein marker (kDa); 2, BL21 (DE3) transformed with pET-Vp4; 3, BL21 (DE3) mock transformed with pETL.

SDS-PAGE analysis of the renaturation rVp4

The results showed that some dimers of the rVp4 were formed during renaturation. The gel scanning analysis of figure indicated that the molecular weight of the dimer is 176.67kDa. Some trimers or polymer were also seen. When the samples were either treated by denaturation/heating at 100℃, all of the dimmers or polymers disappeared (Fig. 6).

Gel scaning analysis of the renatured rVp4 by Imagemaster VDS system

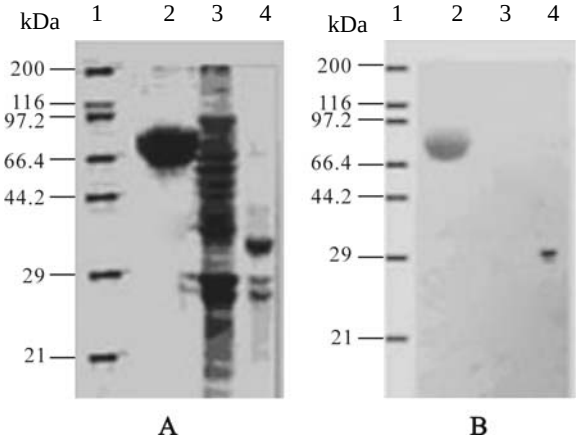

Fig. 4. Western blot of recombinant rVp4 with antibodies against SA11 in guinea pig antiserum. 12% SDS-PAGE and Coomassie brilliant blue R250 stained proteins (A) and Western blot (B). 1, Protein marker; 2, rVp4; 3, BL21 (DE3) cells mock transformed with pETL; 4, Recombinant RV Vp6 (3).

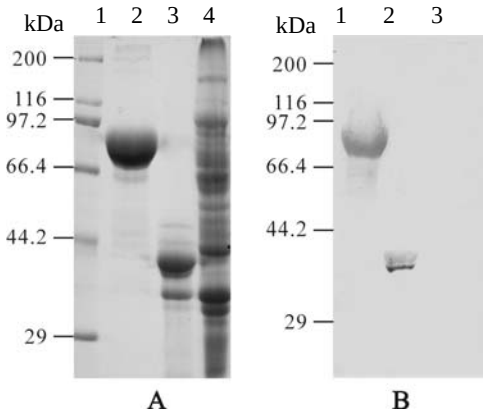

Fig. 5. Western blot of recombinant rVp4 with antibodies against Wa in guinea pig antiserum. 8-10-12% gradient SDS-PAGE and Coomassie brilliant blue G250 stained protein (A) and Western blot (B). 1, Protein marker; 2, rVp4; 3, Recombinant RV Vp6; 4: BL21 (DE3) cells mock transformed with pETL.

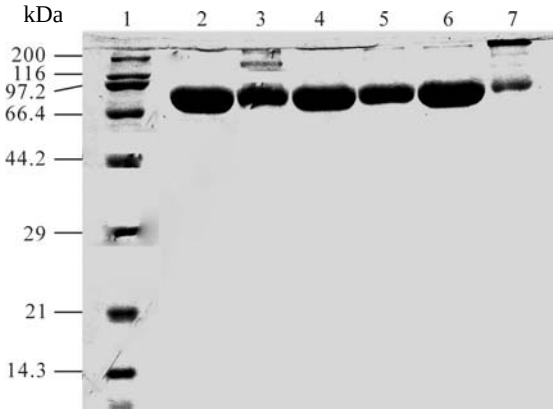

Fig. 6. SDS-PAGE analysis of the renatured rVp4 under different conditions. 1, Protein marker (kDa); 2, Denaturation rVp4, adding 5X loading buffer without heating; 3, Renatured rVp4,treated as in 2; 4, Denaturation rVp4, adding 5X loading buffer and heating 5 min; 5, Renatured rVp4, treated as in 4; 6, Denaturation rVp4, adding 5X loading buffer without mercaptoethanol and without heating; 7, Renatured rVp4, treated as in 6.

It was estimated from above gel (Fig. 6) scanned by Imagemaster VDS system that dimers account for about 9.13% of the total renaturation rVp4. Some trimers and polymers could also be seen at the top line of the separation gel, accounting for about 30.17%. See Table 1.

Western blot analysis of renaturation rVP4

Anti-rVp4 antiserums were used to detect renaturation rVp4, as the results showing, dimers (176.67kDa) and other polymers can be seen (Fig. 7).

