FULL PAPER
Immunology

Immunogenicity and protection against *Glaesserella parasuis* serotype 13 infection after vaccination with recombinant protein LolA in mice

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ABSTRACT. *Glaesserella parasuis* is a pathogen causing Glässer's disease characterized by fibrinous polyserositis, polyarthritis, and meningitis. Owing to the low cross-immunogenicity of different bacterial antigens in commercial vaccines, finding and identifying effective immunoprotective antigens will facilitate the development of novel subunit vaccines. In this study, LolA, identified by bioinformatics approaches, was cloned and successfully expressed as a recombinant protein in *Escherichia coli*, and its immunogenicity and protection were evaluated in a mouse model. The results showed that the recombinant protein LolA can stimulate mice to produce high levels of IgG antibodies and confer 50% protection against challenge with the highly virulent *G. parasuis* CY1201 strain (serotype 13). By testing the cytokine levels of interleukin 4 (IL-4), IL-10, and interferon-γ (IFN-γ), it was found that the recombinant protein LolA can induce both Th1 and Th2 immune responses in mice. These results suggest that the recombinant protein LolA has the potential to serve as an alternative antigen for a novel vaccine to prevent *G. parasuis* infection.

KEYWORDS: *Glaesserella parasuis*, immunogenicity, LolA, protection, vaccine

Glaesserella parasuis is a pleomorphic, gram-negative bacterium that colonizes the upper respiratory tract of pigs. Associated morbidity has been observed in weaned and nursery pigs under stressful conditions [12]. It is the causative agent of Glässer's disease in pigs and is responsible for typical polyserositis, meningitis, bronchial pneumonia, and polyarthritis [36, 38]. For *G. parasuis* serotyping, methods such as the cooperative agglutination test (CA), indirect hemagglutination test (IHA), and polymerase chain reaction (PCR) can be used. Fifteen *G. parasuis* serotypes have been identified using classical and modern approaches. However, some serotypes have not yet been characterized. The virulence of *G. parasuis* strains is related to serotypes, which range from non-virulent to highly virulent [3, 16, 31]. The morbidity rate of Glässer's disease typically ranges from 10–15%, moreover, the mortality rate is as high as 50% in severe cases.

G. parasuis negatively affects the modern swine industry and is an important bacterial disease that should be prevented [29, 30]. Glässer's disease has traditionally been prevented using intensive feeding management techniques, antibiotics, and inactivated vaccines [10, 20]. Antibiotics exert a certain protective effect against Glässer's disease. However, the widespread use of antibiotics has contributed to the emergence of resistance to antibiotics in *G. parasuis*. Furthermore, multi-drug resistance has also been observed [27]. In addition, while inoculation with inactivated vaccines reduces mortality and morbidity to a certain extent, immune failures are still observed because of limited cross-protection across serovars [21]. Therefore, research and development of novel vaccines that are safe, effective, can stimulate long-term immunity, and provide effective cross-protection against *G. parasuis* infection is required [36].

Subunit vaccines have garnered more interest for avoiding limited cross-protection between different strains than conventional bacterins. In addition, combinations of conserved subunits from different serotypes are more effective than bacterial combinations [7, 11, 20]. Secreted proteins and outer membrane proteins (OMPs) are excellent protective antigens, as determined via prediction

and screening performed using a combination of genome-wide, bioinformatics, and proteomics approaches [28]. Various secreted proteins, such as PflA, Gcp, Ndk, HsdS, RnfC, and HAPS 0017, have been selected from serotype 5 of *G. parasuis*. Particularly, Gcp, Ndk, and RnfC demonstrate remarkable potential as vaccine candidates [18]. In addition, recombinant antigens, including GAPDH-OapA-HPS-0675, VtaA, and OPPA, have been tested in a protective swine infection model against homologous challenges [7, 22, 32]. Hence, preventing the spread of *G. parasuis* by screening proteins with good immunogenicity can be beneficial.

LolA encodes the precursor of the carrier protein of the outer membrane lipoprotein, which is involved in the transport of lipoproteins from the inner membrane to the outer membrane. It also acts as a regulator of the RCS-phosphorylation signal transduction pathway [5, 19]. *LolA* is an essential virulence gene in *G. parasuis*. In this study, we analyzed LolA protein using bioinformatics approaches to predict its potential as a predominant antigen. Subsequently, LolA was cloned, expressed, purified, and evaluated to characterize related immunogenicity and protective effects in a mouse model. Our results provide a foundation for the development of a novel *G. parasuis* subunit vaccine.

MATERIALS AND METHODS

Bacterial strains and culture conditions

The highly virulent *G. parasuis* CY1201 strain (serotype 13) was isolated from a diseased pig in Liaoning province, China. *G. parasuis* was cultured in tryptic soy broth (TSB, Solarbio, Beijing, China) or tryptic soy agar (TSA, Solarbio) supplemented with 10 mg/mL of nicotinamide adenine dinucleotide (NAD, Solarbio) and 5% fetal bovine serum at 37°C in an atmosphere enriched with 5% CO_2. *Escherichia coli* BL21 (DE3) was used as the host strain for the protein expression analysis. *E. coli* strains BL21 (DE3) were cultured in the Luria-Bertani (LB, Solarbio) medium containing 100 µg/mL of kanamycin.

