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FULL PAPER

Immunology

Application of mouse model for evaluation of recombinant LpxC and GmhA as novel antigenic vaccine candidates of *Glaesserella parasuis* serotype 13

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ABSTRACT. *Glaesserella parasuis* (*G. parasuis*) has been one of the bacteria affecting the large-scale swine industry. Lack of an effective vaccine has limited control of the disease, which has an effect on prevalence. In order to improve the cross-protection of vaccines, development on subunit vaccines has become a hot spot. In this study, we firstly cloned the *lpxC* and *gmhA* genes from *G. parasuis* serotype 13 isolates, and expressed and purified their proteins. The results showed that LpxC and GmhA can stimulate mice to produce IgG antibodies. Through testing the cytokine levels of interleukin 4 (IL-4), IL-10 and interferon-γ (IFN-γ), it is found that recombinant GmhA, the mixed LpxC and GmhA can stimulate the body to produce Th1 and Th2 immune responses, while recombinant LpxC and inactivated bacteria can only produce Th2 immune responses. On the protection rate for mice, recombinant LpxC, GmhA and the mixture of LpxC and GmhA can provide 50%, 50% and 60% protection for lethal dose of *G. parasuis* infection, respectively. The partial protection achieved by the recombinant LpxC and GmhA supports their potential as novel vaccine candidate antigens against *G. parasuis*.

KEY WORDS: *Glaesserella parasuis*, GmhA, LpxC, vaccine candidate

Glaesserella parasuis, the etiological agent of Glässer's disease, is one of the important bacteria affecting the pathogenesis of pigs [21]. *G. parasuis* can be colonized in the upper respiratory tract of healthy porcine [2]. When the bacterium causes disease, some typical symptoms appear such as polyserositis, meningitis and arthritis. However, we still lack understanding of what triggers the transition between colonization and systemic disease [18]. *G. parasuis* has a variety of genotypes and serotypes with different virulence, of which serotypes 4, 5 and 13 are more common serotypes, followed by serotypes 12 and serotypes 14 [4, 9]. The bacterium are mainly transmitted by means of air, direct contact, contaminated items. Piglets 5–8 weeks old are most susceptible to *G. parasuis* infection. Co-infection with other bacteria and virus is also common [13, 16, 20], which causes substantial economic losses in the pig industry worldwide [25].

Currently, control of Glässer's disease can be carried out by means of drug treatment and vaccination [15, 19]. Drug treatment is effective to a certain extent, but long-term and unreasonable use of antibiotics makes *G. parasuis* more resistant [27]. Vaccination is also the most convenient means of controlling Glässer's disease, in which inactivated vaccines are widely used worldwide. As we know, inactivated vaccines can play a good protective effect, but due to the lack of cross-protection, the situation of immune failure has repeatedly appeared [17]. Compared with inactivated vaccines, some attenuated vaccines and bacterial ghost vaccines have improved immune protection, but the disadvantages are that these vaccines are ineffective against the virulence of the strains, and the production process of that is immature [10, 15]. However, subunit vaccine, to a certain extent, can solve the above problems. Previous studies have shown that some outer-membrane and secreted proteins (OmpP2,D15,PalA,TbpA,TbpB,HPS-06257,HPS-0675, GAPDH,OapA,Gcp,Ndk and RnfC) can provide partial protection against challenging with *G. parasuis* and have strong potential as vaccine candidates [1, 6, 14, 28]. To date, although it has been reported that some subunit vaccines have been

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tested, there are still lack of commercial subunit vaccines against *G. parasuis* infection, thus it is essential to find more effective novel protective antigens [7, 8].

Virulence factor-related proteins are widely used in the research of subunit vaccines. Both *lpxC* and *gmhA* are important virulence genes of *G. parasuis*. In this study, we predicted and identified a few immunogenic proteins of *G. parasuis* by genomics and proteomics analysis. Subsequently, we cloned the *lpxC* and *gmhA* genes, and expressed and purified their corresponding proteins. Mouse models are used to evaluate the immunoprotective effects of LpxC and GmhA proteins on *G. parasuis*, and further to screen out effective recombinant proteins as components of subunit vaccines. The results from this study provide a foundation for developing highly efficient, safe and cross-protective vaccines, and the prevention and treatment of *G. parasuis* infection.

MATERIALS AND METHODS

Bacterial strains and growth conditions

G. parasuis strain CY1201 (serotype 13) was isolated from a pig farm located in Chaoyang County in Liaoning Province of China. The strain was maintained on tryptic soy agar (TSA, Solarbio, Beijing, China) or tryptic soy broth (TSB, Solarbio) containing 10 mg/ml nicotinamide adenine dinucleotide (NAD, Solarbio) and 5% fetal bovine serum at 37°C aerobically. *Escherichia coli* BL21 (DE3) strains were cultured in luria bertani (LB, Solarbio) medium containing 100 µg/ml kanamycin. Strain CY1201 was used as the template for *lpxC* and *gmhA* amplification and the challenge strain in the subsequent experiments.

