Subunit Vaccine Preparation of Bovine Rotavirus and Its Efficacy in Mice

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ABSTRACT

Background: Rotaviruses (RV) are important viral diarrheal agents in calves. Vaccination is an optimum measure to prevent bovine rotaviruses (BRV) infection. However, little research on BRV VP7 vaccine has been done and currently there is no BRV vaccine. **Objective:** To prepare a subunit vaccine of BRV and investigate its efficacy. **Methods:** Total RNA was extracted from MA104 cells infected with bovine rotavirus (BRV) strain GSB01. BRV VP7 gene was amplified using real time fluorescence quantitative PCR (qPCR). The pEASY-T3-VP7 plasmid was digested using *Hind Ⅲ* and *BamHI* restriction endonucleases, then recombined into the prokaryotic expression vector pET32a. The pET32a-VP7 and pET32a-VP7-LTB (heat-labile enterotoxin B subunit) were transformed into BL21 (DE3) competent cells of *Escherichia coli,* respectively, and induced with IPTG, then analyzed using SDS-PAGE. Sixty mice were randomly divided into three groups (n=20). Group A mice was used as His-tag control and mice in group B and C were inoculated with pET32a-VP7 and pET32a-VP7-LTB, respectively. VP7 IgG antibody titers and protection efficiency of pET32a-VP7-LTB were further determined in neonatal mice challenged with GSB01 BRV strain. **Results:** SDS-PAGE analysis showed that the pET32a-VP7 was highly expressed in the BL21 (DE3) cells. PET32a-VP7 and pET32a-VP7-LTB protein could promote VP7 IgG antibody titer（8.33×103 vs. 17.26×103）in mice. Immunization protection ratios of pET32a-VP7 and pET32a-VP7-LTB proteins in the neonatal mice were 86.4% and 91.7%, respectively. **Conclusion:** The fusion protein of pET32a-VP7-LTB had excellent immunogenicity and protected mice from BRV infection. Our findings can be used for further developing of a high-efficiency subunit vaccine of BRV.

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Keywords: Bovine, Rotavirus, Subunit Vaccine, VP7 Structure Protein

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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 and deaths were particularly high in the developing countries (1,2). Even in the developed countries, rotaviruses remain important causes of morbidity. Rotaviruses (RV) are classified into at least eight different groups according to the serological reactivity and genetic variability of VP6. Up to date, RV 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) (3,4).

VP7 (encoded by gene segments 7, 8 or 9 depending on the strain) plays a major role in the stability and virus particle formation of rotavirus (5). VP7 is involved in the initial interactions with cell-surface molecules in the rotavirus entry process (6). VP7 gene is highly conservative at both ends of open reading frame (ORF). VP7 protein (glycoprotein) elicits the production of neutralizing antibodies, and defines the major antigenic specificities to which neutralizing immune responses appear during rotavirus infections (7).

The recombinant VP7 protein was firstly developed in 1987. At present the main structural proteins of VP2, VP4, VP6 and VP7 are prepared using gene recombination technique (8,9). Previous studies reported that recombinant VP7 protein could produce the special antibody to protect the animals from rotavirus infection (10). But the subunit vaccines of the recombinant proteins have obvious disadvantage that they are not endogenous antigens and also cannot effectively stimulate mucosal immunity and prevent rotavirus infection which is essential in the intestinal local immunity Lewis *et al.* (8). Therefore, the roles of the antibodies in effective immunity remain unclear.

Up to date, there is no effective specific treatment for bovine rotavirus diarrheal disease except the symptomatic supportive therapy. Vaccination is an optimum measure to prevent bovine rotaviruses infection. However, currently there is no such vaccine to be applied in practice. It is difficult to develop a rotavirus vaccine because the VP7 epitope is a conformational one with complex structure. Very little research on subunit vaccine of BRV VP7 has been performed (11). Furthermore, the difficulty of developing a new vaccine results in preclinical and clinical failure (12).

Rotavirus candidate vaccine development has followed two views regarding the importance of serotype-specific protection. Many candidates are based on the theory that protection is not solely dependent on neutralizing antibodies. These candidates, such as Rotarix vaccine, contain single rotavirus strains (13). On the other hand, several candidate vaccines are based on the concept that neutralizing antibodies are the primary determinant of protection. These candidates, including RotaTeq, are composed of multiple rotavirus strains representative of the major human rotavirus serotypes (14).

