

A Multivalent Vaccine for Preventing Piglet Diarrhea A New Multivalent Vaccine Candidate Targeting Fimbriae and Enterotoxin of Enterotoxigenic *Escherichia coli* for Preventing Newborn Piglet Diarrhea

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Abstract

In order to develop the K88ac-3ST_a-LT₂ trivalent genetically engineered inactivated vaccine, the recombinant strain BL21(DE3)(pXK88ac3ST_aLT₂) was constructed. ELISA test showed that the K88ac-3ST_a-LT₂ fusion protein could be recognized by ST_a monoclonal antibody, LT₂ and K88ac antibody. The test of intragastric administration in the suckling mouse confirmed that the expressed fusion protein had lost the toxicity of the natural ST_a enterotoxin. The results of immune experiments showed that K88ac-3ST_a-LT₂ fusion protein could induce rabbits to produce serum antibodies, which had the effect of neutralizing the toxicity of natural ST_a enterotoxin. Efficacy test of the K88ac-3ST_a-LT₂ genetically engineered inactivated vaccine showed that the immune protection rate of the newborn piglets could reach 85% on the 1st day after suckling. At the same time, it was determined that the minimum immunization doses for mice and pregnant sows were 0.2 mL and 2.5mL respectively. This study provided scientific parameters for the industrialized production of the vaccine and showed that the K88ac-3ST_a-LT₂ trivalent genetically engineered inactivated vaccine had a broad immune spectrum for *E. coli* diarrhea in newborn piglets. Through this research, a new genetic engineering vaccine candidate strain was provided for more effective prevention of *E. coli* diarrhea in piglets.

Introduction

Enterotoxigenic *Escherichia coli* (ETEC) is the main pathogen causing *E. coli* diarrhea in newborn piglets^[1, 2, 3, 4]. ETEC includes two pathogenic factors: enterotoxin and adhesin, among which adhesin mainly includes K88, K99 and 987P^[5, 6]. ETEC uses these adhesin to settle on the epithelial cells of the host intestinal mucosa, thereby multiplying and producing large amounts of enterotoxin. Enterotoxin cause pathological changes in intestinal mucosal epithelial cells, leading to piglet diarrhea^[7, 8, 9]. The main adhesion of ETEC from pigs is K88ac, which is an indirect diarrhea-causing factor that causes diarrhea in piglets^[10, 11, 12]. Enterotoxin is a direct cause of diarrhea in piglets, including heat-stable enterotoxin (ST) and heat-labile enterotoxin (LT)^[13, 14, 15, 16]. According to antigenicity and host difference, ST is divided into ST_a and ST_b. Among them, ST_a is the most direct pathogenic factor^[17, 18, 19]. The non-immunogenic ST_a is a small peptide of 18 or 19 amino acids that still remains active even if heated to 100°C for 30 minutes^[20, 21]. ST_a can activate guanylate cyclase (GC), and its biological activity is tissue specific. It can increase cyclic guanosine monophosphate(cGMP) in ileal epithelial cells by 10 times while the lower part of the colon only increases by 1.8 times. ST_a is inactive in other tissues and organs, and this specificity may be related to the distribution of receptors. The increased cGMP content in the villi epithelial cells of the small intestine makes the Cl⁻ transporter phosphorylate and the active Cl⁻ transport is inhibited. Eventually, the electrolyte and water retention in the intestinal lumen cause diarrhea^[22]. LT is composed of toxic subunit LT₁ and binding subunit LT₂^[23, 24, 25]. LT or LT₂ has good immunogenicity. The LT₂ subunit binds to the ganglioside receptor on the intestinal epithelial cell membrane. After the LT₁ subunit enters the intestinal epithelial cell, it activates adenylate cyclase in the cytoplasm and increases the level of cAMP. As a result, it changes the permeability of cell membranes to water and salt ions and leads to hypersecretion of glandular epithelial cells. Above changes cause the increase in the secretion of Cl⁻, Na⁺, HCO₃⁻ and water in the small intestine, exceeding the reabsorption capacity of the intestinal tube, and eventually leading to diarrhea^[26, 27].

The onset of *E. coli* diarrhea in newborn piglets is acute and the course of the disease develops rapidly. Because the emergence of drug-resistant strains, the effect of drug treatment is not ideal. Scientists from all over the world

have conducted a lot of research on vaccines against this disease. Due to the large number of ETEC serotypes, most of the serotypes only produce ST, and the immunogenicity of ST is very weak. None of the existing vaccines has solved the problem of the immunogenicity of ST, and therefore all current vaccines have not reached a very high level. At present, more than 50% of the isolates of piglet diarrhea cases contain K88 antigen and ST_a pathogenic factors. Targeting the direct diarrheal factors heat-stable enterotoxin (ST), heat-labile enterotoxin (LT) and the main adhesion factor K88 of ETEC, the *K88ac* gene, *ST_a* mutant gene and *LT₂* gene were amplified from *E.coli* C83902 plasmid to construct *K88ac-3ST_a-LT₂* fusion gene by using genetic engineering technology. At the same time, the genetically engineered strain BL21(DE3) (pXK88ac3ST_aLT₂) expressing K88ac-3ST_a-LT₂ fusion protein was constructed. The fusion protein not only maintained the good immunogenicity of K88ac fimbriae antigen and LT₂ enterotoxin, but also conferred ST_a immunogenicity. In the construction process, ST_a lost its original biological toxicity through site-directed mutation, so that the purpose of immune prevention could be achieved through two ways of fimbriae and enterotoxin. This strain was used to develop the piglet *E.coli* K88ac-3ST_a-LT₂ trivalent genetically engineered inactivated vaccine. The vaccine had the following characteristics: First of all, the genetic engineering vaccine successfully solved the ST_a biological toxicity problem. By PCR site-directed mutagenesis technology, Cys was converted to Ser, resulting in the disappearance of one of the three pairs of disulfide bonds, so that the spatial conformation of ST_a was changed and the biological toxicity of ST_a was lost. Secondly, we successfully solved the problem of weak immunogenicity of heat-resistant enterotoxin ST_a. In this study, three mutant *ST_a* genes were linked together using genetic engineering technology and two antigenic determinants had been added to the expression product, thereby improving the immunogenicity of ST_a. And then, we had successfully constructed recombinant strain BL21(DE3) (pXK88ac3ST_aLT₂) expressing the K88ac-3ST_a-LT₂ fusion protein. It is particularly important that the fusion protein not only maintained the good immunogenicity of K88ac fimbriae antigen and LT₂ enterotoxin, but also conferred ST_a immunogenicity. Moreover, the K88ac-3ST_a-LT₂ trivalent genetically engineered inactivated vaccine also had a wide range of immune spectrum. Therefore, a new genetic engineering vaccine candidate strain was provided for more effective prevention of *E. coli* diarrhea in piglets through this research.