Construction of recombinant plasmids

The primers for amplifying the *lpxC* and *gmhA* genes were designed based on the nucleotide sequence of *G. parasuis* SH0165 (Accession number: CP001321 in GenBank). The forward primer was introduced to form a *BamH* I restriction enzyme site, and the reverse primer was introduced to create an *EcoR* I site (Table 1). The genomic DNA from CY1201 strain was extracted according to the bacterial genomic DNA extraction kit (TaKaRa, Dalian, China). The PCR conditions were as follows: initial denaturation at 95°C for 30 sec, followed by 30 cycles of denaturation for 30 sec at 94°C, annealing at 52°C for 30 sec, and extension at 72°C for 90 sec. Amplification was ended at 72°C for 10 min. The purified PCR products and pET-28a (expression vector) plasmids were digested with the restriction endonuclease *BamH* I and *EcoR* I, and then the processed DNA fragment and expression vector were ligated with T4 DNA ligase (TaKaRa) at 16°C overnight. The ligation mixture was transformed in *E. coli* BL21 (DE3), and *E. coli* transformants containing the genes were used to express the recombinant proteins. The positive plasmids containing the *lpxC* and *gmhA* gene were sequenced by the Sangon Biotech Co., Ltd. (Shanghai, China).

Expression and purification of immunogenic proteins

The *E. coli* BL21 (DE3) transformants containing the LpxC-His and GmhA-His recombination protein were grown in 400 ml LB supplemented with 100 µg/ml kanamycin at 37°C under agitation. When the optical density at 600 nm (OD_{600}) had reached 0.5–0.6, the protein expression was induced by adding 1 mM IPTG (Sangon Biotech, Shanghai, China) at 30°C for 4 hr. Thereafter, the cultures were centrifuged and resuspended in phosphate-buffered saline (PBS). Then the mixture was sonicated on ice (50 cycles of 10 sec, 40% duty cycle, Ultrasonic cell pulverizer, China) and centrifuged. The purity and concentration of the proteins were evaluated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Bradford method, respectively.

The protein suspension was purified using nickel-NTA affinity chromatography, followed by elution with 500 mM imidazole. The purified proteins were detected using the SDS-PAGE and electroporated onto the nitrocellulose filter membrane. The nitrocellulose filter membrane was placed in TBST (PBS containing 0.05% Tween 20) containing 5% skimmed milk powder and blocked overnight. Then we used mouse anti-His (TransGen Biotech, Beijing, China) and rabbit anti-*G. parasuis* as primary antibodies to incubate at 37°C for 2 hr, respectively. Horseradish peroxidase-conjugated goat anti-mouse and goat anti-rabbit IgG (1:3,000) (Sigma, Thousand Oaks, CA, USA) were used as secondary antibodies and incubated at room temperature for 1 hr. After washing the membrane for 3 times with PBST, the ECL luminescent solution (Beyotime Biotechnology, Shanghai, China) was used to expose in the membrane for immunologically bound bands.

Immunization and challenge experiments in mice

One hundred µg of LpxC and GmhA proteins was mixed with Freund's adjuvant (Sigma) in a ratio of 1:2 and stored at 4°C until further use. A total of 70 female KM mice (18–22 g) were purchased from Changsheng Biological Co., Ltd. (Benxi, Liaoning,

Table 1. Primer sequences and restriction sites used for gene amplification

Gene	Primer name	Primer length (nucleotide)	Primer sequence (5′-3′)	Annealing temperature (°C)
lpxC	*lpxC*-F	24	CCGGGATCCATGATTAAACAAAGA	53
	lpxC-R	27	CCGGAATTCTTAAATAAACACCTGTTC	
gmhA	*gmhA*-F	21	CGGGGATCCATGTACCAACAA	52
	gmhA-R	24	CCGGAATTCTTACTTTGCCATTTC	

Underlined bases are restriction sites.

China). All experimental protocols were approved by the Ethical Committee of Shenyang Agricultural University (No. 201806014). Here, the mice were randomly assigned to seven groups with each ten animals. The immunization route is subcutaneous injection on the back. The mice from LpxC group were immunized with purified 100 µg/100 µl LpxC. The mice from GmhA group were immunized with purified 100 µg/100 µl GmhA. The mice from mixed protein LpxC and GmhA group were immunized with purified 50 µg/50 µl of LpxC and 50 µg/50 µl of GmhA. The mice from inactivated vaccine group were immunized with 100 µg/100 µl inactiviated *G. parasuis* (serotype 13). Preparation process of inactivated vaccine are as follows. The well-grown CY1201 strain was cultured on TSA plates. After incubating at 37°C for 18 hr, the bacteria were blown down with PBS and washed twice. The bacteria were inactivated with 0.2% formaldehyde for 24 hr at 37°C. After the inactivation solution was centrifuged, the formaldehyde was discarded, and the precipitate was washed three times with PBS, and finally the concentration of the bacterial solution was adjusted to 4.0×10^9 colony-forming units (CFU)/100 µl. The mice from negative control group were immunized with PBS and complete Freund's adjuvant. The immunization procedure is shown in Table 2. The mice from the immunization group were boosted with the same dose of antigens, and incomplete Freund's adjuvant at day 14 following the primary immunization. The remaining blank control group (Untreated) and positive challenge group (Only attack) were not treated during the immunization process.