The aims of the present study were to express VP7 Gene of BRV in prokaryotic cells, successfully construct a genetic engineered bacteria for expressing VP7 protein within BL21 (DE3) cells, and also to prepare the fusion protein of pET32a-VP7-LTB and evaluated its protection efficiency in mice. The findings promise to serve as a scientific foundation for further development of a genetically engineered vaccine of bovine rotavirus.

MATERIALS 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 (15,16) and the neonatal calf diarrhea virus (NCDV) strain of bovine rotavirus were adapted to MA-104 cell cultures. The cells were cultured for 4 to 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 Design and Synthesis. 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* Ⅰ site). Primers were synthesized by Takara Bio Company (Dalian, China).

The primers of LTB (heat-labile enterotoxin B subunit) of *Escherichia coli* (Accession number: M17873.1) was also designed using above software. The forward primers were as following: 5′-GCCGGATCCATGGCTCCTCAGTCTATTACAGAACTATG.

RNA Extraction. The genomic dsRNA was extracted from purified BRV utilizing a Trizol method (Beijing, China). One milliliter of virus-infected cell was centrifuged at 5000 r/min for 20 min to pellet the suspension for further total RNA extraction. Total RNA was obtained according to the manufacturer's instructions and was re-suspended in DEPC-treated water and stored at -80°C until use. The cDNA was synthesized from the NCDV strain and extracted viral RNA by reverse transcription reaction and utilized for PCR amplification of the VP7 gene. The expected amplicon size was 342 bp.

Reverse Transcription PCR (RT-PCR) of BRV RNA. The extracted total RNA was reversely transcribed with taking BRV as template (16). PCR was done in accordance with manufacturer's instructions for superscript II reverse transcriptase. Following substances were added into a 500 μL Eppendorf tube: 5 μL of 10×PCR Buffer, 1.5 μL of 50 mM magnesium chloride ($MgCl_2$), 1 μL of 10 mM dNTP Mix FP, RP (10 μM), 0.4 μL LA Taq DNA polymerase, the first chain cDNA 2 μL, with coke carbonic acid ethyl ester two (diethypyrocarbonate, DEPC) water added up to 50 μL.

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

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

Construction of pEASY-T3-VP7 and pEASY-T3-LTB.

1-The Ligation and Transformation of BRV VP7. *Escherichia coli* strain DH5α was cultured in Luria-Bertani medium (LB) containing 100 μg/mL ampicillin and grown overnight at 37°C. The recovered PCR products of BRV VP7 were transformed into the

competent DH5α cells of *Escherichia coli* after they were ligated into pEASY-T3 vector. The transformation processes were conducted in the reaction systems of 1 μL pEASY-T3 a vector, 3 μL PCR products, with a total reaction volume of 5μL. Four microliters (4 μL) reaction compounds were added slowly to 100 μL competent DH5α cells of *Escherichia coli*, incubated in ice for 30 min, then bathed in 42°C water for 90 sec, lastly bathed once more on ice for 5 min. 900 μL Luria-Bertani media (LB media) were added. The bacteria liquid of *Escherichia coli* was cultured at 37°C, and rejuvenated for 1h. Bacteria liquid was induced on the plate coated with LB/Amp (50 μg/mL) and isopropyl β-D-1-Thiogalactopyranoside (IPTG). Thereafter they were cultured at 37°C for 12-20h.

The recombinant pEASY-T3-VP7 plasmid was then digested with both restriction endonuclease of *Hind* Ⅲ and *BamH* I (Dalian, China), respectively. The products were assayed on 1.5% agarose gel electrophoresis (Amresco, Solon, USA). The fragments were amplified with the forward and reverse primers using the extracted recombinant plasmid as a template. The amplification conditions were the same as described above. The sequencing was fulfilled by Sangon Biotech (Shanghai, China).

2-The Ligation and Transformation of LTB. The selected colonies of *Escherichia coli* were inoculated into Luria-Bertani medium (LB medium) containing 200 μg/mL of ampicillin and cultured with a slow oscillation at 37°C for 12 to 14h. Then the LTB (heat-labile enterotoxin B subunit) was extracted with pure plasmid mini Kit (Beijing, China). LTB (heat-labile enterotoxin B subunit) was amplified in the PCR reaction 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 45 sec 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). LTB ligation and transformation with pEASY-T3 vector were performed as the processes described above.

