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Preparation of novel trivalent vaccine against enterotoxigenic *Escherichia coli* for preventing newborn piglet diarrhea

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OBJECTIVE
To develop a trivalent genetically engineered inactivated *Escherichia coli* vaccine (K88ac-3STa-LT_B) that neutralizes the STa toxin by targeting fimbriae and entertoxins for the treatment of enterotoxigenic *E coli*.

ANIMALS
18- to 22-g mice, rabbits, pregnant sows.

PROCEDURES
Using PCR, the *K88ac* gene and LT_B gene were cloned separately from the template C83902 plasmid. At the same time, the 3 STa mutant genes were also amplified by using the gene-directed mutation technology. Immune protection experiments were performed, and the minimum immune dose was determined in mice and pregnant sows.

RESULTS
The ELISA test could be recognized by the STa, LT_B, and K88ac antibodies. Intragastric administration in the suckling mouse confirmed that the protein had lost the toxicity of the natural STa enterotoxin. The results of the immune experiments showed that K88ac-3STa-LT_B protein could stimulate rabbits to produce serum antibodies and neutralize the toxicity of natural STa enterotoxin. The efficacy test of the K88ac-3STa-LT_B-inactivated vaccine showed that the immune protection rate of the newborn piglets could reach 85% on the first day after suckling. At the same time, it was determined that the minimum immunization doses for mice and pregnant sows were 0.2 and 2.5 mL, respectively.

CLINICAL RELEVANCE
This research indicates that the K88ac-3STa-LT_B trivalent genetically engineered inactivated vaccine provides a broad immune spectrum for *E coli* diarrhea in newborn piglets and prepares a new genetically engineered vaccine candidate strain for prevention of *E coli* diarrhea in piglets.

Enterotoxigenic *Escherichia coli* (ETEC) is the main pathogen causing *E coli* diarrhea in newborn piglets.[1–4] Diarrhea has been a major cause of mortality and morbidity according to National Swine Surveys in the United States. Although diarrhea has been on the decline in recent years, ETEC remains a frequent agent of porcine diarrhea and continues to be diagnosed in neonatal and postweaning piglets that die from diarrhea in the United States. ETEC has recently been identified as the cause of an increasing number of outbreaks of foodborne infection in the United States.[5–8]

ETEC includes 2 pathogenic factors: enterotoxin and adhesin, among which adhesion mainly includes K88, K99, and 987P. Adhesions produced by *Escherichia coli* are various bacterial surface structures with an adhesion effect. Their essence is a kind of pili protein, which directly mediates bacterial adhesion to host cells, also known as settlement factors. ETEC is adsorbed to receptors on the surface of small intestinal mucosal epithelial cells by K88.[9,10] ETEC uses adhesion to settle on the epithelial cells of the host intestinal mucosa, thereby multiplying and producing large amounts of enterotoxin. Enterotoxin causes pathological changes in intestinal mucosal epithelial cells, leading to piglet diarrhea.[11–13] The main adhesion of ETEC in pigs is K88ac, which is an indirect factor that causes diarrhea in piglets.[14–16]

Enterotoxin is a direct cause of diarrhea in piglets, including heat-stable enterotoxin (ST) and heat-labile enterotoxin (LT).[17–20] According to antigenicity and host difference, ST is divided into STa and ST_b. Among them, STa is the most direct pathogenic factor.[21–23] The nonimmunogenic STa is a small peptide of 18 or 19 amino acids that still remains active even if heated to 100 °C for 30 minutes.[24,25] STa is inactive in other tissues and organs, and this specificity may be related to the distribution of receptors.[26] LT is composed of toxic subunit LT_A and binding subunit LT_B.[27–29] LT or LT_B has good immunogenicity. LT_A can change permeability of cell membranes and lead to diarrhea.[30,31]

The onset of *E coli* diarrhea in newborn piglets is acute, and the course of the disease develops rapidly. At present, the treatment of this disease is mainly drug therapy and vaccination, but due to the high price of drug therapy and the increase of a drug-resistant strain, drug therapy is not effective. Therefore, prevention is the best choice to control the disease instead of drug therapy.