Construction of recombinant plasmids

Primers used to amplify the *lolA* gene were designed with reference to the sequence of *G. parasuis* SH0165 (Accession number: CP001321 in GenBank). *BamH* I restriction enzyme site and protective bases were added to the forward primer, and *Hind* III site and protective bases were added to the reverse primer (Table 1). Genomic DNA from the CY1201 strain was extracted to amplify the *lolA* gene using bacterial genomic DNA extraction kit (TaKaRa, Dalian, China). PCR amplification was performed using a thermal cycler (BIOER, Hangzhou, China). The purified PCR product and pET-28a expression vector were digested with restriction enzymes *BamH* I and *Hind* III, and ligated with T4 DNA ligase (TaKaRa) at 16°C overnight. The ligation mixture was transformed into *E. coli* BL21 (DE3) cells and cultured, followed by PCR amplification and restriction enzyme identification. Positive plasmids were sequenced by Sangon Biotech Co., Ltd. (Shanghai, China).

Expression, purification, and identification of recombinant protein LolA

E. coli containing recombinant positive plasmids were cultured on an incubator in 400 mL of the LB medium containing 100 µg/mL of kanamycin at 37°C with stirring. When the concentration of cell suspension reached an optical density (OD) of 0.5–0.6 at 600 nm, 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sangon Biotech) was added to induce protein expression at 30°C for 4 hr. The cultures were centrifuged at 8,000 rpm for 10 min at 4°C, and the resultant bacterial pellet was resuspended in phosphate-buffered saline (PBS) and sonicated (100 cycles of 10 sec, 50% duty cycle) (Ultrasonic Cell Pulverizer; Zhisun Equipment, Shanghai, China) on ice. The lysate was centrifuged at 12,000 rpm for 10 min. A small sample was analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the remaining lysate was reserved for purification.

A suspension was obtained to purify the target protein using the His-Ni-resin affinity chromatography method (Cwbiotech, Beijing, China). The target proteins were eluted in 500 mM imidazole buffer and stored at −20°C. Subsequently, crude extracts and the unbound and eluted fractions were analyzed using 12% SDS-PAGE. The separated proteins were electrotransferred onto nitrocellulose (NC) membranes (Solarbio). The membrane was blocked with 5% skim milk in TBST (0.05% Tween-20, 20 mM Tris-HCl, and 150 mM NaCl) at 4°C overnight and washed thrice with TBST. Then, rabbit anti-*G. parasuis* polyclonal serum (diluted 1:500 in TBST) was produced by immunizing rabbits with the completely inactivated CY1201 strain, which was used as the primary antibody, incubated for 2 hr at room temperature, and washed thrice with TBST. Horseradish peroxidase-conjugated goat anti-rabbit IgG (1:3,000) (TransGen Biotech, Beijing, China) was used as the secondary antibody and washed thrice with TBST after 1 hr of incubation at room temperature. Finally, ECL luminescence solution (Beyotime Biotechnology, Shanghai, China) was used dropwise on the membrane, which was developed in a dark room to detect the bands.

Table 1. Primer sequences and cloning sites used for gene fragment amplification

Gene	Primer name	Primer length (nucleotide)	Primer sequence (5′-3′)	Annealing temperature (°C)
lolA	*lolA*-F	32	TTT<u>GGATCC</u>ATGAAAAATTTGTTAAAAAAATC	55
	lolA-R	28	TTT<u>AAGCTT</u>TTTTTTTACCACGCTGATCG	

ᵃ Underlined bases are restriction sites.

Animal immunization and challenge experiments

A total of 65 female Kunming mice (18–22 g) were purchased from Liaoning Changsheng Biotechnology Co., Ltd. (Benxi, China). All experimental protocols were in compliance with the necessary guidelines and approved by the Ethical Review Committee and Laboratory Animal Welfare Committee of Shenyang Agricultural University (No. 201806014). The animals were randomly divided into five groups. The immunization group was injected subcutaneously with 100 μg of recombinant protein emulsified with 100 μL of complete Freund's adjuvant. The other group was immunized with *G. parasuis* serotype 13 inactivated with 0.3% formaldehyde at 37°C for 24 hr, and the bacterial concentration was 4×10^9 colony-forming units (CFU)/100 μL. Mice inoculated with equal amounts of PBS emulsified in the same adjuvant were used as negative controls. After primary immunization, on day 14, the mice were boosted with the same dose of antigen and incomplete Freund's adjuvant.