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

Expressions and Identification of Recombinant pET32a-VP7-LTB. The genetically engineered bacteria were cultured in Luria-Bertani media (LB media) containing 200 μg/mL of ampicillin up to 0.6 of the absorbance value (OD_{600}), then added into 1.0 mmol/L IPTG (isopropyl β-D-1-Thiogalactopyranoside) and induced at 30°C for 6h. then the Bacteria liquid was collected.

The recombinant pET32a-VP7-LTB was identified on the SDS-PAGE at constant voltage 120V. Fifty milliliters (50 mL) of the transferred bacteria were induced and cultured in accordance with the method mentioned above. 20 mL of bacterial supernatant was purified in the QIAexpressionist^{TM} purification system labeled with His (Beijing, China) according to the instructions. The purified recombinant protein was subjected to further SDS-PAGE analysis.

Inoculation Tests of Mice. Sixty Kunming mice, 10-weeks-old and body weights of

46.38 ± 2.95 g, were purchased from Experiment Animal Center, Lanzhou University (License No. SCXK (Gansu) 2005-0007), and 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 pET32a-VP7 fusion protein twice in an interval of 14 days. Mice in group C were inoculated with the fusion protein of pET32a-VP7-LTB twice in an interval of 14 days.

The purified protein was emulsified with Freund complete adjuvant and Freund incomplete (Sigma, USA) at a ratio of 1:1 and mice were intramuscularly injected with this emulsion every two weeks for three times. The antigen emulsion with Freund complete adjuvant was injected in the first inoculation, and the emulsion with Freund incomplete adjuvant was injected in the second and third inoculation, respectively. Blood samples were taken from the orbital cavity of each mouse after a week of the third injection. 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°C to 24°C and 30% to 50% relative humidity, with a controlled 12 hours light-dark cycle. Mice received a commercial diet (Lanzhou, China). Water was supplied *ad libitum*. All animal procedures were performed 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 as mentioned above. All neonatal mice were intraperitoneally injected with 100 μL strain GSB01 of bovine rotavirus referring to the early reports (17,18). 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.

Statistical Analysis. Statistical analysis was done using SPSS v. 18.0 (SPSS Inc. Chicago, IL, USA). The incidences of symptoms were calculated by group as percentages of subjects reporting each symptom, with 95% confidence intervals. A paired *t-test* was also used to evaluate the significance of the measures. Statistical analysis of the mean values of immunogenicity in three groups were compared using one-way ANOVA. P values less than 0.05 were considered to be significant (*$p<0.05$ and **$p<0.01$).

RESULTS

PCR Amplification of BRV VP7 Gene. An ampliphied band of 350 bp was observed on the agarose gel after electrophoresis, which was consistent with the expected size of 342 bp (Figure 1).

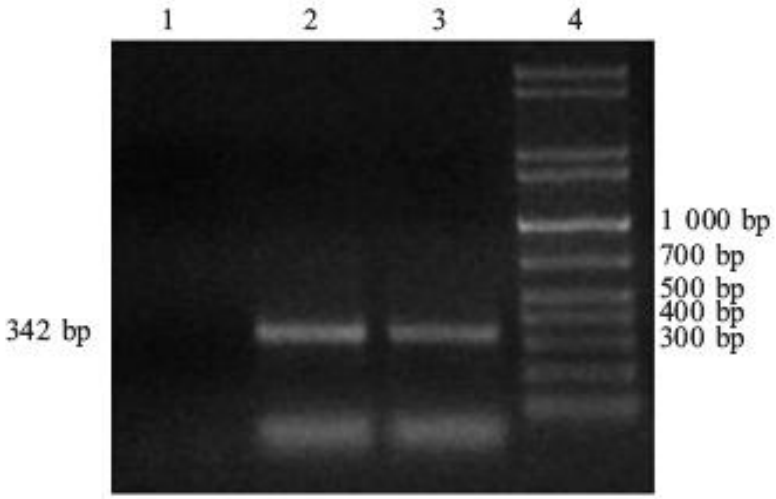

Figure 1. RT-PCR amplification of BRV VP7. Lane 1: negative control; Lane 2: positive samples; Lane 3: NCDV strain; Lane 4: marker.

PCR Amplification of LTB Gene. A band of 310 bp was detected on the agarose gel after electrophoresis, which was similar to the expected size (Figure 2).