After the first immunization two weeks, blood was collected from the tail vein of the mice. Serum was separated and stored at −20°C. In addition, two weeks after the second immunization, serum was also separated for the detection of whole blood sterilization, IgG antibody levels and cytokine levels. After the second immunization two weeks, the mice from experimental groups were challenged with minimum lethal dose of 3×10^9 CFU (minimum lethal dose was determined by pre-testing) of *G. parasuis* CY1201 strain diluted in 0.2 ml of PBS by intraperitoneal injection. All mice were monitored for two weeks (monitor every 6 hr) after challenge and recorded their mortality and clinical symptoms. The dead mice were necropsied and observed for pathological changes in the heart, liver, spleen and lungs. At two weeks after the challenge, the surviving mice were also autopsied, and the pathological changes of organs were observed.

Whole blood sterilization test

CY1201 strain was subjected to TSB culture, washed twice with sterile PBS, and diluted to 1.0×10^9 CFU/ml. All immunized mice were tested, and after the second immunization, the serum was inactivated through a water bath for 30 min at 56°C. Inactivated serum was diluted to 50% with sterile PBS. Ten µl of diluted bacterial solution and 190 µl of diluted serum were mixed in a 1.5 ml centrifuge tube and incubated at 37°C for 30 min. Then 100 µl of unimmunized and heparinized mouse whole blood was added to the centrifuge tube, and cultured at 37°C with shaking for 1 hr. After the culture, all the liquid in the centrifuge tube was diluted 10^7 times and 10^8 times, and 100 µl of the dilution solution was respectively coated on the TSA plate, and the test was repeated in parallel three times. After 12 hr incubation at 37°C, colony counts were performed. The results were calculated based on the following equation: bactericidal rate=(CFU of Negative Control-CFU of Immune Test)/CFU of Negative Control ×100%.

Determination of IgG level by ELISA

An indirect ELISA was used to determine the levels of IgG in mice serum. Ninety-six-well plates were coated with 2 µg/100 µl/well of purified recombinant protein diluted in PBS overnight at 4°C (The IgG levels of the inactivated vaccine group were coated with 3×10^9 CFU/100 µl/well inactivated *G. parasuis* CY1201 strain diluted in PBS). The wells were washed three times with PBST, and then blocked with 5% (w/v) skimmed milk powder in PBST for 2 hr at 37°C. The plate was washed three times and incubated with 100 µl sera diluted in 1:100 for 1 hr at 37°C. After three washes, HRP-labeled 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 three times with PBST, and TMB was added in the dark at room temperature for 20 min. Then the reaction was stopped with 2M H_2SO_4 and OD_{450} was measured on a microplate reader (Bio-Rad, Berkeley, CA, USA).

Determination of cytokines by ELISA assay

According to the instructions of Mouse interleukin 4 (IL-4) ELISA Kit (Elabscience, Wuhan, China), all reagents were equilibrated to room temperature and mixed well. Then 100 µl of each double dilution standard and test sample were added to the bottom of the plate and incubated at 37°C for 90 min. After discarding the supernatant, 100 µl of biotinylated antibody was added to each well and incubated at 37°C for 1 hr. Then 100 µl of enzyme conjugate was added to each well and incubated at 37°C for 30 min. Ninety µl of TMB substrate solution was added to each well, and incubated at 37°C in the dark for 15 min. Finally, 50 µl

Table 2. Experimental design for the primary immunization of mice

Group	No. of mice/group	Antigen	Dosage/vol	Adjuvant	Route
Negative control	10	Phosphate-buffered saline (PBS)	100 µl	Freund's complete	Subcutaneous
Inactivated group	10	Inactivated strain[a]	4.0×10^9 CFU/100 µl	Freund's complete	Subcutaneous
Recombinant protein LpxC	10	LpxC	100 µg/100 µl	Freund's complete	Subcutaneous
Recombinant protein GmhA	10	GmhA	100 µg/100 µl	Freund's complete	Subcutaneous
LpxC+ GmhA mixed protein	10	LpxC+ GmhA	100 µg/100 µl[b]	Freund's complete	Subcutaneous

[a] CY1201 strain (Serotype 13) inactivated with formaldehyde. [b] The mice from LpxC+GmhA mixed protein were immunized with 50 µg of LpxC and 50 µg of GmhA.

of stop solution was added to each well, and OD_{450} was measured on a microplate reader. The IL-4 content in the peripheral blood of mice was obtained according to the standard curve. The detection methods of IL-10 (Elabscience) and interferon-γ (IFN-γ) (Elabscience) are the same as those of IL-4.