Blood samples were obtained by tail bleeding (3 mice per group) on day 7 after each immunization and on day 14 after the second immunization, and serum was separated and stored at −20°C for immunological assays. On day 14 after the second immunization, ten mice from each group were challenged intraperitoneally with a lethal dose (5 LD_{50}) of 4.3×10^9 CFU of log-phase *G. parasuis* CY1201 strain, except for the blank control. All mice were monitored for one week after the challenge, and the clinical symptoms and survival rates of the mice were recorded. Dead and surviving mice were necropsied to observe pathological changes in various tissues and organs. Simultaneously, the liver tissue was aseptically removed, and the number of *G. parasuis* was counted in the liver.

Determination of specific antibody IgG levels

An indirect enzyme-linked immunosorbent assay (ELISA) was used to detect IgG levels in the sera of immunized mice. At 4°C overnight, 96-well plates were coated with 5 μg/100 μL of purified recombinant protein diluted in PBS per well (To determine IgG levels of the inactivated vaccine group, each well was coated with 4×10^9 CFU/100 μL of inactivated *G. parasuis* CY1201 strain diluted in PBS). The wells were washed thrice with PBST (PBS containing 0.05% Tween-20) and then blocked with 5% (w/v) skim milk in PBST for 2 hr at 37°C. The plates were washed for thrice and incubated with 100 μL of sera diluted in 1:400 for 1 hr at 37°C. After three washes, HRP-conjugated goat anti-mouse IgG (TransGen Biotech), diluted 1:3,000 in PBS, was used as the secondary antibody and incubated for 1 hr at 37°C. The plates were washed thrice with PBST, and 100 μL of TMB was added to each well, followed by incubation at room temperature for 30 min in the dark. The reaction was stopped by adding 50 μL of 2 M H_2SO_4 in each well, and OD_{450} was measured using a microplate reader (Bio-Rad, Hercules, CA, USA). Simultaneously, the serum tested on day 14 after the second immunization was diluted 100–204,800 times with PBS as the primary antibody, and the antibody titer was determined.

Determination of cytokines by ELISA

Cytokine levels were measured in isolated serum samples on day 14 after the second booster immunization of mice with the recombinant protein. The levels of interleukin 4 (IL-4), interleukin 10 (IL-10), and interferon-γ (IFN-γ) were determined using ELISA kits (Elabscience, Wuhan, China) according to the manufacturer's instructions. Briefly, 100 μL of each standard or sample was added to each well of a 96-well plate, followed by incubation at 37°C for 90 min. The liquid was discarded, and 100 μL of biotinylated antibody working solution was added to each well. After mixing, the membranes were covered and incubated at 37°C for 1 hr. A total of 350 μL of washing solution was added to each well. After washing thrice, another 100 μL of enzyme conjugate working solution was added, the membranes were covered and incubated at 37°C for 30 min. The liquid was discarded, and washed five times. Next, 90 μL of substrate solution (TMB) was added to each well. The ELISA plate was incubated in the dark for approximately 15 min, followed by the addition of 50 μL of stop solution. The OD value was immediately measured at 450 nm. According to the calculation standard curve, the levels of IL-4, IL-10, and IFN-γ in the peripheral blood of mice were analyzed.

Determination of bacterial load in mice liver

After necropsy, the livers of the mice that died immediately were harvested and weighed (3 mice per group) in a sterile environment. The collected liver tissue was ground on a 100-mesh sterile sieve with a sterile syringe core in 400 μL of PBS. The grinding solution of 100 μL was diluted with sterile PBS, spread evenly on a plate containing TSA, and incubated at 37°C for 12–18 hr. Subsequently, the colonies were counted. In addition, one week after the challenge, the livers of the surviving mice in the euthanized immunization group (3 mice per group) were harvested, and the plate colonies were counted similarly after weighing.

Statistical analysis

The data were analyzed using single factor analysis of variance (ANOVA), and the *t*-test was used as a post hoc test after ANOVA. The experimental data are presented as the mean ± SD. *P*-value <0.05 (or 0.01) was considered statistically significant. Statistical analyses were performed using the SPSS software (version 15.0; SPSS Inc., Chicago, IL, USA).