Materials And Methods

Construction of the recombinant expression strain BL21 (DE3)(pXK88ac3ST_aLT₂)

Based on the *ST_a*, *LT₂* and *K88ac* sequence reported by Chongbo Xu et al^[28,29] and Dykes et al^[30], three *ST_a* mutant genes were amplified using C83902 plasmid DNA as the template and the primer1 5'-CCC**AAGCTT**AACAACACATTTTACTGC-3', the primer2 5'-GGAATTC**CATATGAT** AACTTCCAGCACTGGC-3', the primer3 5'-GGAATTC**CATATG**AACAACACATTTTACTG

C-3', the primer4 5'-CCG**GAATTC**ATAACTTCCAGCACTGGC-3' and the primer5 5'-CCG**GAATTC**AACAACACATTTTACTGC-3', the primer6 5'-CGC**GGATCC**ATAACTTCCAGCA

CTGGC-3'. The primers contained the *Hind* III, *Nde* I, *Eco*R I and *Bam*H I restriction endonuclease sites(italics) and protective bases, respectively. The *K88ac* gene fragment was amplified from the template C83902 plasmid by primer7 5'-CATG**CCATGGC**ATTTACTGAC TATGAAGAA-3' and P8 5'-CCC**AAGCTT**GAGAATATCATTTCTTGATAG-3'. Primer7 and primer8 contained *Nco* I and *Hind* III restriction endonuclease sites(italics)and protective bases, respectively. The *LT₂* gene fragment was amplified from the template C83902 plasmid by

P9 5'-CGC**GGATCCC**AGACTATTACAGAACT A-3' and P10 5'-ATAAGAAT **GCGGCCGC**

AAGCTTGCCCCTCCAGCCTAG C-3'. Primer9 and primer10 contained *Bam*H I and *Not* I restriction endonuclease sites(italics)and protective bases, respectively. The three cloned *ST_a* mutant genes were connected in series and connected with the *K88ac* and *LT₂* genes to construct the fusion gene *K88ac-3ST_a-LT₂*. The fusion gene was cloned into the expression vector pET-28b and the recombinant expression plasmid pXK88ac3ST_aLT₂ was constructed. Then the recombinant plasmid pXK88ac3ST_aLT₂ was transformed to the BL21(DE3) recipient strain and the recombinant expression strain BL21(DE3) (pXK88ac3ST_aLT₂) was constructed. The pXK88ac3ST_aLT₂ plasmid was extracted for restriction digestion identification and nucleotide

sequence analysis.

Induction and SDS-PAGE analysis of BL21(DE3)(pXK88ac3ST_aLT₂) and ELISA detection of K88ac-3ST_a-LT₂ fusion protein

BL21(DE3)(pXK88ac3ST_aLT₂) was coated separately to Kan-containing LB plates and cultured overnight at 37°C. Single colony were picked and inoculated in 5 mL Kan-resistant liquid LB medium, then cultured overnight at 37°C with shaking at 170 r/min. The recombinant bacteria were inoculated into a culture flask containing 250 mL of LB medium at a ratio of 1% to logarithmic growth phase ($OD_{600}=0.4\sim 0.6$). Then, IPTG was added at a final concentration of 1mmol/L and cultured overnight at 37°C with shaking at 170 r/min. Cultures (1mL) of BL21(DE3)(pXK88ac3ST_aLT₂) was centrifuged separately at 12,000 *g* for 10 min at 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 *g* for 10 min at 4°C. Pellets were resuspended in 25 μ L of water. Once the bacteria are dispersed, 25 μ L of 2 \times SDS gel electrophoresis loading buffer was immediately added and shaken for 20 seconds. Boiled in a boiling water bath for 3~5 minutes, and then was centrifuged at 12,000 *g* for 10 min at room temperature. 25 μ L of supernatant was taken for SDS-PAGE analysis. The BL21(DE3) (pXK88ac3ST_aLT₂) cells were lysed with ultrasound and the K88ac, ST_a and LT₂ in the fusion protein were detected by ELISA test kit(Nanjing Jin

Yibai Biological Technology Co. Ltd.).

K88ac-3ST_a-LT₂ antigen preparation

The 50 mL cultures induced by IPTG for 4 hours were centrifuged to collect the bacteria, then resuspended in 5 mL TE (50 mmol/L Tris·Cl, 2 mmol/L EDTA). The lysozyme at a final concentration of 100 μ g/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 twice times for 10 seconds each, and then centrifuged at 12,000 r/min for 15 minutes. The precipitate was the crude inclusion body. After 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 is added to the engineering bacteria culture solution at a final concentration of 0.4% to inactivate the bacteria, and then aluminum hydroxide gel is added to 10% as an antigen for immunization.

Safety test of BL21(DE3)(pXK88ac3ST_aLT₂) and minimum lethal dose test of challenge strain

In order to determine whether the K88ac-3ST_a-LT₂ fusion protein expressed by the recombinant strain had lost the ST_a enterotoxin activity, forty 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)(pXK88ac3ST_aLT₂). The other 4 groups were inoculated orally. The clinical response of the test mice was observed daily, and the necropsy was performed after continuous observation for 3 weeks.

Sixty mice weighing 18-22g were divided randomly into six groups of 10 mice. One of the group was used as a control group and the other groups were challenged separately with 25, 50, 100, 150 and 200 million live bacteria. After 3 days of observation, the minimum lethal dose (MLD) was determined according to the death of the mice. Twelve newborn piglets were divided randomly into four groups of 3 piglets. Each of three groups was challenged separately with 0.2, 2 and 10 billion live bacteria and the last group was used as a control group. After 7 days of observation, the minimum lethal dose (MLD) was determined according to the death of the piglets.

Immune protection test

One-hundred sixty mice weighing 18-22g were divided randomly into four groups of 40 mice. Two groups were injected intraperitoneally with crude inclusion bodies, and the other two groups were injected intraperitoneally with inactivated vaccines of genetically engineered strains. The animals were injected twice with a 14-d 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 forty mice with 20 mice in each group were selected and served as the negative control group.

Five pregnant sows were selected. Two of them were immunized with crude inclusion body via neck muscle on 30 to 35 days and 15 to 20 days before delivery at a dose of 5mL/animal each time. The other two 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 5mL/animal each time. The last one was not inoculated and used as the negative control. After the sows gave birth, one day after the piglets sucked the colostrum, the healthy piglets from the immunized sow and the healthy piglets from the control sow were all challenged, and each piglet was administrated with 1 MLD *E. coli* C83902(K_{88ac}⁺, ST⁺ and LT⁺). All animals were observed for 7 days after challenge and the test results were recorded.

