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Prokaryotic Expression of VP7 Gene and Subunit Vaccine Preparation of Bovine Rotavirus

Research Article

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Abstract

The aims was to construct a prokaryotic expression plasmid and develop a subunit vaccine for bovine rotavirus (BRV), also to investigate the efficacy of this vaccine. BRV total RNA was extracted from M-A104 cell infected by the strain GSB01 of bovine rotavirus. Total RNA as template, BRV VP7 gene was amplified with real time fluorescence quantitative PCR (qPCR), PCR product was cloned into pEASY-T3 vector. The pEASY-T3-VP7 plasmid was recombined into the prokaryotic expression vector pET32a (+) after it was digested using double enzymes. The pET32a-VP7 and VP7-LTB used for prokaryotic expression were constructed, which were converted into Escherichia coli BL21 (DE3) competent cells. After IPTG induction and SDS-PAGE analysis were performed, the fusion proteins of pEASY-T3-VP7 and VP7-LTB with molecular weights of 42.2kD and 53.2kD were acquired respectively. Such a gene engineering bacteria for expressing VP7 protein within BL21(DE3) cells was constructed successfully. The mice immunized using both fusion proteins of pEASY-T3-VP7 and VP7-LTB could promote VP7 IgG antibody production (8.33 vs 17.3). The immunization protection ratio of both fusion proteins in neonatal mice was 86.4% and 91.7%, respectively. These findings laid a foundation for further developing a high-efficiency subunit vaccine of BRV VP7 gene.

Keywords: VP7 gene; Bovine rotavirus; Prokaryotic Expression; Vaccine.

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Introduction

Group A rotaviruses (RVA) are deemed to be important viral diarrheal agents in infants and young animals, including calves. The numbers of the rotavirus-associated mortality were estimated to be 453000 in 2008 [1, 2]. The numbers of deaths were particularly high in the developing countries [3]. Even in the developed countries, rotavirus remains an important cause of morbidity.

Rotaviruses, approximately 70nm diameter in size, are classified into at least eight different groups according to the serological reactivity and genetic variability of VP6 [4] , and consist of three capsid layers including the outer structural proteins (VP7 and VP4), middle structural protein (VP6) and inner core structural proteins (VP 1, 2 and 3). VP4 (encoded by gene segment 4) and VP7 (encoded by gene segment 7, 8 or 9 depending on the strain), both of which are independently responsible for virus neutralization [5, 6]. The neutralization specificity related to VP7 is referred to as the G serotype (for glycoprotein), and that associated with VP4 is referred to as the P serotype (for protease-sensitive protein) [7]. Up to date, the rotavirus species comprises at least 27 G types (according to the nt sequence of VP7) and 37 P types (according to the nt sequence of VP4) [8, 9].

VP7 protein (glycoprotein) and VP4 protein (for protease-sensitive protein) elicit the production of neutralizing antibodies, define the antigenic specificities, referred to as G type (glycoprotein) and P type (protease-sensitive protein), respectively, and are the major antigens neutralizing immune responses during rotavirus infections [10].

The rotavirus VP7 gene is highly conservative at both ends of open reading frame (ORF), such it is feasible to detect rotavirus serotypes utilizing the rotavirus specific primers. VP7 has been shown to be involved in the early interactions with cell-surface molecules, during the rotavirus entry process [11, 12].

VP7, a calcium-binding protein, plays a major role in the stability and morphology and virus particle formation of rotavirus. Previous studies reported that recombinant VP7 protein promoted the antibody production to protect the animals from rotavirus

infection [13, 14]. Although VP7 produce neutralizing antibodies, the role these antibodies play in effective immunity is still unclear.

The real time fluorescence quantitative PCR (qPCR) is not only fast and accurate, and also can test against different target sequences. Its specificity is very strong [15, 16]. Its ability to quantitatively and specifically detect genes has been invaluable for both research and diagnostic applications [17]. So far, little information regarding real time fluorescence quantitative PCR (qPCR) utilized to detect RV VP7 gene has been known.

Vaccination is an optimum measure to prevent BRV infection. But there is no such vaccine up to date. World health organization (WHO) has determined the development of a rotavirus vaccine as a priority project in the future [18]. It is difficult to develop a rotavirus vaccine because the VP7 epitope is conformation and complex structure. Nowadays very little research on BRV VP7 vaccine has been performed [19, 20].