RESULTS

Expression and purification of immunogenic protein

A DNA fragment with a length of 630 bp was amplified from the genome using primers specific to the *lolA* gene, representing the *lolA* gene sequence of *G. parasuis* serotype 13. The amplified fragment was cloned into the pET-28a expression vector and expressed in *E. coli* BL21 (DE3) cells. Analysis via 12% SDS-PAGE showed high expression in *E. coli* BL21 (DE3) cells (Fig. 1A). The His-tagged LolA protein was purified by Ni affinity chromatography, and the molecular mass of LolA was approximately 23 kDa, which corresponded to its predicted size (Fig. 1B). Western blotting performed with a rabbit anti-*G. parasuis* polyclonal serum confirmed
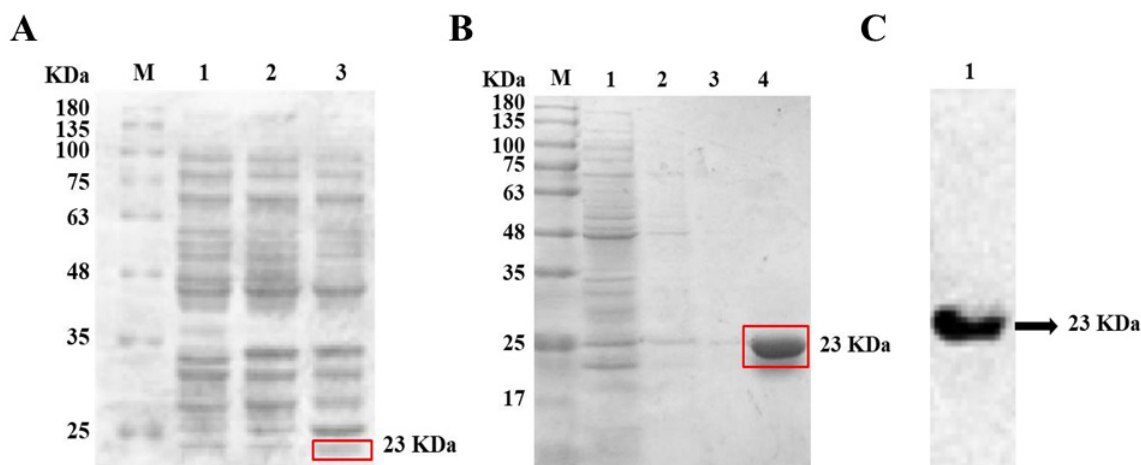
Fig. 1. Expression, purification, and identification of recombinant protein LolA. (**A**) The expression of LolA in *Escherichia coli* (*E. coli*) was analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by staining with Coomassie brilliant blue. Lane M: standard for protein marker; Lane 1: expression product of pET-28a vector; Lane 2: expression product of noninduced recombinant bacterium; Lane 3: expression product of induced recombinant bacterium (23 kDa). (**B**) Purification of recombinant protein LolA after nickel affinity chromatography. Analysis was performed via 12% SDS-PAGE along with staining with Coomassie brilliant blue. Lane M: standard for protein marker; Lane 1, 2, and 3: liquid protein flowing through purified LolA; Lane 4: purified LolA (23 kDa). (**C**) Western blot analysis of purified recombinant protein LolA with anti-serotype 13 *Glaesserella parasuis* (*G. parasuis*) serum. Lane 1: purified LolA protein (23 kDa).

the band as expected (Fig. 1C). This result indicated that the protein had a good specific binding capacity for anti-LolA antibodies and could be used as an immunogenic protein.

Detection of specific antibody IgG production in mice

To determine whether mice immunized with recombinant proteins can induce specific immune responses against recombinant antigens, an indirect ELISA was performed with serum isolated from mouse peripheral blood. After the first immunization, low levels of the antibodies in each group were maintained, which were not significantly different from those in the negative control group ($P>0.05$). After the second immunization, the antibody level in the immunized group was significantly increased compared with the antibody level observed after the first immunization ($P<0.01$); however, no significant differences were observed compared to the antibody levels in the negative control group ($P>0.05$). This shows that with the passage of time in the immunization cycle, the level of antibodies in the immunization group constantly increased. The antibody levels of the recombinant protein LolA group were significantly different from those of the negative control group ($P<0.01$), and the levels of the inactivated group were significantly different from those of the negative control group ($P<0.05$) (Fig. 2A). After the second immunization, serum was diluted to detect the antibody titer. The experimental results showed that the immunized groups had higher antibody titers than the negative control group, and the antibody titer of the recombinant protein LolA group was higher than that of the inactivated group (Fig. 2B).

Evaluation of cytokine production in mice

Serum cytokine levels were measured using an indirect ELISA. The levels of IFN-γ in the recombinant protein LolA group were significantly higher than those in the control group ($P<0.05$) (Fig. 3A). The levels of IL-4 in the recombinant protein LolA and inactivated groups were significantly higher than those in the control group ($P<0.01$) (Fig. 3B). The levels of IL-10 in the recombinant protein LolA group were significantly higher than those in the control group ($P<0.01$) (Fig. 3C). The levels of the cytokines IFN-γ, IL-4, and IL-10 in the recombinant protein LolA group were significantly higher than those in the inactivated group ($P<0.05$) (Fig. 3).

Protective efficacy against G. parasuis challenge

The protective efficacy of the recombinant protein LolA against a lethal challenge with the highly virulent *G. parasuis* CY1201 strain was evaluated in mice. All mice in the positive challenge group and the negative control group died within 2 days after the challenge. The group immunized with the recombinant protein LolA showed 50% protection against the challenge with *G. parasuis* CY1201 strain (Fig. 4).