ST_a enterotoxin preparation and activity determination

Six rabbits were randomly divided into three groups of 2 rabbits and they were all immunized with crude 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 second immunization, after the second immunization, blood was collected for preparation of serum on the 15th day. Group 3 was immunized three times, with an interval of 14 days each time, after the third immunization, blood was collected for preparation of serum on the 10th day. And the above-mentioned sera were respectively subjected to neutralization test of intragastric

administration in the suckling mice. The *E. coli* HB101 strain (pSLM004) producing enterotoxin ST_a was streaked and inoculated on Amp-containing LB plates and cultured at 37°C for 18 hours. Single colony were picked and inoculated in 5 mL Amp-resistant liquid LB medium, then cultured at 37°C for 24 hours with shaking at 170r/min. Cultures (2mL) was inoculated in 200 mL of Amp-containing LB broth at 37°C for 24 hours with shaking at 170r/min. After centrifuged at 5,000g for 20min at 4°C, the supernatant was filtered and sterilized and diluted 10

times with normal saline for activity determination and neutralization test. The prepared ST_a enterotoxin was taken in different doses (10, 12, 15, 17, 20 µL), 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 ST_a enterotoxin of 1 mouse unit.

Neutralization Test of intragastric administration in the suckling mouse

An equal volume of immune rabbit serum was added to ST_a enterotoxin of 1 mouse unit and diluted to 0.1 mL with normal saline. After incubated at 37°C for 1 hour, the activity of ST_a enterotoxin in the mixture was measured by intragastric administration in the suckling mouse. The G/C (intestinal weight/residual corpse weight) value was calculated. The G/C value not less than 0.09 was considered positive for ST_a toxin, and the result of neutralization test was judged as negative. The G/C value not higher than 0.083 was considered negative for ST_a toxin, and the result of neutralization test was judged as positive. Furthermore, the neutralization effect of the immunized rabbit serum antibodies was evaluated.

Selection of the best medium

The prepared LB, modified LB and common broth medium were added separately into the fermentor tank, 100,000 mL per tank, inoculated with the seed solution of *E. coli* strain at a ratio of 2%. After cultured with aeration at 37°C for 6 hours, the glucose solution was added to the final concentration of 0.2%. After culturing for 16 hours, the number of viable bacteria was counted. The test was repeated 3 times for each medium, and the number of bacteria in each medium was recorded and the average value (CFU/mL) was calculated.

Selection of the best inducer

The modified LB medium was added to four fermentation tanks, 100,000 mL per tank, inoculated with the seed solution of *E. coli* strain at a ratio of 2%. After cultured with aeration at 37°C for 4~6 hours, the glucose was added with a final concentration of 0.2% to supplement the carbon source. After culturing for 4 hours, the final concentration of 1mmol/LIPTG, 1mmol/Llactose, 10mmol/Llactose, 100mmol/Llactose were added respectively to each tank for induction. After induced for 6 hours, 1 mL of bacterial solution from each tank was analyzed by SDS-PAGE and the expression of the target protein was detected.

Screening of the best induction conditions for lactose

The seed solution was inoculated into the fermentation tanks at a ratio of 2%. After culturing at 37°C for 4~6 hours, the glucose was added with a final concentration of 0.2% to supplement the carbon source. After continuing to culture with aeration for 4 hours, the final concentration of 100 mmol/L lactose was added to induce 6 hours. During this period, 1 mL of bacterial solution was taken every 1 hour to count the viable bacteria. At the same time, the expression of the target protein was detected by SDS-PAGE.

Selection of the best aeration culture condition

The seed liquid of *E. coli* BL21(DE3) (pXK88ac3ST_aLT₂) strain was inoculated into three fermentation tanks at a ratio of 2%, 100,000 mL per tank, and the aeration volume of the three tanks was 50 L/min, 100 L/min, and 500 L/min. After culturing at 37°C for 6 hours, the glucose was added with a final concentration of 0.2% to supplement

the carbon source. After continuing to culture with aeration for 4 hours, the final concentration of 100 mmol/L lactose was added. After induced for 6 hours, 1 mL of bacterial solution from each tank was analyzed by SDS-PAGE and the expression of the target protein was detected. The experiment was repeated 3 times to improve the credibility of the data and found the most suitable ventilation.

Inactivation test of K88ac-3ST_a-LT₂ genetic engineering bacteria

The *E. coli* bacteria liquid with the bacteria count between $1.15\sim 1.23\times 10^{10}$ CFU/mL was selected from three fermentation tanks. Each tank was divided into a total of 27 parts. The 27 parts of bacteria liquid were randomly divided into 3 groups, each of which had a total of 9 parts. The 9 parts of bacteria liquid were randomly divided into 3 groups, each of which had a total of 3 parts. Each group was inactivated by adding formaldehyde solution with final concentration of 0.4%, 0.6% and 0.8% at 37°C. The inactivation time was 12h, 24h, 48h, respectively. During the inactivation process, the bacterial solution needed to be shaken several times to ensure the complete inactivation of the bacterial solution.

Passive protection test of newborn piglets during the susceptible period

Sixteen pregnant sows were randomly divided into eight groups of 2 sows. Group1, group3, group5 and group7 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 5mL/animal each time. Group2, group4, group6 and group8 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 six groups of 20 mice. Group1, group3 and group5 were used as the immunization group, and each mouse was injected intraperitoneally twice with a 14-d interval. The immunization doses of the three groups were 0.1mL/mouse, 0.2mL/mouse and 0.3mL/mouse of inactivated vaccines each time. Group2, group4 and group6 were used as the control group and were injected with saline only. 14 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. Observed for 7 days, the immune protective effects of different vaccine doses on the mice were recorded.

Determination of the minimum immune dose of pregnant sows

Eight pregnant sows were randomly divided into four groups of 2 sows. Group1 and group3 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 two groups were 2.5mL/sow and 5.0mL/sow of inactivated vaccines each time. Group2 and group4 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 1st 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.

Result

Construction of the expression plasmid pXK88ac3ST_aLT₂

Using PCR site-directed mutagenesis technology, Three 60-bp ST_a genes with the TGT→AGT(Cys→Ser) mutation was amplified by using three pairs of ST_a PCR primers from *E. coli* C83902 plasmid. The three cloned ST_a mutant genes were connected in series into 180bp ST_a - ST_a - ST_a fusion gene. The 330-bp *K88ac* and 500-bp LT_2 gene fragments were amplified separately using C83902 plasmid DNA as the template. The constructed ST_a - ST_a - ST_a fusion gene was connected with the *K88ac* and LT_2 genes to construct the fusion gene *K88ac*-3 ST_a - LT_2 and cloned into pET-28b to generate the recombinant plasmid pXK88ac3 ST_a LT₂. It was transformed into the recipient bacteria BL21(DE3), and the recombinant strain BL21 (DE3) (pXK88ac3 ST_a LT₂) was constructed. Restriction enzyme digestion of pXK88ac3 ST_a LT₂ with *Nco* I/*Not* I confirmed the presence of the *K88ac*-3 ST_a - LT_2 gene. Moreover, the TGT→AGT(Cys→Ser) mutation was confirmed by sequence analysis (Fig. 1).