Determination of bacterial load in mice liver

The livers of challenged mice were removed and weighed under aseptic conditions. The removed liver was placed on a sterilized sieve and fully ground with a sterile syringe core after adding 400 µl of sterile PBS. After multiple dilution of the grinding solution, the diluted solution was evenly spread on a TSA plate containing 10 mg/ml NAD and 5% fetal bovine serum. Then TSA plates were cultured in a 37°C incubator, and colonies were counted 24 hr later. In addition, after challenge two weeks, the livers of surviving mice in the immunization group were taken and the same operation was performed (3 mice in each group).

Statistical analysis

Differences among different groups were analyzed by one-way analysis of variance, followed by the Tukey test. The *P*-value less than 0.05 was considered statistically significant. Statistical analysis were run with SPSS 15.0 software (SPSS Inc., Chicago, IL, USA).

RESULTS

Cloning of lpxC and gmhA genes and expression and purification of their recombinant proteins

The two primer pairs for the *lpxC* and *gmhA* genes resulted in the amplification of DNA fragments of 921 bp and 579 bp in length, respectively, representing the *lpxC* and *gmhA* sequence of 13 serotype strain of *G. parasuis*. The amplified DNA fragment was inserted into the pET-28a expression vector and was highly expressed in *E. coli* BL21 (DE3). The molecular weights of LpxC and GmhA proteins are approximately 34 kD and 21 kD, respectively (Fig. 1a and 1b). After expression, His-tagged LpxC and GmhA were purified using affinity chromatography (Fig. 1c). The purified proteins were identified and the results were the same as the predicted molecular weights. In addition, using the serum of *G. parasuis* of rabbit anti-serotype 13 as the primary antibody,

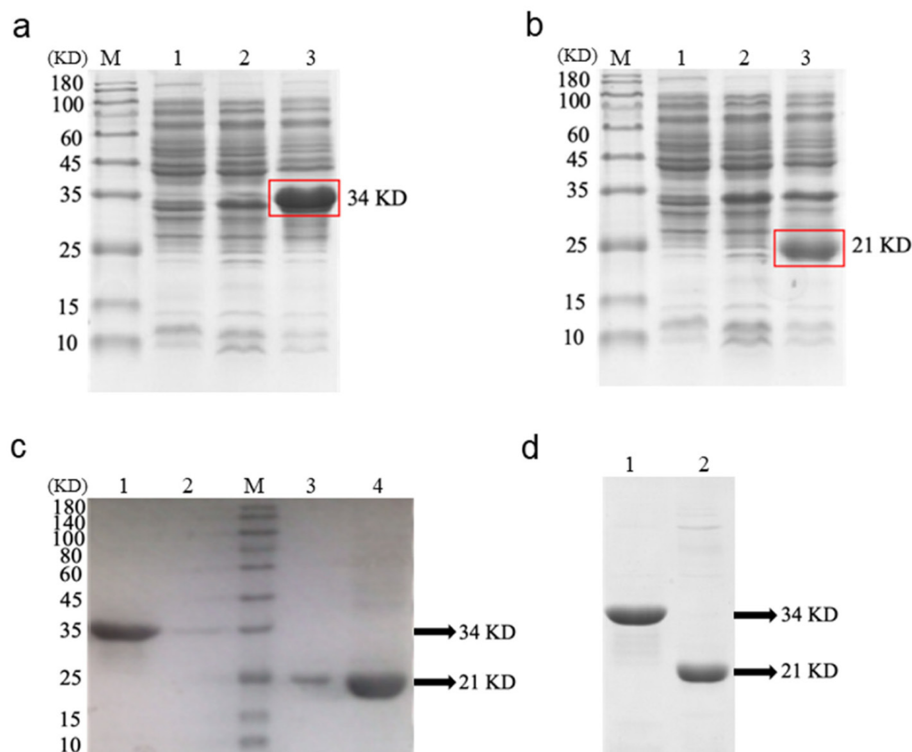

Fig. 1. Expression, purification and identification of recombinant LpxC and GmhA. **(a)** LpxC expression analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Lane M: standard for protein labeling; lane 1: expression product of pET-28a vector; lane 2: expression product of uninduced *Escherichia coli* (*E. coli*); lane 3: expression product of induced *E. coli* (34 kD). **(b)** GmhA expression analyzed by SDS-PAGE. Lane M: standard for protein labeling; lane 1: expression product of pET-28a vector; lane 2: expression product of uninduced *E. coli*; lane 3: expression product of induced *E. coli* (21 kD). **(c)** Purified protein expression was shown by SDS-PAGE test. Lane M: standard for protein labeling; lane 1: purified recombinant LpxC (34 kD); lane 2: liquid protein flowing through purified LpxC; lane 3: liquid protein flowing through purified GmhA; lane 4: purified recombinant GmhA (21 kD). **(d)** Validation of purified recombinant protein by immunoblotting with anti-serotype 13 *Glaesserella parasuis* (*G. parasuis*) sera. Lane 1: purified LpxC protein (34 kD); lane 2: purified GmhA protein (21 kD).