E coli have strain-specific O-lipopolysaccharide antigens on their cell wall and flagella or H antigens. There are also numerous different capsular polysaccharide (K) antigens. *Escherichia coli* are serotyped based on the combination of O, H, and K antigens. Serotyping of *E coli*, together with genome, virulence, and phage typing, is a useful epidemiological tool. Whole-genome sequencing is a method that is becoming commonly used for typing of *E coli* and identification of virulence genes. At present, more than 50% of the isolates of piglet diarrhea cases contain the K88 antigen and STa pathogenic factors from the pig farms in most parts of China.[32,33] The common O serotypes are O5, O8, O60, O115, O138, O139, O141, O149, etc. *E coli* O169:H41 has become the predominant ETEC serotype identified in the United States.[34] The fimbriae and enterotoxins produced by ETEC are different. Some O-serotype virulence factors are mainly K88, some O-serotype virulence factors are mainly ST, some O-serotype virulence factors are mainly LT, and some O-serotype virulence factors even contain the above 2 or 3 kinds. If the inactivated vaccine is prepared with O-serotype bacteria, it can only prevent the diarrhea of newborn piglets caused by this serotype and has a poor effect on the diarrhea of newborn piglets caused by other serotype O. Due to the lack of immunogenicity of STa and limited cross-protection between serotypes O, the genetically engineered vaccine can easily solve the above limitations. Therefore, our research group constructed the trivalent genetically engineered inactivated vaccine. To develop a new genetically engineered vaccine candidate strain for preventing *E coli* diarrhea in piglets, the *K88ac* gene, STa mutant gene, and LT_B gene were amplified from the *E coli* C83902 plasmid to construct *K88ac-3*STa-LT_B fusion gene using genetic engineering technology. Its immunogenicity was then studied, and a trivalent genetically engineered inactivated vaccine was developed. It is particularly important that the trivalent genetically engineered inactivated vaccine not only maintained the strong immunogenicity of K88ac fimbriae antigen and LT_B enterotoxin but also conferred STa immunogenicity. Moreover, the K88ac-3STa-LT_B trivalent genetically engineered inactivated vaccine also had a wide range of immune responses. Therefore, this new genetically engineered vaccine candidate strain was provided for preventing *E coli* diarrhea in piglets and will bring economic benefits.

Materials and Methods

Construction of the recombinant expression strain BL21(DE3) (pXK88ac3STaLT$_B$)

Based on the STa, LT_B, and *K88ac* sequence reported by Xu et al[35,36] and Dykes et al,[37] 3 STa mutant genes were amplified using C83902 plasmid DNA as the template and the primer 1, 5′-CCC***AAGCTT***AACAACACATTTTACTGC-3′; the primer 2, 5′-GGAATTC***CATATG***AT AACTTCCAGCA CTGGC-3′; the primer 3, 5′-GGAATTC***CATATG***AAC AACACATTTTACTGC-3′; the primer 4, 5′-CCG ***GAATTC***ATAACTTCCAGCACTGGC-3′; the primer 5, 5′-CCG ***GAATTC***AACAACACATTTTACTGC-3′; and the primer 6, 5′-CGC***GGATCC***ATAACTTCCAGCACTG GC-3′. The primers contained the *H*ind III, *N*de I, *E*coR I, and *B*amH I restriction endonuclease sites (italics) and protective bases, respectively. The *K88ac* gene fragment was amplified from the template C83902 plasmid by primer 7, 5′-CATG***CCATGG***CATTTACTGAC TATGAAGAA-3′; and primer 8, 5′-CCC***AAGCTT***GA GAATATCATTTCTTGATAG-3′. Primer 7 and primer 8 contained *N*co I and *H*ind III restriction endonuclease sites (italics) and protective bases, respectively. The LT_B gene fragment was amplified from the template C83902 plasmid by primer 9, 5′-CGC***GGATCC***CCAGACTATTACAGAACT A-3′; and primer 10, 5′-ATAAGAAT***GCGGCCGC***AAGCTTGCCC CTCCAGCCTAG C-3′. Primer 9 and primer 10 contained *B*amH I and *N*ot I restriction endonuclease sites (italics) and protective bases, respectively. Using PCR site-directed mutagenesis technology, three 60-bp STa genes with the TGT→AGT(Cys→Ser) mutation were amplified by using 3 pairs of STa PCR primers from *E coli* C83902 plasmid. The 3 cloned STa mutant genes were connected in series into 180-bp STa-STa-STa fusion gene. The 330-bp *K88ac* and 500-bp LT_B gene fragments were amplified separately using C83902 plasmid DNA as the template. The constructed STa-STa-STa fusion gene was connected with the *K88ac* and LT_B genes to construct the fusion gene *K88ac-3*STa-LT_B and cloned into pET-28b to generate the recombinant plasmid pXK88ac3STaLT$_B$. It was transformed into the recipient bacteria BL21(DE3), and the recombinant strain BL21(DE3)(pXK88ac3STaLT$_B$) was constructed. Restriction enzyme digestion of pXK88ac3STaLT$_B$ with *Nco* I/*Not* I confirmed the presence of the *K88ac-3*STa-LT_B gene. Moreover, the TGT→AGT(Cys→Ser) mutation was confirmed by sequence analysis.