SDS-PAGE analysis and ELISA detection of *K88ac*-3 ST_a - LT_2 fusion protein

BL21(DE3)(pXK88ac3 ST_a LT₂) culture was induced with IPTG for 4 h at 37°C, and SDS-PAGE analysis showed that *K88ac*-3 ST_a - LT_2 was expressed highly in the *E. coli* strain BL21(DE3). The *K88ac*-3 ST_a - LT_2 proteins accounted for 73.53% of total cellular protein(Fig.2). The *K88ac*-3 ST_a - LT_2 fusion protein could be recognized by the ST_a monoclonal antibody, *K88ac* and LT_2 antibody through ST_a , *K88ac* and LT_2 ELISA detection kits.

Safety test of recombinant strain BL21(DE3)(pXK88ac3 ST_a LT₂)

In order to determine whether the *K88ac*-3 ST_a - LT_2 fusion protein expressed by the recombinant strain had lost the ST_a enterotoxin activity, Recombinant strain BL21(DE3)(pXK88ac3 ST_a LT₂) was inoculated to mice 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 very safe.

Mice and piglet challenge protection test

The test determined that the minimum lethal dose (1 MLD) for mice was 50 million live bacteria, and the minimum lethal dose (1 MLD) for piglets was 200 million live bacteria. The mice immunized with the crude inclusion body and the engineered strain inactivated vaccine were challenged with the *E. coli* C83902 and both obtained better immune protection.

With 1 MLD challenge, 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, 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).

Two sows immunized with crude inclusion body produced 25 piglets and the other two sows immunized with engineered strain inactivated vaccines produced 24 piglets. As a control group, the sow produced 12 piglets. All piglets were healthy and were challenged. After the newborn piglets were challenged, 23 piglets immunized with crude inclusion body survived and the protection rate was 92% (23/25) and 21 piglets immunized with the inactivated vaccine crude survived and the protection rate was 87.5% (21/24). In the control group, 11 of the 12 piglets died, and the mortality rate was 91.67% (11/12) (Table 2).

Table1 Results of the mouse challenge protection test

challenge dose(MLD)	survival/immune number			immunity way	immunity frequency	immunity dose	immunization interval (day)
	inclusion body group	inactivated vaccine group	control group				
1	38/40	39/40	2/20	intraperitoneal injection	twice	0.2mL	fourteen
2	36/40	38/40	0/20	intraperitoneal injection	twice	0.2mL	fourteen

Table2 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
challengeway	oral administration	oral administration	oral administration
immunity way	neck muscle	neck muscle	-
immunity dose	5mL	5mL	-
second immunization time	15 to 20 days before delivery	15 to 20 days before delivery	
first immunization time	30 to 35 days before delivery	30 to 35 days before delivery	

Neutralization Test of intragastric administration in the suckling mouse

Different doses (10, 12, 15, 17, 20 μ L) of ST_a enterotoxin were administrated intragastrically to suckling rats, and the G/C values were 0.072, 0.081, 0.093, 0.108, 0.124, respectively. Therefore, it was determined that the ST_a enterotoxin of 1 murine unit was 15 μ L.

The ST_a enterotoxin of 1 murine unit (15 μ L) was added with 15 μ L of immune rabbit serum and diluted with normal saline to 0.1 mL. After induction at 37 °C for 1 hour, the test of intragastric administration in the suckling mouse was carried out. The result showed that the G/C value of the control group(ST_a+Normal Saline, ST_a+Sera of healthy rabbit) is not less than 0.09, while the G/C value of the test group(ST_a+Sera of immunized rabbit) is close to the G/C value of sera of healthy rabbit and LB broth. The G/C value of the test group is not higher than 0.083. This indicates that the serum antibodies induced by the inclusion bodies of the K88ac-3ST_a-LT₂ fusion protein in rabbits can neutralize the ST_a enterotoxin produced by pathogenic bacteria (Table 3).

Table3 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
ST _a + normal Saline	9	0.095
ST _a +sera of healthy rabbit	9	0.103
ST _a +sera of immunized of group 1	9	0.075
ST _a +sera of immunized of group 2	9	0.071
ST _a +sera of immunized of group 3	9	0.061

Effect of different media on the growth of strains

The three configured mediums had a greater impact on the growth of bacteria. Among them, the number of bacteria in the modified LB medium compared with the LB medium increased significantly ($P < 0.01$), and the number of bacteria in the normal broth medium compared with the LB medium increased significantly ($P < 0.01$), there was little difference in the number of bacteria between the normal broth medium and the modified LB medium ($P > 0.05$). From the perspective of the stability of the strain culture environment and the reduction of industrial production costs, the modified LB medium was the most suitable medium (Table 4).

Table.4 Number of recombinant strains cultivated in different media

group	culture medium	batch	live bacteria (1×10^9 CFU/mL)	average number of bacteria (1×10^9 CFU/mL)
group1	LB	1	8.91	8.46±0.69
		2	7.85	
		3	8.63	
group2	modified LB	1	11.60	11.11±3.21**
		2	10.43	
		3	11.31	
group3	normal broth medium	1	11.92	11.45±0.41**
		2	10.84	
		3	11.61	

** $P < 0.01$

Effect of different inducers on the growth of strains

The final concentrations of 1 mmol/L IPTG and 100 mmol/L lactose were used to induce expression, respectively. SDS-PAGE and gel imaging analysis showed that the expression levels of the two were 29.43% and 33.24%, respectively. Considering the production cost, lactose should be used as an inducer(Fig.3).

Effect of different induction time on the growth of strains

As the induction time increased, the pH value decreased first and then increased, and the total bacterial count and protein expression also increased accordingly. When the induction time was 6h, the protein expression reached the maximum value. SDS-PAGE and gel imaging analysis showed that the protein expression level was 30.02% (Fig.4).

Influence of different aeration on the growth of strains

The BL21(DE3)(pxK88ac3ST_aLT₂) strain was aerated and cultivated with modified LB medium. The ventilation volume is 50 L/min, 100 L/min, 500 L/min. Among them, the number of bacteria under the culture condition of 500 L/min was the highest, and there was no significant difference in the expression of the target protein in the three cases .