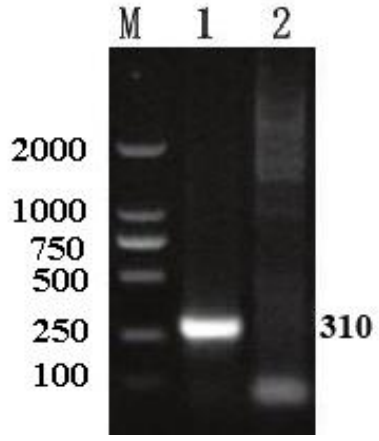

Figure 2. PCR Amplification products of LTB gene M-DL2000 Maker; Lane 1: LTB gene; Lane 2: negative control of redistilled water

Identification of the Recombinant Plasmid. The recombinant pEASY-T3-VP7 plasmid and pET32a vector were digested with both *Hind* III and *BamH I*. 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 and the recombinant pET32a-VP7 plasmid contained the full length VP7.

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

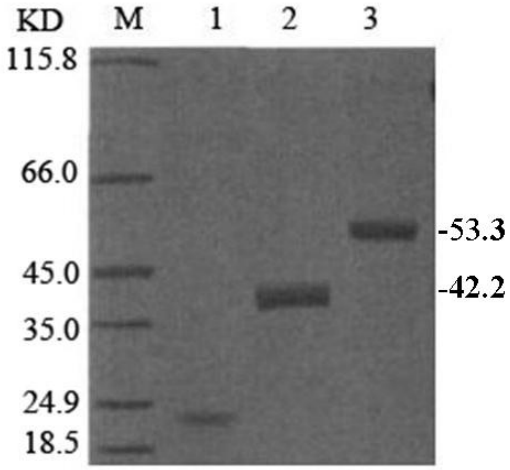

Figure 3. Purity of recombinant protein. Lane M: DNA marker; Lane 1. pET32a His-tag protein Lane 2. pET32a-VP7 protein; Lane 3. pET32a-VP7-LTB protein The purity of the purified protein was assayed by SDS-PAGE. The molecular weights of pEASY-T3-VP7 and pET32a-VP7-LTB proteins were 42.2kD and 53.2kD,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

Detection of Serum BRV VP7 IgG Antibody. As shown in Table 1, serum BRV VP7 IgG antibody in all mice was not detectable before the fusion protein inoculation. VP7 IgG antibodies in groups A and B were significantly higher than that of group A ($p<0.05$ or $p<0.01$) following the inoculation. This illustrated that BRV VP7 gene had excellent immunogenicity. However, the pET32a-VP7-LTB fusion protein could induce a stronger immunological response.

Table 1. Serum IgG antibody titer of mice.

Group	IgG Antibody Titer （×1000）	Before Inoculation	After Inoculation
A	His-tag protein	0	0.61×10^3
B	pET32a-VP7 fusion protein	0	$8.33^{*}\times 10^3$
C	pET32a-VP7-LTB fusion protein	0	$17.26^{**}\times 10^3$

Note: *p＜0.05 when compared to group A; **p＜0.01 when compared to group A.

Immunization Protection Efficiency in Neonatal Mice. Based on the clinical observation, 20, 3 and 2 mice in groups A, B and C showed the typical diarrhea symptoms after intraperitoneal injection of 100 μL strain GSB01 of bovine rotavirus, respectively. The neonatal mice in His-tag control group displayed the obvious diarrhea symptoms in 3 to 7 days after the GSB01 strain injection. The frequency of diarrhea and death among neonatal mice in pET32a-VP7 and pET32a-VP7-LTB fusion protein groups were significantly less than that of His-tag control group ($p<0.01$). The protection ratios of pET32a-VP7 and pET32a-VP7-LTB fusion proteins in the neonatal mice were 86.36% and 91.67%, respectively (Table 2), which indicated that both fusion proteins were inducing the antibody of rotavirus 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	pET32a-VP7 fusion protein	22	3	86.36**
C	pET32a-VP7-LTB fusion protein	24	2	91.67**

Note: ** $P < 0.01$ 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 (19,20). The prevention strategies in calves are based on the increasing levels of passive immunity. The protection against bovine rotavirus (BRV) infection is only related to the passive immunity levels acquired by consumption of colostrum and milk (21).

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 (22). However, there are a lot of failures during vaccination because of instability of the attenuated vaccine and insufficient immune responses stimulated by the inactivated vaccine (23). As such, it is urgently necessary to develop a new vaccine which could produce high protection efficiency (24,25).