Induction and SDS-PAGE analysis of BL21(DE3)(pXK88ac3STaLT$_B$) and ELISA detection of K88ac-3STa-LT$_B$ fusion protein

BL21(DE3)(pXK88ac3STaLT$_B$) was coated separately to Luria broth (LB) plates containing 30 µg/mL kanamycin and cultured overnight at 37 °C. A single colony was picked, inoculated in 5 mL liquid LB medium containing 30 µg/mL kanamycin, and then cultured overnight at 37 °C with shaking at 170 rpm. The recombinant bacteria were inoculated into a culture flask containing 250 mL of LB medium at a ratio of 1% to logarithmic growth phase (optical density at 600 nm = 0.4 to approx 0.6). Then, IPTG was added at a final concentration of 1 mmoL/L and cultured overnight at 37 °C with shaking at 170 rpm. Cultures (1 mL) of BL21(DE3)(pXK88ac3STaLT$_B$) was centrifuged separately at 12,000 X *g* for 10 minute sat 4 °C, and the supernatants were discarded. The pellets were resuspended in 0.5 mL of 50 mmoL/L Tris-HCl pH 7.4 and centrifuged at 12,000 X *g* for 10 minutes at 4 °C. Pellets were resuspended in 25 µL of water. Once the bacteria are dispersed, 25 µL of 2X SDS gel electrophoresis loading buffer was immediately added and shaken for 20 seconds, boiled in a boiling water bath for 3 to approximately 5 minutes, and then was centrifuged at 12,000 X *g* for 10 minutes at room temperature. Twenty-five microliters of supernatant was taken for SDS-PAGE analysis. SDS-PAGE analysis showed that K88ac-3STa-LT$_B$ was expressed highly in the *E coli* strain BL21(DE3). The K88ac-3STa-LT$_B$ proteins accounted for 33.53% of total cellular protein. The K88ac-3STa-LT$_B$ fusion protein could be recognized by the STa monoclonal antibody and K88ac and LT$_B$ antibody through STa, K88ac, and LT$_B$ ELISA detection kits (Nanjing Jin Yibai Biological Technology Co Ltd).

K88ac-3STa-LT$_B$ antigen preparation

The 50-mL cultures induced by IPTG for 4 hours were centrifuged to collect the bacteria and then resuspended in 5 mL TE (50 mmoL/L Tris-HCl and 2 mmoL/L EDTA). The lysozyme at a final concentration of 100 µg/mL and 5 mL of 1% TritonX-100 were added and incubated at 30 °C for 15 minutes. The lysate was treated with an ultrasonic machine (Beidi-IIYJ with 2-mm probe) 2 times for 10 seconds each and then centrifuged at 12,000 X *g* for 15 minutes. The precipitate was the inclusion body. The inclusion bodies were washed twice with 0.5 M urea and 20 mM Tris-HCl pH 8.0. Then, the pellets were resuspended with 500 mL of 20 mM Tris-HCl pH 8.0 and 0.5 M urea, and denaturing solution (8 M urea, 20 mM Tris-HCl pH 8.0, and 0.1% β-mercaptoethanol) was added at a ratio of 1:40. After being denatured at 37 °C for 2 h, the inclusion bodies were resuspended again and centrifuged at 12,000 X *g* for 10 minutes at 4 °C. The supernatant was aspirated and transferred to a dialysis bag containing 150 mL of 20 mM Tris-HCl pH 8.0 with a urea concentration gradient of 6 M, 4 M, and 2 M. The renaturation solution was replaced every 6 hours. After being diluted 10 times, the aluminum hydroxide gel was added at a final concentration of 10% and the mixture was used as an antigen for immunization. In addition, formaldehyde was added to the engineering bacteria culture solution at a final concentration of 0.4% to inactivate the bacteria, and then aluminum hydroxide gel was added to 10% as an antigen for immunization.