Inactivation test

Formaldehyde solutions of three different concentrations (0.4%, 0.6%, 0.8%) were added separately to the cultured bacteria for inactivation. The results showed that 0.4% formaldehyde solution for 48h can achieve a good inactivation effect.

Passive protection test of newborn piglets during the susceptible period

The healthy piglets produced by the sows were selected for a challenge test. Through this test, it was determined that the protection efficiency of piglets could reach over 80% on the 1st day after suckling and the protective effect was 90% on the 7th after suckling, which was the highest in the efficacy test(Table 5).

Tab.5 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(%)
group1	immune group	2	22	20	17	1	85
group2	control group	2	19	19	0	1	0
group3	immune group	2	21	20	18	7	90
group4	control group	2	19	18	0	7	0
group5	immune group	2	17	17	15	14	88.2
group6	control group	2	23	20	0	14	0
group7	immune group	2	21	20	17	28	85
group8	control group	2	18	16	0	28	0

Determination of the minimum immune dose in mice

After the 1MLD challenge, the immune protection rates of the group1, group3 and group5 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 0.2mL/mouse (Table 6).

Tab.6 Results of minimum immunization dose of mice

group	reagent	immunization dose(mL)	challenge dose(MLD)	amount	number of survivor	protection rate(%)
group1	inactivated vaccine	0.1	1	30	19	63.3
group2	normal saline	0.1	1	10	0	0
group3	inactivated vaccine	0.2	1	30	26	86.7
group4	normal saline	0.2	1	10	0	0
group5	inactivated vaccine	0.3	1	30	27	90
group6	normal saline	0.3	1	10	0	0

Determination of the minimum immune dose of pregnant sows

After the 1MLD challenge, the immune protection rates of the Group1 and group3 were 84.5%(49/58) and 91.7% (55/60) in immunized piglets. However, all piglets in the control group died. The results showed that the minimum

immunization dose for pregnant sows was 2.5 mL/sow(Table7).

Tab.7 Results of minimum immunization dose of pregnant sows

group	reagent	immunization dose(mL)	challenge dose(MLD)	amount	Number of survivor	protection rate(%)
group1	inactivated vaccine	2.5	1	58	49	84.5
group2	normal saline	2.5	1	20	0	0
group3	inactivated vaccine	5.0	1	60	55	91.7
group4	normal saline	5.0	1	20	0	0

Discussion

The prevention and treatment of neonatal piglet diarrhea caused by enterotoxigenic *Escherichia coli* (ETEC) is a worldwide problem. The disease is widespread and has high morbidity and

mortality^[31,32,33]. Even the piglet recovers from illness and survives, their growth and

production performance indicators are still severely affected resulting in serious economic

losses to the pig industry^[34,35]. At present, the prevention and treatment of the disease mainly adopts drug treatment and different types of whole cell vaccines and specific fimbriae vaccines for immunization^[36,37,38]. However, due to the complex and diverse pathogen serotypes and the poor broad-spectrum of traditional vaccines, the effect of drug treatment and vaccine prevention 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 ST_a and give it immunogenicity. The ST_a enterotoxin contains six cysteine residues and forms three pairs of intrachain disulfide bonds. The three pairs of disulfide bonds are vital to the biological toxicity of ST_a. If these disulfide bonds are destroyed, the biological toxicity of ST_a can be lost^[39,40]. According to the characteristics of ST_a, we used PCR and gene-directed mutation technology to amplify the *K88ac* gene, *ST_a* mutant gene and *LT₂* gene from the plasmid of *E. coli* C83902, and constructed the recombinant strain BL21(DE3)(pXK88ac3ST_aLT₂). The recombinant strain expressed the K88ac-3ST_a-LT₂ fusion protein in the form of inclusion bodies and exists in the bacteria. The fusion protein retained the original antigenicity of K88ac and LT₂, and at the same time endowed ST_a immunogenicity which was not available before. Studies on the immunogenicity of the K88ac-3ST_a-LT₂ 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⁺, LT⁺). In addition, the immune rabbit serum could neutralize the toxicity of natural ST_a enterotoxin, which fully showed that the vaccine retained the good immunogenicity of K88ac and LT₂ and at the same time conferred ST_a immunogenicity. The research results showed that the vaccine was safe and effective, so the constructed

engineering strain BL21(DE3) (pXK88ac3ST_aLT₂) could be used as a candidate strain for the genetically engineered inactivated vaccine to prevent *E. coli* diarrhea in newborn piglets.

At the same time, this study regulated the expression of strains from the aspects of culture medium, inducer, ventilation and culture time. The selection of the medium for vaccine production must first ensure the immunogenicity, convenient preparation and stable quality of the vaccine, and secondly consider the production cost. In the experiment, the number of bacteria cultured in the modified LB medium was significantly higher than that of the LB medium, but slightly lower than that of the ordinary broth medium. The production cost of the modified LB medium was lower than that of the ordinary broth medium by more than 50%, and the modified LB medium was convenient to prepare. It was especially important that the difference between batches was small, so the modified LB medium was used for the industrial production of vaccines. The use of lactose inducers could greatly reduce the cost of industrial production. Studies had shown that the fusion protein expression was the highest when induced by 100 mmol/L lactose for 6 hours.

E. coli is a facultative anaerobic bacteria that can grow and reproduce vigorously under aerobic conditions. Blowing sterile air into the culture medium during the culture process can increase and maintain a certain degree of oxygen solubility in the culture medium. There is a critical value for the oxygen solubility of each bacterial culture, and excessive oxygen solubility is not conducive to the growth of bacteria. Three different aeration experiments were carried out on 100,000 mL of culture medium. The results showed that the number of bacteria cultured at 500 L/min aeration was the highest, and it had no significant effect on the relative expression of the target protein. Therefore, it was determined that the optimal ventilation rate was 500 L/min under industrial production conditions. By screening the culture conditions of BL21(DE3)(pXK88ac3ST_aLT₂) strain, the best industrialized production process was determined. The test results showed that the inactivated vaccine was safe and effective and confirmed the scientificity and feasibility of the industrialized production process of the vaccine. Considering the pXK88ac3ST_aLT₂ plasmid contained a kanamycin resistance gene, the inactivated vaccine must be prepared even if the engineered strain BL21(DE3) (pXK88ac3ST_aLT₂) was non-toxic and safe. Through this experiment, the best condition was the inactivation with the final concentration of 0.4% formaldehyde solution at 37 °C for 48 hours.