The DNA vaccine, characterized by many advantages over the pure antigen including less side-effects, easy preparation, is a good candidate for BRVvaccine (26). However, the studies on the developing such a vaccine are scarce (27).

Gonzalez *et al*. (2010) demonstrated that a recombinant subunit vaccine containing the VP6 protein induced a passive protective immune response in the calves (28). It is likely that this subunit vaccine had exerted its function through a mixed Th1/Th2 immune response (21,28).

Previous studies reported that recombinant VP7 protein could produce the specifi antibody to protect the animals from rotavirus infection (10). In this study, BRV pET32a-VP7 and pET32a-VP7-LTB fusion proteins were constructed and expressed successfully. They could produce BRV VP7 IgG antibody in mice and lead to immunological response. The protection ratio of recombinant pET32a-VP7 and pET32a-VP7-LTB fusion proteins inoculation was 86.4% and 91.7% in the neonatal mice who were infected with the GSB01 strain of bovine rotavirus. The findings demonstrated that the immunogenicity of pET32a-VP7-LTB fusion protein was greater than that of pET32a-VP7. Our results are consistent with the early studies (8,29). But these findings need to be further evaluated in calves infected by bovine rotavirus.

Our results highlight the possibility of developing a high-efficiency subunit vaccine and DNA vaccine of the VP7 gene for prevention and control of bovine rotavirus (24,25,30).

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REFERENCES

1. Tate JE, Haynes A, Payne DC, Cortese MM, Lopman BA, Patel MM, et al. Trends in national rotavirus activity before and after introduction of rotavirus vaccine into the national immunization program in the United States 2000 to 2012. Pediatr Infect Dis J. 2013; 32:741-4.
2. Abe M, Ito N, Morikaw S, Takasu M, Murase T, Kawashima T, et al. Molecular epidemiology of rotaviruses among healthy calves in of a novel bovine rotavirus bearing new P and G genotypes. Virus Res.2009; 144:250-7.
3. Desselberger U. Rotaviruses. Virus Res. 2014; 190:75-96.
4. Matthijnssens J, Ciarlet M, McDonald SM, Attoui H, Bányai K, Brister JR, et al. Uniformity of rotavirus strain nomenclature proposed by the Rotavirus Classification Working Group (RCWG). Arch Virol. 2011; 156:1397-413.
5. Beg SA, Wani SA, Hussain I, Bhat MA. Determination of G and P type diversity of group A rotaviruses and detection of a new genotype from diarrhoeic calves in northern and southern states of India. Lett Appl Microbiol. 2010; 51:595-9.
6. Martha NC, Fanny G, Orlando A, Carlos AG. Rotavirus VP4 and VP7-Derived Synthetic Peptides as Potential Substrates of Protein Disulfide Isomerase Lead to Inhibition of Rotavirus Infection Int J Pept Res Ther. 2012; 18:373-82.
7. Aminu M, Page NA, Ahmad AA, Umoh JU, Dewar J, Steele AD. Diversity of Rotavirus VP7 and VP4 Genotypes in Northwestern Nigeria. J Infect Dis. 2010; 202:S198-S204.
8. Yen C, Tate JE, Patel MM, Cortese MM, lopman B, Fleming J, et al. Rotavirus vaccines. Hum Vaccin. 2011; 7:1282-90.
9. Albert Z. Kapikian. A Hexavalent Human Rotavirus–Bovine Rotavirus (UK) Reassortant Vaccine Designed for Use in Developing Countries and Delivered in a Schedule with the Potential to Eliminate the Risk of Intussusception. J Infect Dis. 2005; 192: 22-9.
10. Wang L, Huang JA, Nagesha HS, Smith SC, Phelps A, Holmes I, et al. Bacterial expression of the major antigenic regions of porcine rotavirus VP7 induces a neutralizing immune response in mice. Vaccine. 1999; 17:2636-45.
11. Gouvea V, Glass RI, Woods P, Taniguchi K, Clarke HF, Forrester B, et al. Polymerase chain reaction amplification and typing of rotavirus nucleic acid from stool specimens J Clin Microbiol. 1990; 28: 276-82.
12. Bohles N, Bohles N, Busch K, Busch K, Hensel M, Hensel M. Vaccines against human diarrheal pathogens: Current status and perspectives. Hum Vaccin Immunother. 2014; 10:1522-35.
13. Zade JK, Kulkarni PS, Desaei SA, Sabale RN, Naik SP, Dhere RM. Bovine rotavirus pentavalent vaccine development in India. Vaccine. 2014; 32: A124-8.
14. Ward RL, Clark HF, Offit PA. Influence of potential protective mechanisms onthe development of live rotavirus vaccines. J Infect Dis. 2010; 202: S72-9.
15. Wei Suocheng, Feng Ruofei, Gong Zhuandi, Tian Fengling. Researches of Isolation and Cell Cultivation of Bovine Rotavirus in Vero and MA-104 Cells. Journal of Northwest University for Nationalities. 2010; 31:71-5.
16. Wei S, Gong Z, Che T, Ayimu G, Tian F. Genotyping of calves rotavirus in China by reverse transcription polymerase chain reaction. J Virol Methods. 2013; 189:36-40.
17. Wang Zhiming, Meng Yajuan, He Cheng and Gao Jian. Status and Progress of Research on Mucosal Vaccines Chin Pharm J. 2013; 49:1-6.
18. Yang S, He H, Yang H, Wang C, GaoY, Ma Y, Zhong J. Genetic engineering subunit vaccine of group A bovine rotavirus. Chinese J Zoonoses. 2011; 27:505-10.
19. Suocheng W. Epidemiology and integrated control of animal rotavirus disease Animal Husbandry and Veterinary in Gansu. 2005; 27:27-8.