Safety test of BL21(DE3)(pXK88ac3STaLT$_B$) and minimum lethal dose test of challenge strain

To determine whether the K88ac-3STa-LT$_B$ fusion protein expressed by the recombinant strain had lost the STa enterotoxin activity, 40 mice were selected and randomly divided into 8 groups of 5 mice, of which 4 groups were injected intraperitoneally with the recombinant strain BL21(DE3)(pXK88ac-3STaLT$_B$). The other 4 groups were inoculated orally. The clinical response of the test mice was observed daily, and a necropsy was performed after continuous observation for 3 weeks.

Sixty mice weighing 18 to 22 g were divided randomly into 6 groups of 10 mice. One of the group was used as a control group and the other groups were challenged separately by using different gradients of challenge strains (0.25 X 10^8 CFU, 0.5 X 10^8 CFU, 1.0 X 10^8 CFU, 1.5 X 10^8 CFU, and 2.0 X 10^8 CFU). After 3 days of observation, the minimum lethal dose (MLD) was determined according to the death of the mice. MLD is the minimum dose that can cause individual death in a group of test animals. Twelve newborn piglets were divided randomly into 4 groups of 3 piglets. Each of 3 groups was challenged separately by using different gradients of challenge strains (0.2 X 10^{10} CFU, 2 X 10^{10} CFU, 5 X 10^{10} CFU, and 10 X 10^{10} CFU), and the last group was used as a control group. After 7 days of observation, the MLD was determined according to the death of the piglets.

Immune protection test

One hundred and sixty mice weighing 18 to 22 g were divided randomly into 4 groups of 40 mice. Two groups were injected intraperitoneally with the inclusion bodies, and the other 2 groups were injected intraperitoneally with inactivated vaccines of genetically engineered strains. The animals were injected twice at a 14-day interval at a dose of 0.2 mL per animal. Fourteen days after the second immunization, the mice were challenged with 1 MLD and 2 MLD virulent strains of *E coli* C83902, and the death of the mice was observed daily. Another 40 mice with 20 mice in each group were selected and served as the negative control group.

Five pregnant sows were selected. Two of them that produced a total of 25 piglets with an average body weight of 1.51 kg were immunized with the inclusion body intramuscularly in the neck on 30 to 35 days and 15 to 20 days prefarrowing at a dose of 5 mL/animal each time. The other 2 that produced a total of 24 piglets with an average body weight of 1.52 kg were immunized with inactivated vaccines of genetically engineered strains via neck muscles

on 30 to 35 days and 15 to 20 days before delivery at a dose of 5 mL/animal each time. The last 1 that produced a total of 12 piglets with an average body weight of 1.50 kg was not inoculated and used as the negative control.

After the sows gave birth, 1 day after the piglets consumed colostrum, the healthy piglets from the immunized sow and the healthy piglets from the control sow were all challenged, and each piglet was administrated 1 MLD (2.0 X 10^{10} CFU) *E coli* C83902 (K_{88ac}^+, ST^+, and LT^+). All animals were observed for 7 days after challenge, and the test results were recorded.