The aim of the present study was to express VP7 Gene of BRV in prokaryotic cells, construct successfully a genetically engineered bacteria for expressing VP7 protein within BL21 (DE3) cells, and also to produce the fusion protein of pEASY-T3-VP7, furthermore to testify to the protection efficiency of fusion proteins inoculation. The findings hope to serve as a scientific foundation for the further development of genetic engineering vaccine of bovine rotavirus.

Material and Methods

Cell cultures of Bovine rotavirus

The strain GSB01 of bovine rotavirus isolated in our laboratory from the fecal samples, collected from 195 Holstein calves (1-30 days old) with diarrhea [21, 22] and the neonatal calf diarrhea virus (NCDV) strain (AV-51, purchased from Chinese veterinary drugs supervisor institute, Beijing, China) of bovine rotavirus were adapted to MA-104 cell (Ganges River monkey kidney cells, Chinese supervision of veterinary drugs, Beijing, China) cultures. The cells were cultured for 4-6 days at 37°C in the incubator containing 5% carbon dioxide (CO_2). The process stopped when the cytopathogenic effects (CPE) were greater than 90%. Then the cells were frozen and thawed 2 to 3 times. The viral supernatant was collected for RNA extraction or stored -80°C until processed.

Primers designs and synthesis

For RT-PCR, the specific primers were designed using Primer Premier 5.0 software according to the highly conserved regions of BRV VP7 based on the deposited genome sequences of BRV VP7 in GenBank (Accession number No. GQ433985.1 and EU873015.1). The restriction enzymes of *Hind* III and *BamH* I were added at the 5' end of the forward primer (N-terminal primer) and reverse primer (C-terminal primer), respectively. Forward primer : 5'-GTAAAGCTTTGGTATTGAATATACCAC-3' (The underlined bases were the *Hind* III site). Reverse primer: 5'- GATGGATCCCCTGTTGGCCATCC -3' (The underlined bases were the *BamH* III site). Primers were synthesized by Takara Bio Company (Dalian, China).

RNA extractions and cDNA synthesis

To obtain virus RNA from fecal samples, genomic dsRNA was extracted from purified BRV using a Trizol method (Beijing, China). For preparing RNA from virus-infected cell culture, 0.5 to 1.0 mL of virus-infected cell supernatant was pelleted by centrifugation at 5000 rpm for 20 min to pellet the virus-cell suspension for further total RNA extraction. Total RNA was obtained according to the manufacturer's instructions and was resuspended in DEPC-treated water and stored at -80°C until use.

PCR was performed on a 25μL system which contained 15.5μL diethylpyrocarbonate (DEPC) water, 0.5μL (10mM) deoxyribonucleotide triphosphate (dNTPs), 2.5μL 10×PCR buffer, 0.5μL Taq polymerase, 0.5μL BRV forward primer, 0.5μL BRV reverse primer, 5μL cDNA. The reaction conditions were as follows: primary denaturation for 5 min at 94°C with 35 cycles of 1min at 94°C, annealing for 30 at 50°C, and 2min at 72°C, and a final elongation of 10 min at 72°C. RNA samples were quantified using a Nanodrop spectrophotometer (Shanghai, China). The absorbance ratios of 260/280nm in all samples were greater than 1.9, indicating the high purity of RNA. Five microliters of the PCR products were assayed on 1.5% agarose gel electrophoresis (Solon, USA) containing 1×Gel Red (Hayward, USA) and then analyzed with the software CS Analyzer Ver 3.0 (Tokyo, Japan).

The cDNA was synthesized from the NCDV strain and extracted viral RNA by reverse transcription reaction and utilized for PCR amplification of the VP4 gene. Expected amplicons were 890 bp sizes.

RT-PCR of theVP7 gene

The reverse transcription-PCR (RT-PCR) assay was performed by using total RNA from fecal samples with BRV as template [22]. The qPCR was done in accordance with manufacturer's instructions for superscript II reverse transcriptase. Following substances were added into a 500μL Ependorff tube: 5μL of 10×PCR Buffer, 1.5μL of 50mM magnesium chloride (MgCl2), 1μL of 10mM dNTP Mix FP, RP (10 μM), 0.4μL LA Taq DNA polymerase, the first chain cDNA 2μL, with coke carbonic acid ethyl ester two (diethypyrocarbonate, DEPC) water added up to 50μL.

The conditions for qPCR were as follows: initial denaturation at 95°C for 15 min followed by 40 cycles of 94°C for 15sec, 55°C for 30 sec and 72°C for 40sec, and a final extension at 72°C for 10 min.