20. Fukai K, Takahashi T, Tajima K, Koike S, Iwane K, Inoue K. Molecular characterization of a novel bovine group A rotavirus. Vet Microbiol. 2007; 123:217-24.
21. Gonzalez DD, Rimondi A, Perez Aguirreburualde MS, Mozgovoj M, Bellido D, Wigdorovitz A, et al. Quantitation of cytokine gene expression by real time PCR in bovine milk and colostrum cells from cows immunized with a bovine rotavirus VP6 experimental vaccine. Res Vet Sci. 2013; 95:703-8.
22. Lokeshwar MR, Bhave S, Gupta A, Goyal VK, Walia A. Immunogenicity and safety of the pentavalent human-bovine (WC3) reassortant rotavirus vaccine (PRV) in Indian infants. Hum Vaccin Immunother. 2013; 9:172-6.
23. Clark HF, Offit PA, Plotkin SA, Heaton PM. The New Pentavalent Rotavirus Vaccine Composed of Bovine (Strain WC3) -Human Rotavirus Reassortants. Pediatr Infect Dis J. 2006; 25:577-83.
24. Chen Qian, Zhang Ying, Qian Zhi-yong. Case-control Study on Effect of Oral Rotavirus Attenuated Live Vaccine against Rotavirus Diarrhea Chinese Journal of Vaccines and Immunization. 2014; 20:55-5.
25. Gökçe G, Ran Z, Lennart H, Harold M. Engineered Lactobacillus rhamnosus GG expressing IgG-binding domains of protein G: Capture of hyperimmune bovine colostrum antibodies and protection against diarrhea in a mouse pup rotavirus infection model. Vaccine. 2014; 32:470-7.
26. Rajtak U, Leonard N, Bolton D, Fanning S. A real-time multiplex SYBR Green I polymerase chain reaction assay for rapid screening of salmonella serotypes prevalent in the European Union. Foodborne Pathog Dis. 2011; 8:769-80.
27. Baoming J , Xiaoming Y, Deqi X. The current status and development trend of rotavirus vaccine. China Journal of Biologicals. 2012; 25:251-3.
28. Gonzalez DD, Mozgovoj MV, Bellido D, Rodriguez DV, Fernandez FM, Wigdorovitz A, et al. Evaluation of a bovine rotavirus VP6 vaccine efficacy in the calf model of infection and disease. Vet Immunol Immunopathol. 2010; 137:155-60.
29. Oberle D, Jenke AC, von Kries R, Mentzer D, Keller-Stanislawski B. Rotavirus vaccination: A risk factor for intussusception? Bundesgesundheitsblatt Gesundheitsforschung Gesundheitsschutz. 2014; 57:234-41.
30. Gaimei Z, Wenjin W, Zuoshen F. Progresses on human rotavirus vaccine. Chinese Journal of Zoonose. 2015; 31: 83-588.