STa enterotoxin preparation and activity determination

Six rabbits were randomly divided into 3 groups of 2 rabbits, and they were all immunized with the inclusion body. Group 1 was immunized once, and blood was collected on the 20th day to separate serum. Group 2 was immunized twice, with an interval of 14 days for the 2nd immunization, and after the 2nd immunization, blood was collected for preparation of serum on the 15th day. Group 3 was immunized 3 times, with an interval of 14 days each time, after the 3rd immunization, blood was collected for preparation of serum on the 10th day. The above-mentioned sera were, respectively, subjected to a neutralization test of intragastric administration in the suckling mice. The *E coli* HB101 strain (pSLM004) producing enterotoxin STa was streaked and inoculated on LB plates containing 50 µg/mL ampicillin and cultured at 37 °C for 18 hours. A single colony was picked and inoculated in 5 mL liquid LB medium containing 50 µg/mL ampicillin and then cultured at 37 °C for 24 hours with shaking at 170 rpm. Cultures (2 mL) were inoculated in 200 mL of Amp-containing LB broth at 37 °C for 24 hours with shaking at 170 rpm. After centrifuging at 5,000 X *g* for 20 minutes at 4 °C, the supernatant was filtered, sterilized, and diluted 10 times with normal saline for an activity and neutralization test. The prepared STa enterotoxin was taken in different doses (10, 12, 15, 17, and 20 µL) and diluted with normal saline to 0.1 mL, and then the intragastric administration was performed in the suckling mice to determine the minimum amount of STa.

Neutralization test of intragastric administration in the suckling mouse

An equal volume of immune rabbit serum was added to STa enterotoxin of 1 mouse unit and diluted to 0.1 mL with normal saline. After being incubated at 37 °C for 1 hour, the activity of STa enterotoxin in the mixture was measured by intragastric administration in the suckling mouse. A G/C (intestinal weight/residual corpse weight) value was calculated. The G/C value not < 0.09 was considered positive for STa toxin, and the result of the neutralization test was judged as negative. A G/C value not higher than 0.083 was considered negative for STa toxin, and the result of the neutralization test was judged as positive. The G/C values were 0.072, 0.081, 0.093, 0.108, and 0.124 after administration of different doses (10, 12, 15, 17, and 20 µL) of STa enterotoxin.

Inactivation test of K88ac-3STa-LT_B genetic engineering bacteria

The liquid medium containing *E coli* with the bacteria count between 1.15 and approximately 1.23 X 10^{10} CFU/mL was selected from the fermentation tank. Formaldehyde solutions with final concentrations of 0.4%, 0.6%, and 0.8% were added for inactivation at 37 °C. Each inactivation concentration was tested at 3 separate inactivation times (12 h, 24 h, and 48 h). A total of 9 different inactivation concentration levels and times were performed for 3 replicates.

Passive protection test of newborn piglets during the susceptible period

Sixteen pregnant sows were randomly divided into 8 groups of 2 sows. Group 1, group 3, group 5, and group 7 were used as the immunization group, and each sow was immunized twice via neck muscles on 30 to 35 days and 15 to 20 days before delivery at a dose of 5 mL/animal each time. Group 2, group 4, group 6, and group 8 were not immunized as the control group. The piglets produced by the sows in each group were challenged on the 1st, 7th, 14th, and 28th day after suckling.

Determination of the minimum immune dose in mice

One hundred twenty mice were randomly divided into 6 groups of 20 mice. Group 1, group 3, and group 5 were used as the immunization group, and each mouse was injected intraperitoneally twice at a 14-day interval. The immunization doses of the 3 groups were 0.1 mL/mouse, 0.2 mL/mouse, and 0.3 mL/mouse of inactivated vaccines each time. Group 2, group 4, and group 6 were used as the control group and were injected with saline only. Fourteen days after the second immunization, each mouse in the immunization group and the control group was injected intraperitoneally with 1 MLD of the virulent strain C83902. The immunoprotective effects of different vaccine doses on the mice were recorded over 7 days of observation.

Determination of the minimum immune dose of pregnant sows

Eight pregnant sows were randomly divided into 4 groups of 2 sows. Group 1 and group 3 were used as the immunization group, and each sow was immunized twice via neck muscles on 30 to 35 days and 15 to 20 days before delivery. The immunization doses of the 2 groups were 2.5 mL/sow and 5.0 mL/sow of inactivated vaccines each time. Group 2 and group 4 were used as the control group and were injected with saline only. The piglets were challenged with 1 MLD of the virulent strain C83902 on the first day after suckling. After the challenge, the clinical response was observed daily for 7 days. The piglets with diarrhea were not treated until they healed or died. The immune protection effects of different vaccine doses on the newborn piglets were recorded.