Table 1. Results of gel scanning analysis of the renatured rVp4

	1	2	3
	MW (Amount)	MW Amoung	MW Amoung
1	200 (4.98)		242 (30.1)
2	116 (6.37)		176.(9.13)
3	97.2 (14.61)	88.3 (92.07)	88. (29.39)
4	66.4 (9.94)		
5	44.3 (7.75)		
6	29 (8.55)		
7	20.1(3.72)		
8	14.3(34.48)		
9	6.5 (12.29)		
Sum	(102.67)	(92.068)	(68.69)

Detection of neutralizing antibodies

The neutralization test showed that the anti-rVp4 antibodies in guinea pig serum could protect MA104 cells from infection by RV strains Wa and SA11. The neutralization titer was 1:256 against Wa strain, and 1:160 against SA11. While the neutralization titers of antisera from Wa virus inoculated guinea pigs were

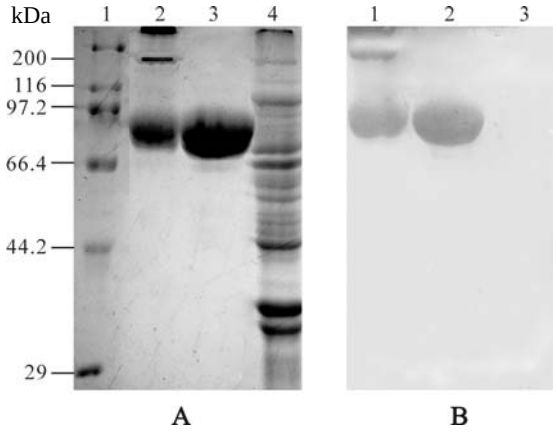

Fig.7. Western blot of renaturated rVp4 with antibodies against rVp4. G250 stained protein (A) and Western blot (B). 1, Protein marker (kDa); 2, Renaturated rVp4; 3, rVp4; 4: BL21(DE3) cells mock transformed with pETL.

1:2 560 against Wa infection and 1:640 against SA11 infection. The neutralization titers of antisera from SA11 virus inoculated guinea pigs were 1:2560 against SA11 infection and 1:640 against Wa infection.

DISCUSSION

RV can be classified into P serotype and G serotype according to the neutralization characteristics of the outer capsid protein Vp4 and Vp7 (1). At present, G1-G4 is the most prevalent epidemic (2). Vp4, expressed in eukaryotic or prokaryotic systems has neutralization activity (8, 12, 16), such works have been done by Sun Maoshen (11) and Huo Yunwen *et al* in China (9). Our previous studies showed that antigen epitopes of the Vp4 expressed in prokaryotic and eukaryotic systems have wide cross protecting activities in rat (4). VP4 can also significantly improve the protective efficacy of VLPs (viral like particles) administered intramuscularly against a rotavirus challenge in rabbits (5). Many studies suggested that immune responses to VP4 and VP7 might be a significant component of protective immunity against rotavirus infection (13, 17).

In the present study we cloned the Vp4 gene from the TB-Chen strain RV and highly expressed it in *E.coli* strain BL21 (DE3). Our findings confirmed that the rVP4 protein could be expressed in the system. Western blot results showed that expressed rVp4 could be specifically recognized by anti-SA11 and anti-Wa antibodies. Nucleoside sequence analysis revealed that the Vp4 gene of TB-Chen strain (G2 type) have 86% and 76.5% identity to that of SA11 (G3 type) and Wa (G1 type), respectively. Amino acid homology analysis revealed that the Vp4 protein of TB-Chen possesses 71% and 89% identity to SA11 and Wa, respectively. Western blot results showed that the homologies are not very high, while the Vp4 proteins possess very strong cross reactivities among these viruses. Furthermore, the results of the neutralization test indicated that the anti-rVp4 serum can protect the MA104 cells from infection by RV strains Wa or SA11.

In order to get the proper S-S bond of the rVp4 proper conformation, the conditions of denaturation and renaturation were strictly controlled through adjust the ratio of oxidized-glutathione and reduced-glutathione. This procedure can provide the time and space required for rVp4 to form the proper conformation (10). When the rVP4 was dissolved into 8M urea from inclusion body, it could have been changed into linear forms. Since the Vp4 exists as dimers in viral structure (18), it is better to renature it to resume its properties. The renaturation results showed that some dimers and trimers were formed which can be specifically recognized by anti-rVp4 antibodies, but whether it is the proper conformation or required further study. Additionally, when the renatured rVp4 was treated in different conditions, all of the polymers disappeared and resumed their monomer form. Therefore, some other way has to be found to maintain the stability of the dimers.

The results reported here indicated that a strong basis for further study on the structure and function of the rVp4 and its application as a RV recombinant protein vaccine have been set up.

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