Clinical symptoms and pathological changes in mice

At 12 hr after the challenge, all mice in the positive challenge group died, and other mice showed symptoms of varying degrees of lethargy, loss of appetite, decreased movement, increased eyelid secretions, and covering of the entire eye. Simultaneously, some mice developed severe dyspnea and congestion in the ears and tail veins. At 24 hr after the challenge, all mice in the negative control group died, and the symptoms of the mice in the immunized group were relieved, with observations such as a reduction in eyelid
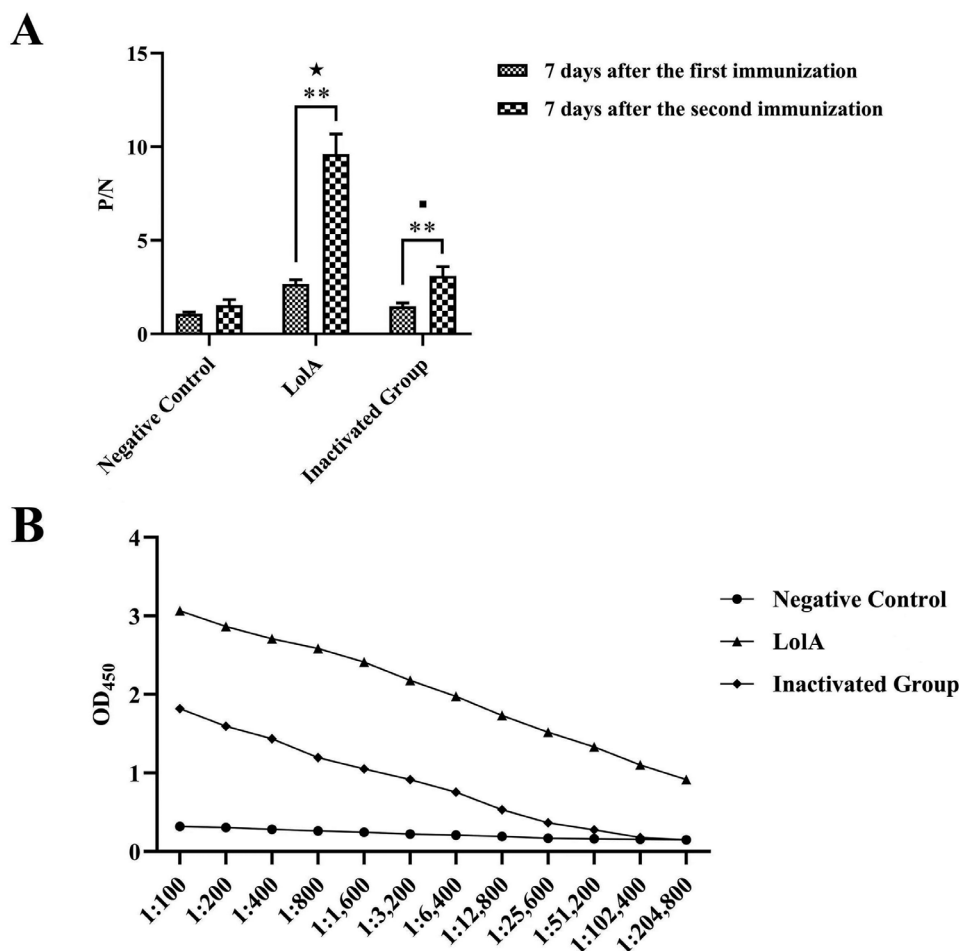
Fig. 2. Detection of levels of antibody after the production was induced by recombinant protein in mice by indirect ELISA. (**A**) Results of antibody monitoring after two immunizations in mice performed via indirect ELISA. Serum samples from each group were collected 7 days after each immunization. A total of 200 ng of purified recombinant protein was used to coat 96-well plates. "P/N" indicates the ratio of OD_{450} of the immunized group or the negative control group to the OD_{450} of the blank control group; the asterisk "**" indicates that the antibody level after 7 days of the second immunization was significantly different from that after 7 days of the first immunization ($P<0.01$); "★" indicates that the antibody level was significantly different from that of the negative control group after 7 days of the second immunization ($P<0.01$); "■" indicates that the antibody level was significantly different from that of the negative control group after 7 days of the second immunization ($P<0.05$). (**B**) Antibody titer detection of serum IgG in mice by ELISA. Serum samples from each group were collected 14 days after the second immunization. A total of 200 ng of purified recombinant protein was used to coat 96-well plates.

secretions, minor eyes opening movement, and gradual recovery of appetite. In addition, mice who experienced sudden death stage showed neurological symptoms such as convulsions, uncontrollable body, and opisthotonus. The main clinical symptoms observed in each group are presented in Table 2. At 48 hr after the challenge, the clinical symptoms of the tolerant mice in each group disappeared, and the physiological state was normal.

Pathological examination was performed on all mice that died after the challenge. The tissues and organs of mice in each group showed different degrees of pathological damage. Significant hyperemia of the heart was observed. The pathological changes in the liver manifested as adhesion of the liver lobules and apparent bleeding spots on the surface. The lungs of the positive challenge group and the negative control group were congested. The recombinant protein group had shrimp-like lesions, and the whole bacteria-inactivated group had dull lungs. Pathological changes involving different degrees of swelling and infarction at the edge were observed in the spleen. Pathological damage to the spleen was only detected in surviving mice immunized with the recombinant protein LolA after the challenge (Table 3). These results show that the recombinant protein LolA can significantly reduce histopathological damage after *G. parasuis* infection in mice, which also proves that the recombinant protein LolA exerted a certain protective effect against *G. parasuis* infection.