Results

Safety test of recombinant strain BL21(DE3)(pXK88ac3STaLT$_B$)

To determine whether the K88ac-3STa-LT$_B$ fusion protein expressed by the recombinant strain had lost the STa enterotoxin activity, mice were inoculated with recombinant strain BL21(DE3) (pXK88ac3STaLT$_B$) by intraperitoneal injection and oral administration. All the mice survived after 3 weeks without clinical symptoms and no pathological changes during necropsy, indicating that the strain was not pathogenic and safe for mice.

Mice and piglet challenge protection test

The test determined that the minimum lethal dose (1 MLD) for mice was 1.5 X 10^8 CFU, and the minimum lethal dose (1 MLD) for piglets was 2 X 10^{10} CFU. The mice immunized with the inclusion body and the engineered strain inactivated vaccine were challenged with *E coli* C83902. With 1 MLD challenge to mice, the protection rate of the inclusion body immunization group was 95% (38/40), and the protection rate of the inactivated vaccine immunization group was 97.5% (39/40). With 2 MLD challenge to mice, the protection rate of the inclusion body immunization group was 90% (36/40), and the protection rate of the inactivated vaccine immunization group was 95% (38/40; **Table 1**).

The results showed that the protection rate in the inclusion body immunized piglets was 92%92% (23/25), and the protection rate in the inactivated vaccine immunized piglets was 87.5% (21/24). In control group, the mortality rate was 91.67% (11/12; **Table 2**).

Neutralization test of intragastric administration in the suckling mouse

The G/C value of the control group (STa + normal saline, STa + sera of healthy rabbit) was not less than 0.09, while the G/C value of the test group (STa + sera of immunized rabbit) was close to the G/C value of sera of healthy rabbit and LB broth. The G/C value of the test group was not > 0.083 **(Table 3)**.

Inactivation test

When the bacterial count reached 1.15 to approximately 1.23 X 10^{10} CFU/mL, no bacterial growth was detected in formaldehyde solution with a final concentration of 0.4% after 48 hours of inactivation, while bacterial growth was detected at both 12 hours and 24 hours of inactivation. No bacterial growth was detected in formaldehyde solution with final concentrations of 0.6% and 0.8% after 24 hours and 48 hours of inactivation, while bacterial growth was detected at for both concentrations at 12 hours of inactivation.

Passive protection test of newborn piglets during the susceptible period

The piglets produced by the sows were selected for a challenge test. A total of 4 piglets in the immunized group developed very mild diarrhea, while the control group developed severe diarrhea and eventually all died. Through this test, it was determined that the protection efficacy of piglets could reach over 80% on the first day after suckling and the protective effect was 90% on the seventh after day suckling, which was the highest in the efficacy test **(Table 4)**.

Determination of the minimum immune dose in mice

After the 1MLD challenge, the immune protection rates of the group 1, group 3, and group 5 were 63.3% (19/30), 86.7% (26/30), and 90% (27/30) in immunized mice. However, all mice in the control group died. The results showed that the minimum immune dose of the vaccine to mice was 0.2 mL/mouse in group 3 **(Table 5)**.

Table 1—Results of the mouse challenge protection test.

	Survival/immune number						
Challenge dose (MLD)	**Inclusion body group**	**Inactivated vaccine group**	**Control group**	**Immunity way**	**Immunity frequency**	**Immunity dose**	**Immunization interval (day)**
1	38/40	39/40	2/20	Intraperitoneal injection	Twice	0.2 mL	14
2	36/40	38/40	0/20	Intraperitoneal injection	Twice	0.2 mL	14

Table 2—Results of the piglet challenge protection test.