Five microliters of the PCR products were assayed on 1.5% agarose gel electrophoresis (Solon, USA) containing 1×Gel Red (Hayward, USA) and subsequently analyzed with the software CS analyzer Ver 3.0 (Tokyo, Japan).

Ligation, transformation and induction of VP7 gene and pEASY-T3 vector

After the recovered fragments of PCR products were ligated into pEASY-T3 vector and cloned, they were transformed into competent DH5α cells of *Escherichia coli*. *Escherichia coli* strain DH5α was cultured in Luria-Bertani medium (LB) containing 100 μg/mL ampicillin and grown overnight at 37°C and 180 r/min. The transformation processes were conducted in the following reaction systems, which consisted of 1μL pEASY-T3 vector, 3μL PCR products, total reaction volume of 5μL. The ligation process was completed at room temperature for 5 min.

Four microliters (4μL) reaction compounds were added slowly

to 100μL competent DH5α cells of E. Coli, incubated in ice for 30min, then bathed in 42°C water for 90sec, then bathed once more on ice for 5min. 900μL Luria-Bertani media (LB media) were added. The bacteria liquid was cultured in the incubator at 37°C, and rejuvenated for 1h. Bacteria liquid was induced on the plate coated with LB/Amp (50μg/mL) and isopropyl β-D-1-Thiogalactopyranoside (IPTG). Thereafter they were cultured at 37°C for 12-20h. The recombinant pEASY-T3-VP7 was then constructed.

The B subunit gene of Escherichia coli heat-labile enterotoxin (LTB, Accession number No. : M17873.1) was amplified with the forward preimer (5'-GCCGGATCCATGGCTCCTCAGTCT-ATTACAGAACTATG). The PCR cycle parameters were as follows: initial denaturation for 5 min at 94°C followed by 32 cycles consisting of denaturation for 45 sec at 94°C, annealing for 45sec at 53°C, extension for 45sec at 72°C and final extension for 10 min at 72°C. The amplification products were assayed on 1.2% agarose gel electrophoresis (Amresco, Solon, USA).

The ligation and transformation of LTB (lymphotoxin beta) with pEASY-T3 vector were performed as described above.

Construction and PCR of recombinant plasmid

The selected single colony of bacterium was inoculated into LB media containing 200μg/mL of ampicillin and cultured with a slow oscillation at 37°C for 12 to 14h. Then the plasmids were extracted with the pure plasmid mini Kit (Beijing, China). The recombinant pEASY-T3-VP7 plasmid was digested with both restriction endonuclease of *Hind* III and *BamH* I (Dalian, China), respectively. The products were assayed on 1.5% agarose gel electrophoresis (Solon, USA). The fragments were amplified with the forward primer and reverse primer using the extracted recombinant plasmid as template. The amplification conditions were as the same described above. The sequencing was fulfilled by Sangon Biotech (Shanghai, China).

Prokaryotic expression plasmid constructions, identification and sequencing

The recombinant pEASY-T3-VP7 plasmid and pET32a vector for prokaryotic expression were digested with both restriction endonuclease of *Hind* III and *BamH* I (Dalian, China), respectively. The fragments were recovered and ligated according to the manufacturer's instruction of T4-DNA ligase (Dalian, China), then stayed overnight at 16°C. The ligation products were transformed into the BL21 (DE3) competent cells of E. Coli. Selected colonies were identified by PCR and digested with both *Hind* III and *BamH* I. The strain of positive clones were sequenced and preserved.

Protein expressions and purity identification

The recombinant genetically engineered bacteria were cultured in Luria-Bertani media (LB media) containing 200μg/mL of ampicillin up to 0.6 of the absorbance value (OD_{600}), then added into 1.0mmol/L IPTG and induced at 30°C for 6h. Bacteria liquid was collected.

The recombinant proteins were identified on the SDS-PAGE at constant voltage 120V. Fifty milliliters (50mL) of the transferred bacteria were induced and cultured in accordance with the method mentioned above. 20mL of bacterial supernatant was purified in the QIAexpressionistTM purification system labeled with His (Beijing, China) according to the instructions. The purified recombinant protein was subjected to further SDS-PAGE analysis.

Inoculation of mice with recombinant proteins

Sixty mice of 10-weeks-old with a body weight of 46.38±2.95g, were bought from Experiment Animal Center, Lanzhou University (License No. SCXK (Gansu) 2005-0007), were randomly divided into three groups (n=20). Mice in group A were used for the control group of His-Tag prokaryotic expression vector. Mice in group B were inoculated with PEASY-T3-VP7 fusion protein. Mice in group C were inoculated with the fusion protein of VP7-TB.