The vaccine in this study is used to prevent *E. coli* diarrhea disease in newborn piglets, and the disease is clinically susceptible only within 3 weeks after the piglet is born. After more than 3 weeks, the susceptibility is greatly reduced and even the piglets are not infected. Therefore, the purpose of the K88ac-3ST_a-LT₂ inactivated vaccine for piglet diarrhea is to target the susceptible period of the piglet, so that the piglet could obtain the best protection and reduce the morbidity and mortality. The suckling time of the piglets selected in this experiment was 1, 7, 14, 28 days, and the challenge time covered the entire susceptible period of the piglets. The experimental data obtained from the experiment showed that the protection effect of piglets was best when the milking time was 7 days, and the protection rate was more than 85% when the milking time was 1, 14 and 28 days. In addition, the protective effect of challenged piglets during the entire susceptibility period of piglets was higher than 80%. With an average protection rate of 87.05%, the vaccine met the original design requirements. In this experiment, the protection rate of 1 day of milking was 85% which was similar to the average protection rate of the whole susceptible 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, when the dosage of 2.5 mL/head for pregnant sows was used, the protective effect was 84.5%. In order to obtain better immune prevention effects, we determined that the dosage for pregnant sows was 5 mL/head. And mice were used as the model animals of the vaccine efficacy test in order to facilitate production and testing. Through this experiment, it was determined that the minimum immunization dose for mice was 0.2 mL/mouse. When the dose was 0.1 mL/mouse, the protective effect was only 63%, which did not meet the protection requirement of 80%. When the dose was 0.3 mL/mouse, the protective effect was increased by 3% compared with the dose of 0.2 mL/mouse. Therefore, the dose of 0.2 mL/mouse was selected. Safety and efficacy tests showed that the safety and efficacy test between mice and sows had a good parallel relationship. Therefore, it was feasible and scientific to use mice instead of sows for safety inspections. The above research showed that the K88ac-3ST_a-LT₂ trivalent genetically engineered inactivated vaccine for *E.coli* diarrhea in newborn piglets provided a new genetic engineering vaccine candidate strain for more effective prevention of *E.coli* diarrhea in piglets.

Conclusions

Efficacy test of the K88ac-3ST_a-LT₂ genetically engineered inactivated vaccine showed that the immune protection rate of the newborn piglets could reach 85% on the 1st 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 scientific parameters of the vaccine, showed that the K88ac-3ST_a-LT₂ trivalent genetically engineered inactivated vaccine had a broad immune spectrum for *E. coli* diarrhea in newborn piglets, and provided a new genetic engineering vaccine candidate strain for prevention of *E. coli* diarrhea in piglets.

Declarations

Availability of data and materials

The datasets supporting the conclusions of this article are available from the corresponding author on reasonable request.

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Contributions

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

Ethics declarations

Ethics approval and consent to participate

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.

Consent for publication

Not applicable.