Group	**Inclusion body group**	**Inactivated vaccine group**	**Control group**
Survival/immune number	23/25	21/24	1/12
Challenge dose (MLD)	1	1	1
Challenge way	Oral administration	Oral administration	Oral administration
Immunity way	Neck muscle	Neck muscle	–
Immunity dose	5 mL	5 mL	–
Second immunization time	15–20 days before delivery	15–20 days before delivery	–
First immunization time	30–35 days before delivery	30–35 days before delivery	–

Table 3—Results of neutralization test of intragastric administration in the suckling mouse.

Treatment	Amount of sucking mice	G/C average value
Normal saline	9	0.064
LB medium	9	0.065
sera of healthy rabbit	9	0.068
STa + normal saline	9	0.095
STa + sera of healthy rabbit	9	0.103
STa + sera of immunized of group 1	9	0.075
STa + sera of immunized of group 2	9	0.071
STa + sera of immunized of group 3	9	0.061

Table 4—The results of the challenge test of newborn piglets during the susceptible period.

Group	Reagent	Sow	Number of piglet	Number of challenge	Number of survivor	Time of suckling (day)	Protection rate (%)
Group 1	Immune group	2	22	20	17	1	85
Group 2	Control group	2	19	19	0	1	0
Group 3	Immune group	2	21	20	18	7	90
Group 4	Control group	2	19	18	0	7	0
Group 5	Immune group	2	17	17	15	14	88.2
Group 6	Control group	2	23	20	0	14	0
Group 7	Immune group	2	21	20	17	28	85
Group 8	Control group	2	18	16	0	28	0

Table 5—Results of minimum immunization dose of mice.

Group	Reagent	Immunization dose (mL)	Challenge dose (MLD)	Amount	Number of survivor	Protection rate (%)
Group 1	Inactivated vaccine	0.1	1	30	19	63.3
Group 2	Normal saline	0.1	1	10	0	0
Group 3	Inactivated vaccine	0.2	1	30	26	86.7
Group 4	Normal saline	0.2	1	10	0	0
Group 5	Inactivated vaccine	0.3	1	30	27	90
Group 6	Normal saline	0.3	1	10	0	0

Table 6—Results of minimum immunization dose of pregnant sows.

Group	Reagent	Immunization dose (mL)	Challenge dose (MLD)	Amount	Number of survivor	Protection rate (%)
Group 1	Inactivated vaccine	2.5	1	58	49	84.5
Group 2	Normal saline	2.5	1	20	0	0
Group 3	Inactivated vaccine	5.0	1	60	55	91.7
Group 4	Normal saline	5.0	1	20	0	0

Determination of the minimum immune dose of pregnant sows

After the 1MLD challenge, the immune protection rates of the group 1 and group 3 were 84.5% (49/58) and 91.7% (55/60) in immunized piglets. Only 2 piglets had very mild diarrhea in the immunized group. However, all piglets in the control group died. The results showed that the minimum immunization dose for pregnant sows was 2.5 mL/sow in group 1 **(Table 6)**.

Discussion

The prevention and treatment of neonatal piglet diarrhea caused by ETEC are worldwide problems. The disease is widespread and has high morbidity and mortality.[38–40] At present, the treatment of the disease adopts drug treatment. However, more effective is vaccination.[41–43] Due to the complex and diverse pathogen serotypes and the poor broad spectrum of traditional vaccines, the effect of treatment is not satisfactory. The disease has always been the focus and difficulty of scholars at home and abroad.

The difficulty lies in how to eliminate the biological toxicity of STa and give it immunogenicity. The STa enterotoxin contains 6 cysteine residues and forms 3 pairs of intrachain disulfide bonds. The 3 pairs of disulfide bonds are vital to the biological toxicity of STa. If these disulfide bonds are destroyed, the biological toxicity of STa can be lost.[7,44] According to the characteristics of STa, we used PCR and gene-directed mutation technology to amplify the *K88ac*