When the purified protein was emulsified with adjuvant at a ratio of 1:1, the mice were intramuscularly injected with this emulsion every two weeks for three times. Blood samples were taken after a week of the last injection from the orbital cavity of each mouse. Serum IgG antibody titers were detected with Mlbio® Elisa Kit for mice (Shanghai, China), according to the manufacturer's instruction.

All mice were raised in subgroup and kept in mice cages equipped with automatic water dispensers under the same conditions in the stable room maintained at 22-24°C and 30% to 50% relative humidity, with a controlled 12-hour light-dark cycle. Mice received a commercial diet (Lanzhou, China), depending on their physiological condition. Water was supplied ad libitum. All animal procedures were carried out in strict accordance with the Gansu province committee of experimental animal care and use of China, which meets the European Union Directive 2010/63/EU on the protection of animals used for scientific purposes.

Protection tests of neonatal mice

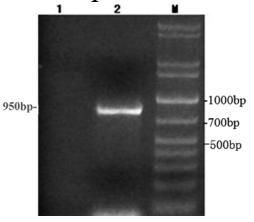
Sixty six neonatal mice (3 to 4 days old) born from the female mice were randomly allocated into three groups according to described above. All neonatal mice were intraperitoneally injected with 100μL strain GSB01 of bovine rotavirus referring to the early reports [23, 24]. Diarrhea symptoms were observed and death numbers were calculated after 3-7 days. All neonatal mice were raised under the same conditions by using the same methods described above. All procedures referring to animal treatments were approved by Gansu province committee of experimental animal care and use of China.

Results

PCR amplification of BRV VP7 gene

A band of 950bp was amplified on Agarose gel electrophoresis, which was consistent with the expected size (Figure 1).

Figure 1. PCR amplification of BRV VP7



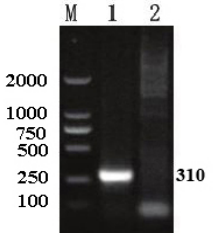
A 950bp band was found on Agarose gel electrophoresis, which was consistent with the expected size.

Lane 1-negative control; Lane 2-positive samples; Lane 3-NCDV strain; 4-marker.

PCR amplification of LTB gene

A band of 310bp was amplified on Agarose gel electrophoresis, which was similar to the expected size (Figure 2).

Figure 2. PCR Amplification products of LTB gene



M-DL2000 Maker; 1-LTB gene; 2- negative control of redistilled water

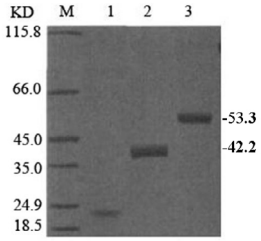
Identification of the recombinant pEASY-T3-VP7 plasmid and pET32a-VP7

Following the recombinant pEASY-T3-VP7 plasmid and pET32a vector was digested with both *Hind* III and *BamH* I, respectively. The results showed that the VP7 gene had been cloned into the pEASYT3 vector. The cloned VP7 gene was introduced into pET32a vector. The recombinant pET32a-VP7 plasmid was achieved. The recombinant plasmid was named as pET32a-VP7 containing the full length VP7 cDNA.

SDS-PAGE analysis and purification

SDS-PAGE analysis showed that the pET32a-VP7 was highly expressed in the BL21 (DE3) cells, and the molecular weights of pEASY-T3-VP7 and VP7-LTB (heat-labile enterotoxin B subunit) proteins were 42.2 kDa and 53.2 kDa, respectively. They were consistent with the expected size. (Figure 3).

Figure 3. Purity of recombinant protein



Lane M: DNA marker; Lane 1. pET32a tag protein; Lane 2. pEASY-T3-VP7 protein;
Lane 3. VP7-LTB protein;
The purity of the purified protein was assayed by SDS-PAGE. The molecular weights of pEASY-T3-VP7 and VP7-LTB proteins were 42.2kD and 53.2kD, respectively.

Detection of serum BRV VP7 IgG antibody

As shown in Table 1, serum BRV VP7 IgG antibody in all mice was not detected before the fusion protein inoculation. VP7 IgG antibodies in groups A and B were significantly higher than that in group A ($P<0.05$ or $P<0.01$) following the inoculation. This illustrated that BRV VP7 gene had excellent immunogenicity. However, the VP7-LTB fusion protein could arouse a stronger immunological response.