Western Blot tests were performed on the LpxC and GmhA. The results showed bands expected, indicating that both the LpxC and GmhA have good immunogenicity (Fig. 1d).

Protective efficacy after immunization and whole blood killing bacteria assay

At 2 weeks after the second immunization, challenge was performed with the lowest lethal dose of serotype 13 of *G. parasuis* CY1201 strain (3×10^9 CFU). At 12 hr after challenge, the mice began to develop morbidity and death. Within 30 hr, all mice have died in the positive challenge group and the negative control group. The death of mice was recorded for two week. As a result, the whole inactivated bacteria, recombinant LpxC, recombinant GmhA, and mixed LpxC and GmhA could provide 40%, 50%, 50% and 60% protection against lethal dose of CY1201 infection, respectively (Fig. 2a). In order to verify whether the recombinant protein can induce antibody-mediated bactericidal effect, the serum extracted after immunization was inactivated and identified on TSA plates. According to the calculation of the sterilization rate, the *G. parasuis* in the negative control group grew well, and the sterilization rate was only 2.5%. However, the growth of the other four groups of *G. parasuis* was severely inhibited, with bactericidal rates of 49.5%, 62%, 57.5%, and 73.83%, respectively (Fig. 2b). Compared with the negative control group, the bactericidal rate was significantly different ($P<0.01$), which indicates that the induced immune response can play a bactericidal effect through antibody mediation.

Clinical and pathological results in mice

After immunization, the mice did not show any obvious clinical symptoms, and the feeding, coat and movement were normal. At 12 hr after challenge, the mice developed shortness of breath, no food intake, and a large amount of yellow secretions around the eyes. At 24 hr after challenge, the clinical symptoms of some mice began to lighten, eye secretions began to decrease, exercise began, and a small amount of food and water were consumed. However, some mice began to experience neurological symptoms, such as tremors and convulsions and died within 1 hr. A summary of the main clinical symptoms found in the six experimental groups is shown in Table 3. At 72 hr after challenge, the clinical symptoms of the tolerant mice disappeared, and feeding and exercise returned to normal.

After autopsy, it was found that the organs of the mice in the positive challenge group had some congestion and bleeding, no other lesions. The mice that died after immunization had more severe lesions, liver bleeding points, and severe adhesion to the peritoneum. There were bleeding spots in the lungs and shrimp-like lesions appeared. Heart lesions are congestion and adhesions in the lungs. The volume of the spleen became significantly larger, and the congestion was severe, and infarcts appeared at the edge of the liver. In addition, the mice survived after immunization showed that the spleen was swollen, and there were no other obvious symptoms (Table 4).

Antibody testing results in mice

In order to detect antibody levels after the first and second immunizations, an ELISA test was performed. The antibody level was lower at 2 weeks after the first immunization, and there was no significant difference compared with the negative control group. After a booster, the antibody levels in each group increased significantly. The antibody levels of the inactivated vaccine group, recombinant LpxC group, recombinant GmhA group, and the mixed LpxC and GmhA group were significantly different from 2 weeks after the first immunization ($P<0.01$) (Fig. 3a).

Cytokine expression in mice peripheral blood

Cytokines play an important role in regulating innate and acquired immunity, and can cooperate to kill pathogens. In this study, serum was collected from peripheral blood of mice two weeks after the second immunization, the cytokine detection kit was used to measure the levels of IFN-γ, IL-4 and IL-10 (Fig. 3b). The levels of IFN-γ in the recombinant GmhA group and the mixed LpxC and GmhA group were significantly increased compared to in the blank and negative control groups ($P<0.05$). Compared with the blank and negative control groups, the levels of IL-4 in the recombinant protein immunized groups and the whole bacteria inactivated group were significantly increased ($P<0.05$). The IL-10 levels of the three recombinant protein immunization groups and the whole bacterial inactivation group were significantly increased compared with the blank and negative control groups ($P<0.01$). In addition, the levels of IL-4 in the recombinant LpxC group, the recombinant GmhA group and the whole bacteria inactivated group were significantly increased compared to in the mixed LpxC and GmhA group ($P<0.05$).