Competing interests

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

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Figures

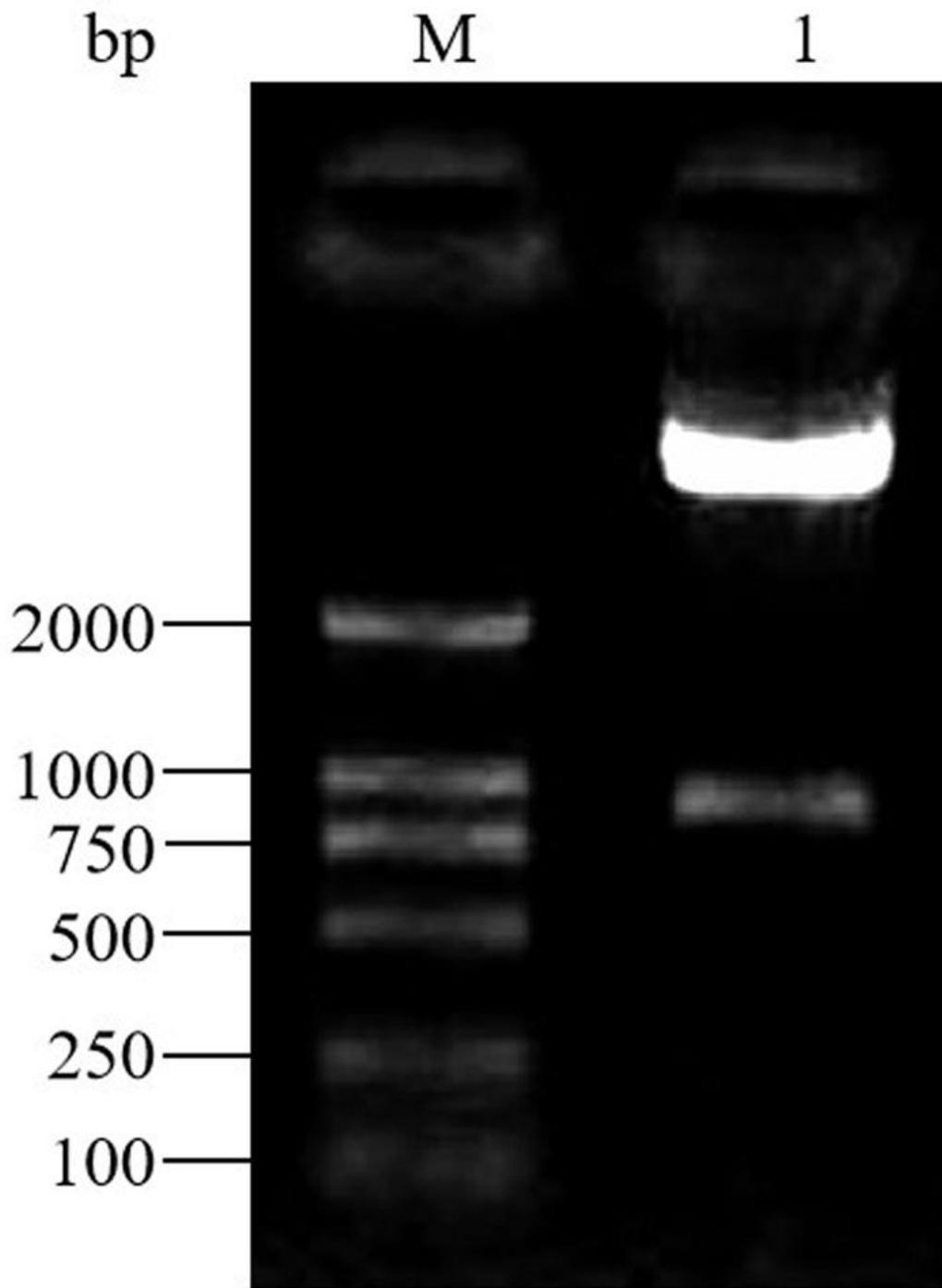


Figure 1

Enzyme digestion identification of pXK88ac3ST_aLT₂

M. DL2000DNA markers; 1. pXK88ac3ST_aLT₂ digested by *Nco* I/*Not* I

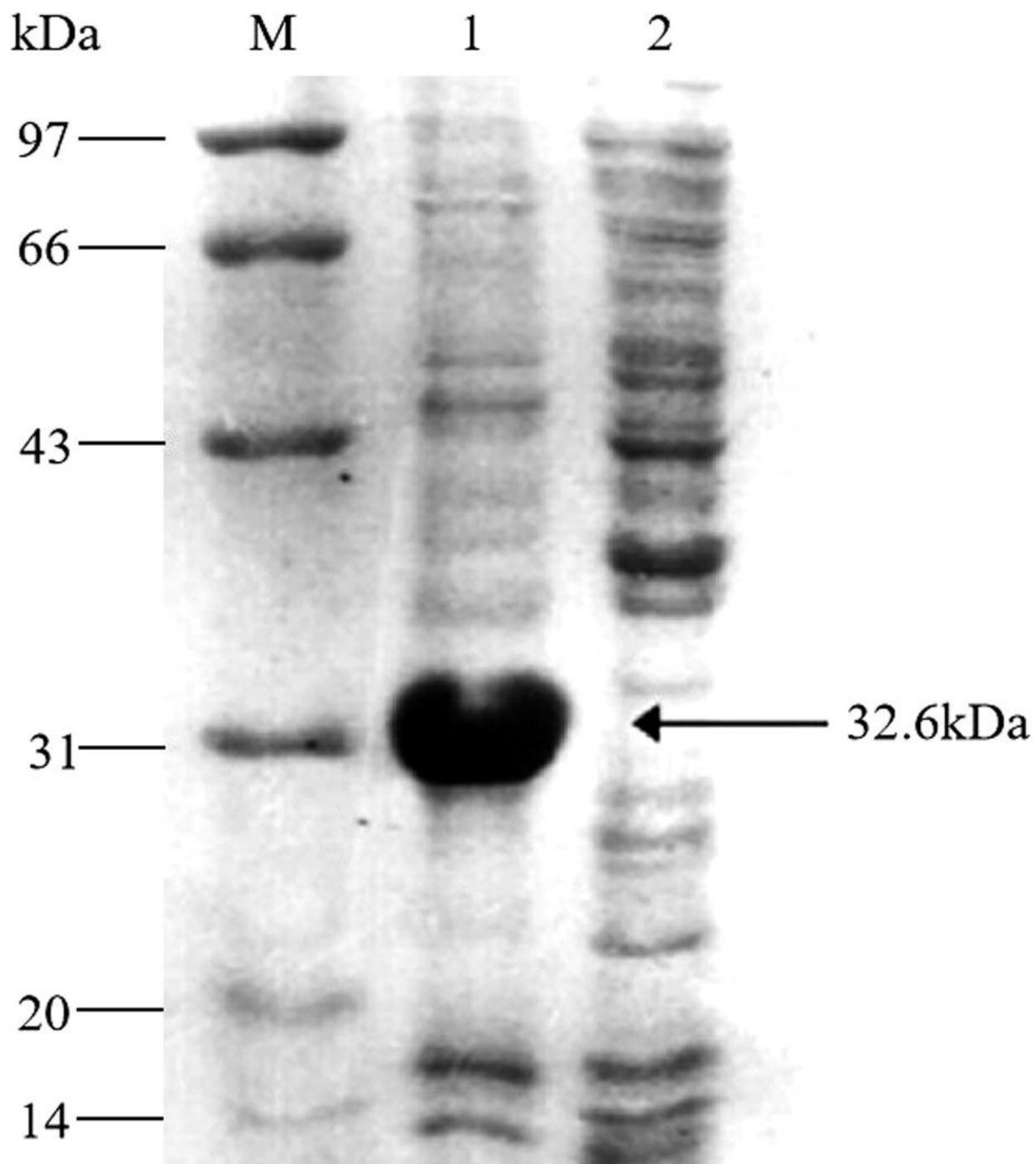


Figure 2

SDS-PAGE analysis of BL21(DE3)(pXK88ac3ST_aLT₂) Expression

M.Low molecular protein marker; 1.Total cell lysate of BL21(DE3)(pXK88ac3ST_aLT₂);

2.Total cell lysate of BL21(DE3)(pET-28b)