Bacterial load in mice liver

In the immunized and negative control groups, the livers of the dead mice were weighed (3 mice per group) after the challenge, and the colonies were counted. The immunized group showed a significantly reduced bacterial load in the liver compared to the negative control group ($P<0.01$) (Fig. 5), indicating while *G. parasuis* reproduced in the liver of mice after the challenge, immunization
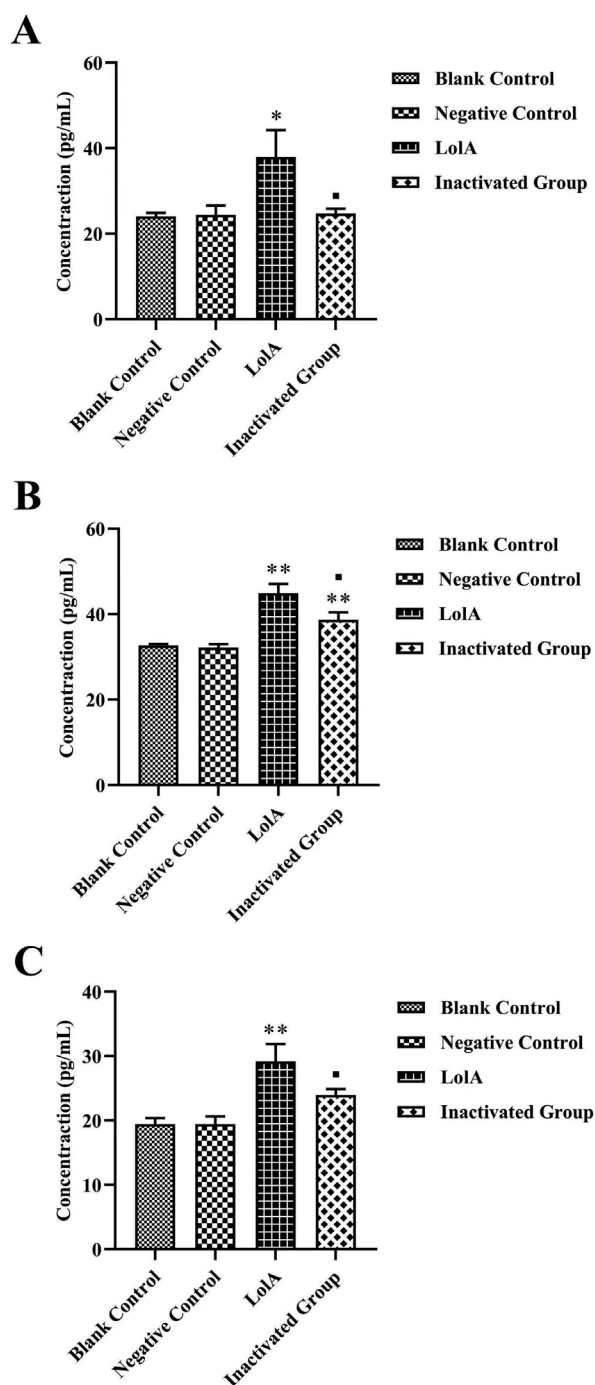
A

B

C

Fig. 3. Detection of the levels of interferon-γ (IFN-γ), interleukin 4 (IL-4), and IL-10 in peripheral blood of mice by indirect ELISA. (**A**) IFN-γ. "*" indicates that the cytokine level was significantly different from those of the blank and negative control groups (*P*<0.05); "■" indicates that the cytokine level was significantly different from that of the recombinant protein LolA group (*P*<0.05). (**B**) IL-4. "**" indicates that the cytokine level was significantly different from those of the blank and negative control groups (*P*<0.01); "■" indicates that the cytokine level was significantly different from that of the recombinant protein LolA group (*P*<0.05). (**C**) IL-10. "**" indicates that the cytokine level was significantly different from those of the blank and negative control groups (*P*<0.01); "■" indicates that the cytokine level was significantly different from that of the recombinant protein LolA group (*P*<0.05).
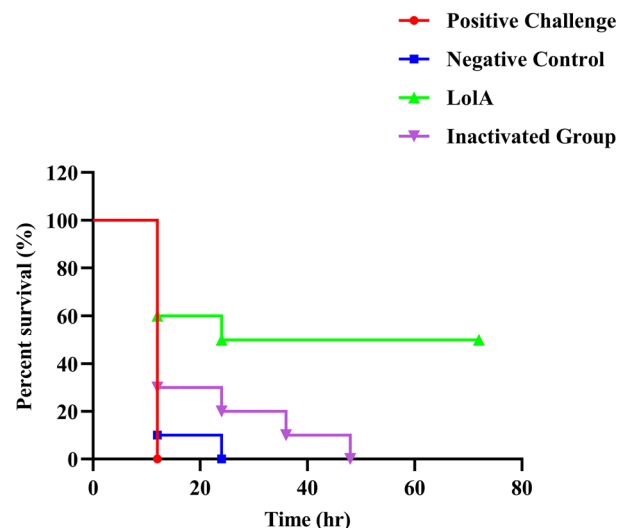