Table 3. Clinical symptoms found in each of the six groups infected with the *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	8/8[c]	5/5	8/8	5/5	7/8	5/5	6/8	5/5	4/8	5/5
Negative control	8/8	6/6	8/8	6/6	6/8	6/6	5/8	6/6	4/8	6/6
Inactivated group	8/9	8/8	9/9	8/8	7/9	8/8	5/9	4/8	2/9	4/8
Recombinant protein LpxC	7/10	6/9	5/10	5/9	4/10	4/9	3/10	4/9	1/10	4/9
Recombinant protein GmhA	7/9	4/7	7/9	4/7	5/9	2/7	5/9	2/7	2/9	2/7
LpxC+ GmhA mixed protein	6/9	4/8	7/9	4/8	3/9	2/8	3/9	2/8	1/9	2/8

[a] 12 hr after mice challenge. [b] 24 hr after mice challenge. [c] Number of mice with clinical symptoms among surviving mice.

Table 4. Pathological changes found in each of the six groups infected with the *Glaesserella parasuis* CY1201 strain

Group	Positive challenge[a]	Negative control	Inactivated group		Recombinant protein LpxC		Recombinant protein GmhA		LpxC+ GmhA mixed protein	
	Dead[b]	Dead	Survivors[c]	Dead	Survivors	Dead	Survivors	Dead	Survivors	Dead
Heart haemorrhages	++(10)[d]	++(10)	+(1), -(3)	++(4), +(2)	-(5)	+(5)	-(5)	+(5)	-(6)	+(4)
Lung congestion	+++(5), ++(5)	+++(4), ++(6)	+(1), -(3)	+++(5), ++(1)	-(5)	+++(3), ++(2)	-(5)	+++(3), ++(2)	-(6)	+++(2), ++(2)
Lung haemorrhages	+++(8), ++(2)	+++(6), ++(3), +(1)	-(4)	++(4), +(2)	-(5)	++(3), +(2)	-(5)	++(3), +(2)	-(6)	++(2), +(2)
Liver congestion	++(7), +(3)	++(5), +(5)	+(2), -(2)	++(3), +(3)	-(5)	++(4), +(1)	-(5)	++(3), +(2)	-(6)	++(3), +(1)
Liver haemorrhages	++(4), +(6)	++(3), +(7)	-(4)	++(2), +(4)	-(5)	++(3), +(2)	-(5)	++(4), +(1)	-(6)	++(2), +(2)
Splenomegaly	+(5), -(5)	+(4), -(6)	+++(1), ++(3)	+++(2), ++(4)	++(3), +(2)	++(3), +(2)	+++(2), ++(3)	++(4), +(1)	+++(1), ++(4), +(1)	++(2), +(2)
Adhesion of liver to pleura	+(3), -(7)	+(2), -(8)	+(1), -(3)	+++(4), ++(2)	-(5)	++(1), +(4)	-(5)	++(2), +(3)	-(6)	++(1), +(3)

[a] There were no changes in the organs of the mice in the positive group and the negative group that survived after two weeks of challenge. [b] Mice that died after being challenged. [c] Mice that survived after two weeks of challenge. [d] Lesion severity: -, no changes; +, mild changes; ++, moderate changes; +++, severe changes. The number in brackets indicates the number of mice experiencing this change in each experimental group.

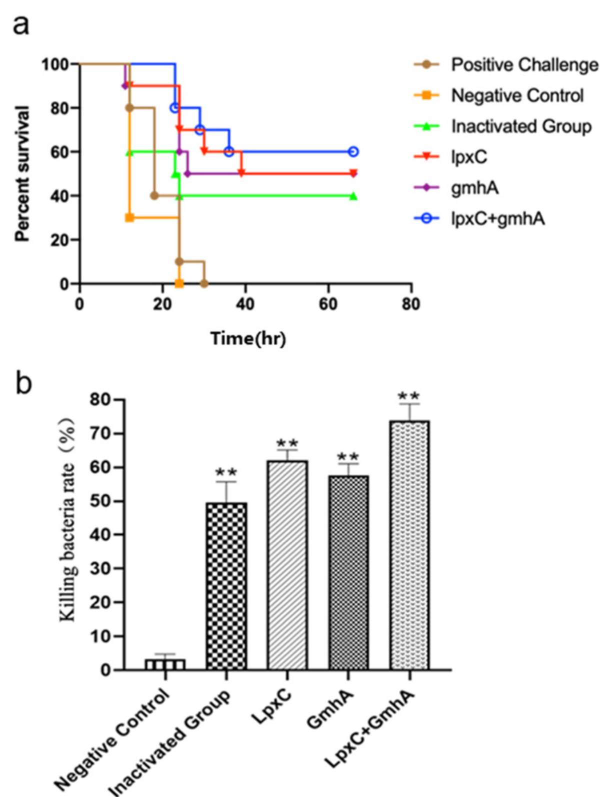

Fig. 2. Survival rate of *Glaesserella parasuis* (*G. parasuis*) challenged mice and results of whole blood killing bacteria test. **(a)** Survival rate of *G. parasuis* challenged mice. Positive Challenge: non-immunized, challenging directly; Negative Control: phosphate-buffered saline (PBS) immunized; Inactivated Group: immunized with whole inactivated bacteriocins; lpxC: immunized with recombinant LpxC; gmhA: immunized with recombinant GmhA; lpxC + gmhA: immunized with recombinant LpxC and GmhA. **(b)** Results of whole blood killing bacteria test. "**" indicates that the killing bacteria rate is significantly different from that of the negative control group (P<0.01).