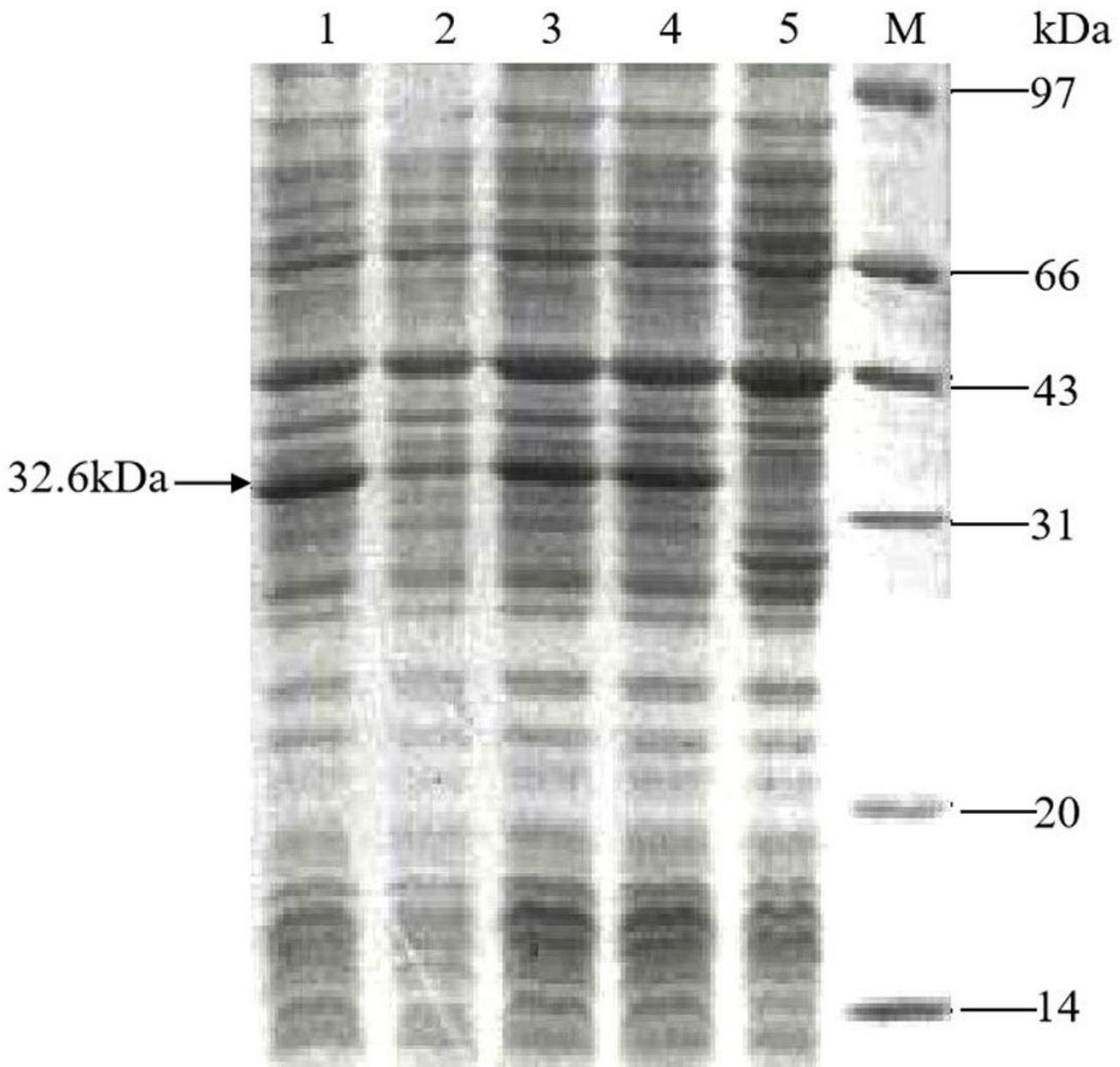


Figure 3

The induced test results of IPTG and lactose

1.1mmol/LIPTG BL21(DE3)(pxK88ac3ST_aLT₂) cell lysate;

2.1mmol/Llactose BL21(DE3)(pxK88ac3ST_aLT₂) cell lysate;

3.10mmol/Llactose BL21(DE3)(pxK88ac3ST_aLT₂) cell lysate;

4.100mmol/Llactose BL21(DE3)(pxK88ac3ST_aLT₂) cell lysate;

5.100mmol/Llactose BL21(DE3)(pET-28b) cell lysate;

M.Low molecular protein marker

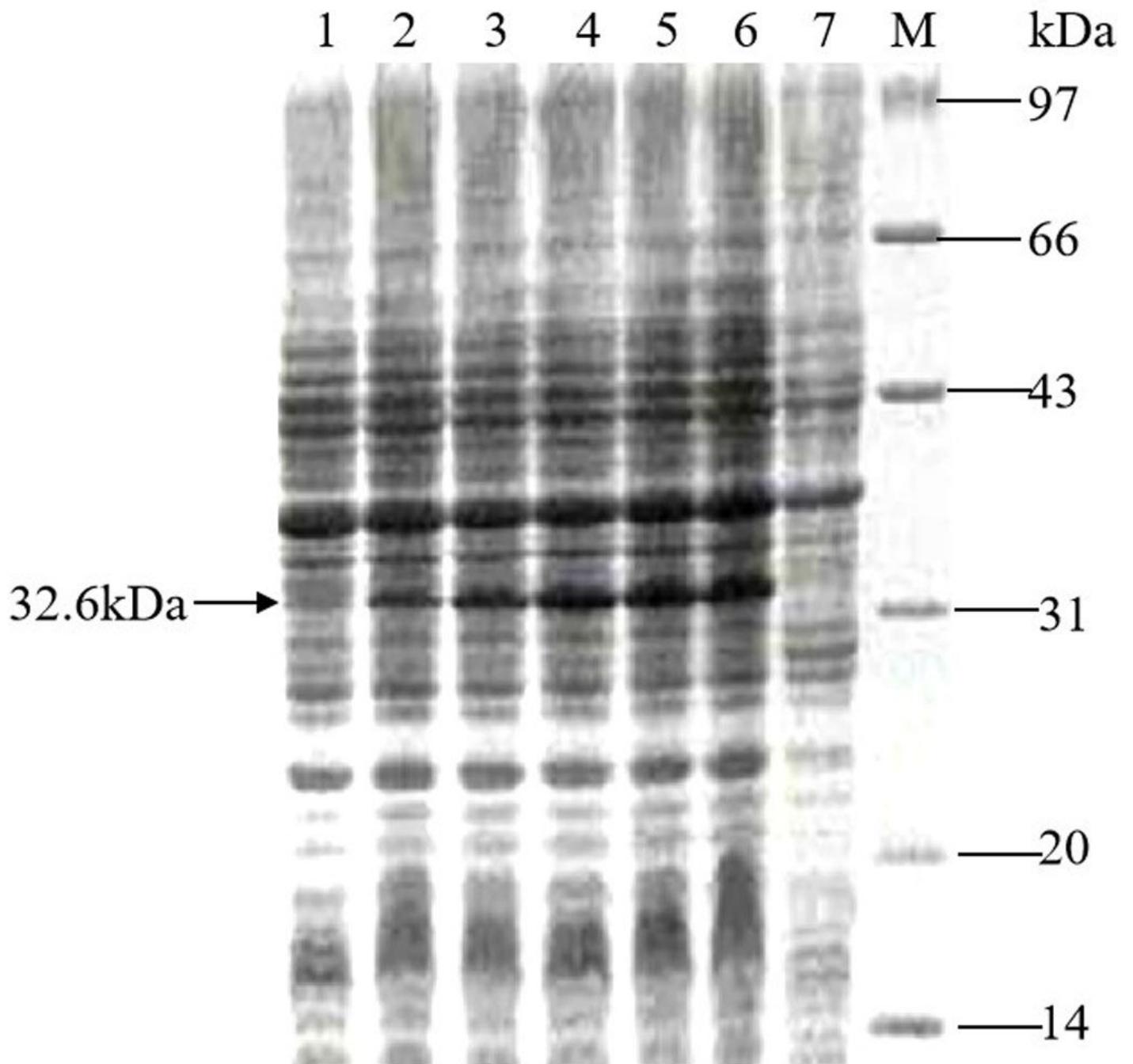


Figure 4

Effects of different induced time on the expression of the target protein

1. Induction 1h BL21(DE3)(pxK88ac3ST_aLT₂) cell lysate;
2. Induction 2h BL21(DE3)(pxK88ac3ST_aLT₂) cell lysate;
3. Induction 3h BL21(DE3)(pxK88ac3ST_aLT₂) cell lysate;
4. Induction 4h BL21(DE3)(pxK88ac3ST_aLT₂) cell lysate;
5. Induction 5h BL21(DE3)(pxK88ac3ST_aLT₂) cell lysate;

6. Induction 6h BL21(DE3)(pxK88ac3ST_aLT₂) cell lysate;

7. Induction 6h BL21(DE3)(pET-28b)cell lysate;

M. low molecular protein marker