Fig. 4. Survival rates of mice challenged with *Glaesserella parasuis* CY1201 strain. Positive Challenge, non-immunized, challenging directly; Negative Control, immunized with phosphate-buffered saline (PBS); LolA, immunized with recombinant protein LolA; Inactivated Control, immunized with whole inactivated bacteria.

could play a certain role in clearing it. One week after the challenge, the livers of the surviving mice in the immunized group were used for colony counting after euthanasia. The number of colonies was zero, indicating that *G. parasuis* was substantially cleared in the mice in the immunized group one week after the challenge (Table 4).

DISCUSSION

Infection with *G. parasuis* is a major factor responsible for piglet mortality and critically jeopardizes advances and developments in the swine industry [13, 35]. Hence, it is essential to take effective measures to reduce the threat to pig herds [29]. Vaccination is an important preventive and treatment method, and further studies are crucial. At present, the prevention of *G. parasuis* infection mainly depends on inactivated vaccines in China, but the effect is not satisfactory because of the large number of *G. parasuis* serotypes and large differences in antigens between serotypes. Subunit vaccines prepared by screening immunogenicity-related proteins are an effective alternative to traditional vaccines for avoiding the lack of cross-protection between serotypes and have demonstrated the advantages and potential [15, 20]. The SH0165 strain of serotype 5 is the most studied strain in China with complete genome sequencing [7, 9, 23]. However, few studies have examined the serotype 13 strain, which is also more prevalent and highly virulent. Therefore, in this study, serotype 13 of the CY1201 strain isolated in our laboratory was selected for further study.

LolA is a precursor of the carrier protein of the outer membrane lipoprotein, which is involved in lipoprotein transporter-related activity and chaperone-mediated translocation of proteins across the periplasmic space. Random

Table 2. Clinical symptoms of mice in each group infected with *Glaesserella parasuis* CY1201 strain

Group	Unwilling to eat		Rough hair		Difficulty breathing		Secretions around the eyes		Neurological symptoms	
	12 hr[a]	24 hr[b]	12 hr	24 hr	12 hr	24 hr	12 hr	24 hr	12 hr	24 hr
Positive challenge	-	-	-	-	-	-	-	-	-	-
Negative control	1/1[c]	-	1/1	-	1/1	-	1/1	-	1/1	-
Inactivated group	3/3	2/2	3/3	2/2	3/3	2/2	3/3	2/2	3/3	2/2
Recombinant protein LolA	4/6	3/5	4/6	3/5	3/6	3/5	2/6	2/5	1/6	1/5

[a] 12 hr after mice were challenged. [b] 24 hr after mice were challenged. [c] Number of surviving mice that developed clinical symptoms after the challenge.

Table 3. Pathological changes in tissues and organs of mice in each group infected with *Glaesserella parasuis* CY1201 strain

Group	Positive challenge	Negative control	Inactivated group	Recombinant protein LolA	
	Dead[a]	Dead	Dead	Survivors[b]	Dead
Heart congestion	+++(6)[c], ++(4)	++(7), +(3)	+(10)	-(5)	++(2), +(3)
Lung congestion	+++(4), ++(5), +(1)	++(6), +(4)	+(5), -(5)	-(5)	-(5)
Lung carnification	-(10)	-(10)	++(3), +(2), -(5)	-(5)	+(4), -(1)
Liver haemorrhages	++(7), +(3)	++(8), +(2)	++(3), +(7)	-(5)	+(5)
Splenomegaly	++(3), +(7)	++(2), +(8)	+++(8), ++(2)	+++(3), ++(2)	+++(4), ++(1)

[a] Mice that died within a week of the challenge. [b] Mice that survived one week after the challenge. [c] Numbers in parentheses represent the number of mice with this characteristic lesion. The severity of tissue organ lesions: -, no changes; +, mild changes; ++, moderate changes; +++, severe changes.
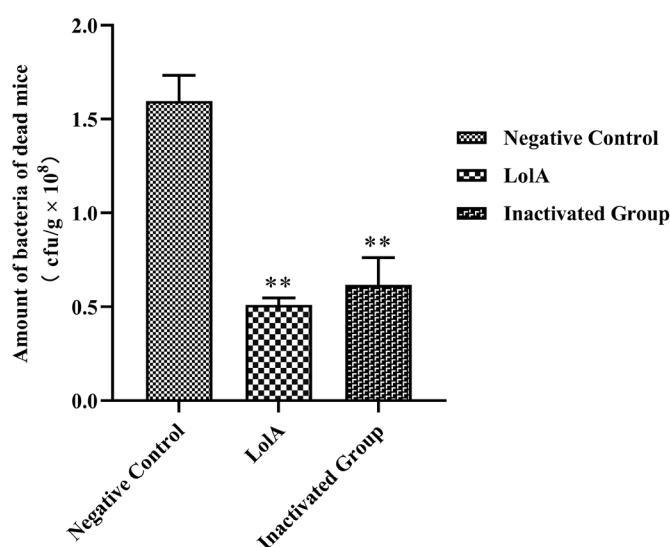