Protection efficiency of fusion proteins inoculation in neonatal mice

Base on the clinical observation,the neonatal mice in His-tag control group showed the typical diarrhea symptoms in 3 to 7 days after intraperitoneal injection of 100μL strain GSB01 of bovine rotavirus. Mice numbers of diarrhea and death neonatal mice of pEASY-T3-VP7 and VP7-LTB fusion protein group were highly significantly less than that of His-tag control group ($P<0.01$). The protection efficiency of neonatal mice to pEASY-T3-VP7 and VP7-LTB fusion proteins was 86.36% and 91.67%, respectively (Table 2), which indicated that the protein was reacting with the antibody of rotavirus A.

Table 1. Serum IgG antibody titer of mice.

Group	IgG antibody Titer (×1000)	Before inoculation	After inoculation
A	His-tag protein	0	0.61
B	pEASY-T3-VP7 fusion protein	0	8.33*
C	VP7-LTB fusion protein	0	17.26**

* $P<0.05$ when compared to group A;
** $P<0.01$ when compared to group A.

Table 2. Protective efficacy of neonatal mice.

Group	Treatment	Neonatal mice	Diarrhea and dead mice	Protection rate
A	His-tag protein	20	18	10.00
B	pEASY-T3-VP7fusion protein	22	3	86.36**
C	VP7-LTB fusion protein	24	2	91.67**

Note: Twenty, three and two mice in groups A, B and C showed the typical diarrhea symptoms in 3 to 7 days after the strain GSB01 injection, respectively. Mice numbers of diarrhea and dead neonatal mice of groups A and B were highly significantly less than that of group A ($P<0.01$). The protection efficiency of pEASY-T3-VP7 and VP7-LTB fusion proteins for the neonatal mice was 86.36% and 91.67%, respectively.

** $P<0.01$ when compared to His-tag control group

Discussion

Rotaviruses are the major cause of acute viral gastroenteritis in infants and young children, as well as in young animals of several species, including strains of bovine, equine, porcine and canine origin [25, 26]. Worldwide prevalence rates in BRV infection range from 7% to 94%, with an average of 30%-40% [27]. BRV VP7 gene plays an important role in the stability and morphology and virus particle formation of rotavirus and takes part in the initial interactions with cell-surface molecules, during the rotavirus entry process [12]. Previous studies demonstrated that recombinant VP7 protein promoted the antibody production [13].

Vaccination is an effective measure for preventing from rotavirus infection in calves. At present, the conventional vaccines (including the live attenuated vaccine and virulent virus inactivated vaccine) are widely used [18, 28]. However, there are a lot of failures during vaccination because of very unstable of the attenuated vaccine and insufficient immune responses stimulated by the inactivated vaccine [29]. Current inactivated and attenuated vaccines have been limited owing to their high costs and inconvenience [30]. As such, it is urgently necessary to develop a new vaccine which could produce high protection efficiency [31, 32]. Successful expression of VP4 or VP7 proteins in a highly immunogenic form would be a first step towards making an effective subunit vaccine to protect against rotavirus infection [33].

The DNA vaccine is characterized with many advantages of the pure antigen, less side-effects, easy preparation, and will become the main methods of preventing BRV infection. It is a hot topic field of vaccine research and development [34]. However, there lacks in information regarding of genetic engineering vaccine research of BRV [23, 35].

In this study, the pEASY-T3-VP7 plasmid was obtained and introduced into pET32a vector. The pET32a-VP7 was also prepared and transformed to BL21 (DE3) competent cells of E. Coli. Immunogenicity of BRV VP7 and LTB were investigated preliminarily at the molecular level. BRV pEASY-T3-VP7 fusion protein and VP7-LTB fusion protein had been constructed and expressed successfully. In the neonatal mice infected with the strain GSB01 of bovine rotavirus, the protection ratio of recombinant pEASY-T3-VP7 and VP7-LTB fusion proteins inoculation was 86.4% and 91.7%, respectively. The findings demonstrated that the immunogenicity of VP7-LTB fusion protein was greater than that of pEASY-T3-VP7.

Conclusion

The genetically engineered bacteria were harvested. The pEASY-T3-VP7 and VP7-LTB fusion proteins could promote the formation of IgG antibody in inoculated mice. The results lay a solid foundation for further studies on developing a high-efficiency subunit vaccine and DNA vaccine of the VP7 gene for prevention and control of bovine rotavirus [30, 32].

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