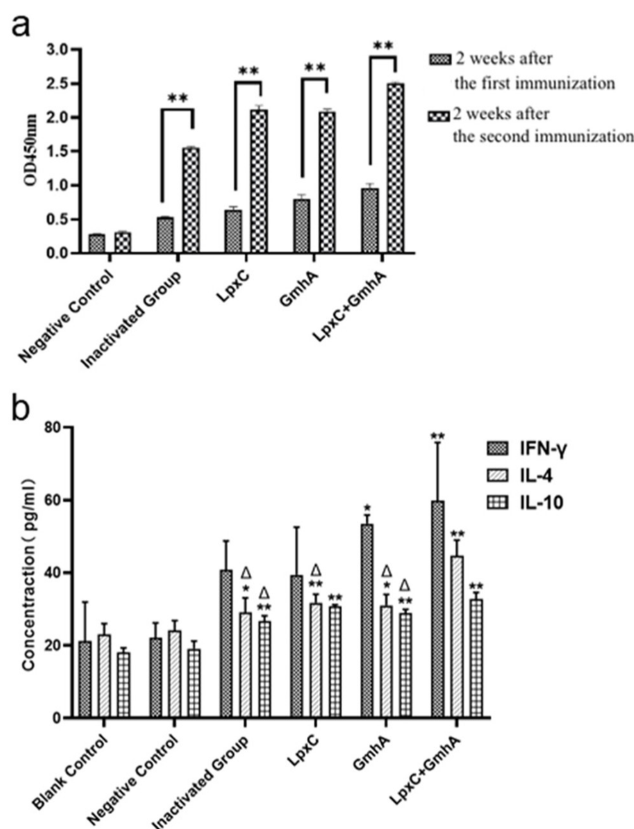

Fig. 3. Indirect ELISA detects protein-induced serum antibody levels and the concentration of interferon-γ (IFN-γ), interleukin 4 (IL-4), IL-10 in mice. **(a)** Detection of antibody levels in each group 2 weeks after the first immunization and 2 weeks after the second immunization. "**" indicates that the antibody level after 2 weeks of the second immunization is significantly different from the antibody level after 2 weeks of the first immunization (P<0.01). **(b)** Indirect ELISA detected the concentration of IFN-γ, IL-4, IL-10 in peripheral blood of mice. "**" indicates that the cytokine level is significantly different from the blank and negative control groups (P<0.01); "*" indicates that the cytokine level is significantly different from the blank and negative control groups (P<0.05); "Δ" indicates that the cytokine level is significantly different from the LpxC+GmhA mixed protein group (P<0.05).

Table 5. Results of mice liver-loaded bacteria experiment

Group	Number of surviving mice	Number of dead mice	Attack amount	Colony-forming units of dead mice (3 mice in each group)	Colony-forming units of surviving mice (3 mice in each group)
Negative control	0	10	3.0×10^9	9.57×10^7	-
				8.66×10^7	-
				1.05×10^8	-
Inactivated group	4	6	3.0×10^9	8.36×10^6	0
				8.56×10^6	0
				8.18×10^6	0
Recombinant protein LpxC	5	5	3.0×10^9	6.98×10^6	0
				7.05×10^6	0
				7.01×10^6	0
Recombinant protein GmhA	5	5	3.0×10^9	6.85×10^6	0
				6.69×10^6	0
				6.75×10^6	0
LpxC+ GmhA mixed protein	6	4	3.0×10^9	5.07×10^6	0
				4.92×10^6	0
				4.70×10^6	0

Bacterial load in mice liver

After challenge, livers of mice that died or survived for one week (3 mice per group) were taken, and colonies were counted after weighing (Table 5). The bacteria load of dead mice in each immunization group is different from that of the control group. However, the mice survived after immunization and challenge were measured for liver bacterial load and colony count. The results showed that the colonies were 0, indicating that *G. parasuis* in the surviving mice was basically eliminated.

DISCUSSION

With the large-scale development of pig industry, Glässer's disease has become more and more serious, and it has been an important bacterial disease [5]. Due to the pathogenic mechanism of the disease has not been fully clear, the prevention and treatment of the disease is also facing great difficulties. In addition, there are many serotypes of *G. parasuis*, and the antigens of each serotype are quite different. Moreover, the vaccine cross-immunization effect is poor. At present, the trivalent inactivated vaccine has been developed and used on the market, but its protection rate is not high, which has brought great obstacles to the prevention and control of the disease [26, 29]. At present, through the epidemiological investigation of *G. parasuis* in China, serotypes 4, 5 and 13 are the most prevalent serotypes. Besides, the only recently reported *G. parasuis* for genome sequencing is the SH0165 strain of serotype 5, so more studies have been performed on serotype 5 strains. Especially in the research of vaccines, Li *et al*. used immunoproteomics to identify serotype 5 of *G. parasuis* candidate vaccines, and Guo *et al*. evaluated *G. parasuis* SH0165 recombinant protein superoxide dismutase in a mouse model evaluation of vaccine candidates [7, 12]. However, there are few studies on serotype 13 strains of the same highly virulent strains with serotype 5. Therefore, in this study, the serotype 13 of CY1201 strain was selected for vaccine identification.