Fig. 5. Analysis of the bacterial load in the liver of dead mice. "**" indicates that the bacterial load in the liver of dead mice was significantly different from that of the negative control group ($P<0.01$).

mutagenesis of highly conserved residues results in loss of the ability to bind to lipoproteins [24–26]. The involvement of the LolA system in the transport of lipoproteins from the inner membrane to the outer membrane is essential for the growth of *E. coli* and is widely conserved in gram-negative bacteria. Therefore, it is considered a promising target for the development of anti-gram-negative bacteriostatic agents [19]. At present, the function of the LolA protein in *G. parasuis* has not been completely elucidated. However, the function of the protein predicted by bioinformatics-based analysis can be used as a reference for further analysis [4, 6, 33]. In this study, the same method was used to predict that LolA protein as an antigen may elicit a strong immune response in the host body. Simultaneously, LolA was cloned and expressed in *E. coli*. It showed high specific binding capacity to convalescent sera, further indicating that the recombinant protein LolA can be used as an immunogenic protein.

As a model organism for immunological research, mice have the advantages of known genes, a short reproductive cycle, typical mammalian representative physiology, and cost-effectiveness and can be used to evaluate the immune efficacy of recombinant proteins in the study of subunit vaccines [17, 37]. Many factors, such as the choice of adjuvant and antigen concentration, may affect the immunological efficacy of subunit vaccines [1, 34]. Montanide Gel 01 and Freund's adjuvants are more effective for the development

Table 4. Results of the detection of bacterial load in the liver of mice

Group	Attack amount	Number of bacteria in dead mice (cfu/g)	Number of bacteria in surviving mice (cfu/g)
Negative control	4.3×10^9	1.72×10^8	-
		1.45×10^8	-
		1.62×10^8	-
Recombinant protein LolA	4.3×10^9	5.1×10^7	0
		4.7×10^7	0
		5.4×10^7	0
Inactivation group	4.3×10^9	6.8×10^7	-
		7.2×10^7	-
		4.5×10^7	-

of *G. parasuis* vaccines, and the use of Freund's adjuvant has a protective effect in a mouse model of *G. parasuis* infection [2, 18]. In our study, we also selected the same adjuvant and recombinant protein to co-immunize mice and preliminarily evaluate their immune response. Injecting the adjuvant and antigen together into the body can enhance the immune response against the antigen. Furthermore, the process is useful for selecting different adjuvants to evaluate the immunogenicity of the recombinant protein. Therefore, different immune adjuvants should be selected to analyze immune response levels in the future.

IgG activates complement system, mediates bacteriolysis and phagocytosis, and plays an important role in resisting bacterial infection and invasion [8]. In addition, IgG, as a major component of antibodies, is produced by the re-immune response. It shows a wide ranging of long-lasting effects and can completely reflect the level of humoral immunity. Therefore, when screening candidate proteins for subunit vaccines, the first consideration is whether they can stimulate the body to produce an immune response. Here, the immunization group could induce the body to produce high levels of IgG antibodies, and the antibody level of the recombinant protein LolA group was higher than that of the whole bacteria-inactivated group. The protective effect was also more pronounced, as vaccinated animals exhibited better clinical presentation and fewer pathological lesions. Mice subjected to the challenge showed a protection rate of 50% attributed to the recombinant protein LolA. Antibody levels were consistent with our challenge results, indicating that the recombinant antigen had a protective effect.

Cytokines can mediate the interaction between cells, play an important role in regulating the innate and adaptive immunity of the body, synergistically eliminate pathogens, and demonstrate a variety of immune regulatory functions. Recombinant proteins can induce the body to produce a systemic response to secrete cytokines, and the type of immune response can be inferred and evaluated based on the type of cytokine (Th1 and Th2) [14]. In this study, the levels of IFN-γ, IL-4, and IL-10 cytokines in the recombinant protein LolA group were significantly higher than those in other groups to varying degrees, so it can be inferred that recombinant protein LolA can induce Th1 and Th2 immune responses in mice.

In conclusion, this study shows that the recombinant protein LolA can induce cellular and humoral immunity in a mouse model, and demonstrate good protection against homologous *G. parasuis*. Hence, it can be used as a potential candidate vaccine against *G. parasuis* infection. Since weaning and nursery pigs are susceptible to Glässer's disease, further evaluation of immune protection in piglets should be considered.

CONFLICT OF INTEREST. The authors declare that there are no conflicts of interest in this paper.

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