gene, STa mutant gene, and LT_B gene from the plasmid of *E coli* C83902 and constructed the recombinant strain BL21(DE3)(pXK88ac3STaLT_B). The recombinant strain expressed the K88ac-3STa-LT_B fusion protein in the form of inclusion bodies and exists in the bacteria. The fusion protein retained the original antigenicity of K88ac and LT_B, and at the same time endowed STa immunogenicity, which was not available before. Studies on the immunogenicity of the K88ac-3STa-LT_B fusion protein showed that both the fusion protein inclusion body and the engineered strain inactivated vaccine could induce good immune protection in animals and could resist the virulent strain of *E coli* C83902 (K88ac$^+$, ST$^+$, and LT$^+$). In addition, the immune rabbit serum could neutralize the toxicity of natural STa enterotoxin, which fully showed that the vaccine retained the good immunogenicity of K88ac and LT_B and at the same time conferred STa immunogenicity. The research results showed that the vaccine was safe and effective, so the constructed engineering strain BL21(DE3) (pXK88ac3STaLT_B) could be used as a candidate strain for the genetically engineered inactivated vaccine to prevent *E coli* diarrhea in newborn piglets.

The test results showed that the inactivated vaccine was safe and effective and confirmed the feasibility of the industrialized production process of the vaccine. This experiment showed that the best condition was the inactivation with the final concentration of 0.4% formaldehyde solution at 37 °C for 48 hours.

Piglet gains passive immunity by feeding colostrum after birth. Colostrum contains a large number of immune antibodies, which makes the piglet acquire immune antibodies and the ability to resist certain diseases. The gut of the newborn piglet has the ability to absorb these immunoglobulins, and this ability disappears after 48 hours. Therefore, the piglet test should be done after 48 hours. The vaccine in this study was used to prevent *E coli* diarrhea disease in newborn piglets. The purpose of the K88ac-3STa-LT_B-inactivated vaccine for piglet diarrhea is to target the piglet so that the piglet could obtain the best protection and reduce morbidity and mortality. The suckling time of the piglets selected in this experiment was 1, 7, 14, and 28 days, and the challenge time covered the entire susceptible period of the piglets. In this experiment, the protection rate of 1 day of milking was 85% which was similar to the average protection rate of the whole period. Therefore, the piglets were selected to challenge after 1 day of milking in the vaccine efficacy test. From the data point of view, it was reasonable and better reflected the true protective effect of the vaccine.

In this study, mice and sows were used as the model animals for the vaccine efficacy test to facilitate production and testing. Although 5 mL/sow could obtain a high immune protection rate, considering the practical application, 2.5 mL/sow was selected as the minimum immune dosage. Safety and efficacy tests showed that the safety and efficacy test between mice and sows had a good parallel relationship. The immune protection test in mice can be used to test the efficacy and quality of the vaccine in the vaccine production process. If the immunized pregnant sows and newborn piglets are used as the detection indicator, the cost of quality inspection and monitoring in vaccine production is very high. The use of mice for the immune challenge protection test can greatly save the production cost of vaccine manufacturers. Therefore, it was feasible to use mice instead of sows for safety inspections. The above research showed that the K88ac-3STa-LT_B trivalent genetically engineered inactivated vaccine for *E coli* diarrhea in newborn piglets provided a new genetically engineered vaccine candidate strain for preventing *E coli* diarrhea in piglets.

The efficacy test of the K88ac-3STa-LT_B genetically engineered inactivated vaccine showed that the immune protection rate of the newborn piglets could reach 85% on the first day after suckling. At the same time, it was determined that the minimum immunization doses for mice and pregnant sows were 0.2 and 2.5 mL, respectively. Overall, this research provided a new type of vaccine, showed that the K88ac-3STa-LT_B trivalent genetically engineered inactivated vaccine had a broad immune spectrum for *E coli* diarrhea in newborn piglets, and provided a new genetically engineered vaccine candidate strain for the prevention of *E coli* diarrhea in piglets.

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The authors declare that there are no conflicts of interest associated with the publication of this article.

This animal study was approved by the Institutional Animal Care and Use Committee and complied with the principles of Laboratory Animal Management of China. All procedures performed in studies involving animals were in accordance with the ethical standards of the Ethics Committee of Shaoguan University.

ChongBo Xu and Yimin Lin designed the research. ChongLi Xu and Yuhan She performed the research. Kun Peng, Fengyang Fu, and Qinhong Shi analyzed the data. All authors contributed to the writing and revision of the manuscript. All authors reviewed the manuscript.

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