Lypooligosaccharide (LOS) is an important virulence factor of *G. parasuis*, in which *lsgB*, *opsX*, *rfaF*, *waaQ*, *lgtB* and *lex-1* are genes encoding lypooligosaccharide. Some researchers have constructed deletions of the above genes and found that heptose residues can reduce *G. parasuis* adhesion and invasion to host cells, and are more sensitive to serum effects [22, 23]. In this study, we also selected lypooligosaccharide as a candidate factor for future vaccine research. By sequencing comparison and function analysis of the genome on *G. parasuis* of SH0165 strains, *lpxC* and *gmhA* were screened and identified as key genes encoding lypooligosaccharides, and found that they were highly conserved among *G. parasuis* [24]. GmhA is a heptose phosphate isomerase which is mainly involved in the synthesis of core oligosaccharides and LpxC is UDP-3-O-[3-hydroxymyristoyl] N-acetylglucosamine deacetylase, mainly involved in the synthesis of lipid A [23]. At the same time, the authors verified the conservation of the *lpxC* and *gmhA* among serotype 5, 9, and 13 of *G. parasuis*. After nucleotide homology comparison, it was found that the *lpxC* and *gmhA* are more than 95% homologous to the genes of other strains.

The best experimental animal for evaluating the immune efficacy of *G. parasuis* related vaccines is pigs. However, due to its high cost, complicated experimental operations, and limited breeding environment, convenient and easy-to-operate mice are often used as animal models in the laboratory. Also, we evaluated the immunoprotective effects of LpxC and GmhA on *G. parasuis* in mice model [7]. In the experiment of protective effect in mice, the protection rate of the mixed protein is higher than that of the single protein, indicating that the mixed protein can stimulate the mouse body to produce more antibodies. The whole bacteria inactivated vaccine has a poor protection effect on mice, probably because the inactivated vaccine is absorbed more slowly by the body than the subunit vaccine and requires a longer immune cycle [15]. Various antigenic components can induce different protective efficacy, but the type of adjuvant is also one of the main reasons [22]. It can strengthen the body's immune response and

promote the absorption of vaccines. Studies have shown that antiserum from mice immunized with Freund's adjuvant or Montanide Gel 01 adjuvant TbpB-derived antigen can activate the classical complement pathway (CCP) and kill *G. parasuis*, and the type of adjuvant can regulate TbpB Functional responses induced by derived antigens [3].

In the detection of antibody experiment, it was found that the antibodies of each immune group were at a lower level after the first immunization, but the antibody level of each immunization group increased after the second immunization, demonstrating that the recombinant LpxC and GmhA can induce high level IgG antibodies, especially mixed recombinant proteins group were much higher. In previous experiments, the antibody level of antigen-induced was consistent with its protection rate, which was also confirmed in this experiment [14]. The recombinant protein showed better protection rate (LpxC, 50%; GmhA, 50%; the mixture, 60%, respectively) than others, indicating that the recombinant protein can provide protection against infection of *G. parasuis*.

Cytokines play an important role in the immune system. A small amount of secretions can respond to invading pathogens, and pathogens are killed by lymphocytes in the body through cell communication [11]. In this experiment, the expression levels of IL-4 and IL-10 in the recombinant LpxC group and the inactivated bacteria group were significantly increased compared to the control group, and the expression level of IFN-γ in the recombinant GmhA group and the mixed LpxC and GmhA group was significantly increased compared to the control group. Therefore, it is inferred that recombinant GmhA, mixed LpxC and GmhA can stimulate the body to produce specific Th1 and Th2 immune responses, while recombinant LpxC and the inactivated bacteria can only induce the body to produce Th2 immune responses. If the adjuvant and formulation are changed, the final data is likely to change accordingly.

In conclusion, we successfully expressed recombinant LpxC and GmhA of CY1201 of *G. parasuis* (serotype 13), and evaluated the protective efficacy of the two recombinant proteins in mouse model. The results show that the two recombinant proteins can induce humoral immunity in mice and produce protective effects against infection of homologous *G. parasuis*. This experiment investigated for the first time the protective effects of these two proteins against *G. parasuis* in mice, suggesting that the proteins can be used as candidate vaccines against *G. parasuis* infection. Next, these two proteins will be further studied and evaluated for their immunoprotective effects on caesarean section and weaned piglets.

CONFLICTS OF INTEREST. There are no conflicts of interest.

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