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NOTE

Bacteriology

Evaluation of glutathione-binding protein A of *Haemophilus parasuis* as a vaccine candidate in a mouse model

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ABSTRACT. The virulent strains of *Haemophilus parasuis* are the causative agents of Glässer's disease, which can cause systemic infection and result in polyserositis, meningitis and arthritis. The development of novel, effective vaccines would be beneficial to preventing *H. parasuis* infections. Here, we report a novel immunogenic protein, glutathione-binding protein A (GbpA), which can elicit a significant humoral antibody response and confer significant protection against challenge with a lethal dose of a highly virulent *H. parasuis* strain. The *H. parasuis* strain can be fully eliminated in the immunized mice. The results indicate that GbpA has the potential to be used as an effective component of a new vaccine against *H. parasuis*.

KEY WORDS: *Haemophilus parasuis*, glutathione-binding protein A (GbpA), vaccine candidate

Haemophilus parasuis (*H. parasuis*), a gram-negative bacterium, is a member of the normal microbiota in the upper respiratory tract of pigs [10]. Using heat-stable antigens and by gel diffusion test, 15 serotypes of *H. parasuis* have been identified with apparent differences in virulence. Notably, up to 26% of the clinical strains are nontypeable. Serotypes 1, 5, 10, 12, 13 and 14 are usually virulent strains, whereas serotypes 6, 7, 9 and 11 are considered avirulent [2, 6, 9, 11]. The virulent strains of *H. parasuis* are the causative agents of Glässer's disease, which can cause systemic infection and result in polyserositis, meningitis and arthritis [1]. In recent years, *H. parasuis* infection has led to considerable economic losses in the pig industry worldwide.

To date, many researchers have tried to develop a vaccine to effectively prevent *H. parasuis* infections. An example is formalin-inactivated bacterin, but the protection was either serotype- or strain-dependent [8, 12]. Immunization with other members of the *Pasteurellaceae* family and attenuated Actinobacillus pleuropneumoniae strains has also been reported to offer some cross-protection against virulent strains [2, 3]. However, the potential side effects of these vaccines can not be ignored. Recently, interest has shifted towards protein antigens of *H. parasuis* as vaccine candidates. Although some outer-membrane and secreted proteins have been confirmed to offer good protection [4, 7, 17], it is necessary to identify additional, novel protective antigens to develop *H. parasuis* vaccines for pigs.

In a previous work, we had identified a few secreted immunogenic proteins of *H. parasuis* by immunoproteomics (data not shown). Among these proteins, glutathione-binding protein A (GbpA), showed very strong reaction with convalescent serum, indicating that it has potential as a candidate vaccine. In this study, the potential of developing GbpA as a novel vaccine antigen against *H. parasuis* serotype 5 infection was investigated.

Bacterial strains and growth conditions: *H. parasuis* serotype 5 strain Nagasaki was maintained on tryptic soy agar (TSA; Difco Laboratories, Detroit, MI, U.S.A.) containing 10% bovine serum and 0.01% nicotinamide adenine dinucleotide (NAD). It was cultured in tryptic soy broth (TSB) medium (Difco Laboratories) containing 10% bovine serum and 0.01% NAD at 37°C aerobically.

Laboratory *Escherichia coli* strain DH5α was used for gene cloning, whereas *E. coli* strain BL21 (DE3) was used to produce the recombinant GbpA (rGbpA). The *E. coli* strains were cultured on Luria-Bertani (LB) agar. When necessary, kanamycin (25 μg/m*l*) was added to the agar.

Gene amplification and construction of expression vectors: Primers for the *gbpA* gene of *H. parasuis* serotype 5 strain Nagasaki (gi: 219691582) were designed to generate recombinant protein that contained *Nco*I and *Xho*I sites at the 5′ and 3′ ends, respectively. The primers were as follow: GbpA-F (5′-ATACCATGGAAAACGTTTATTAACTG-3′) and GbpA-R (5′-ATACTCGAGTTAATCCGCCAACT-3′). PCR was performed in a thermal cycler, using genomic DNA of *H. parasuis* strain Nagasaki as a template with the following conditions: denaturing at 95°C for 30 sec, annealing at 50°C for 30 sec and extension at

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72°C for 1.5 min, for a total of 30 cycles. The 1596-bp PCR product of *gbpA* was cloned into expression vector pET-28a in frame with the N-terminal 6×His tag. The derivative plasmid, pET-gbpA, was introduced into *E. coli* strain BL21 (DE3) to produce the recombinant protein.

Purification of recombinant GbpA: To produce rGbpA, overnight cultures of *E. coli* strain BL21 (DE3) harboring pET-gbpA were subcultured (1:1,000) in fresh LB medium with 25 μg/m*l* kanamycin and incubated at 37°C. When the optical density at 600 nm (OD_{600}) had reached 0.6, the cultures were induced with 1 mM isopropyl β-D-1-thiogalactopyranoside and incubated at 28°C for 3 hr. Thereafter, the bacteria were harvested by centrifugation. The pellet was resuspended in 5 m*l* of 50 mM sodium phosphate buffer (pH 8.0) containing 300 mM NaCl, 10 mM imidazole, 1 mM phenylmethyl sulfonylfluoride and 5 mg of lysozyme, and incubated on ice for 30 min. After the bacterial cells had been disrupted by ultrasonication on ice, the lysates were centrifuged at 10,000 ×*g* for 30 min. The rGbpA containing the N-terminal 6×his tag was isolated from the supernatant by Ni^{2+}-nitrilotriacetic acid affinity chromatography, as described in the QIAexpress manual. The rGbpA was stored at −80°C until use.

SDS-PAGE and western blot analysis: The purified rGbpA (2 μg) was separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene difluoride membrane. The membrane was blocked overnight by incubation with 0.5% bovine serum albumin (BSA) in TBST (0.05% Tween-20, 20 mM Tris-HCl and 150 mM NaCl) at 4°C. The membrane was then incubated with convalescent swine serum (1:200) against *H. parasuis* at room temperature for 1 hr and then washed three times with TBST. This was followed by an incubation with horseradish peroxidase (HRP)-conjugated goat anti-porcine IgG (H + L) (1:5,000) (Southern Biotech, Birmingham, AL, U.S.A.) at room temperature for 1 hr. After washing three times with TBST, the membrane was developed with the ECL Plus Western Blotting Detection System (GE Healthcare, Piscataway, NJ, U.S.A.).

Immunization and challenge studies: All experimental protocols were approved by the Animal Ethics Committee of Harbin Veterinary Research Institute and were performed accordingly. Twenty 4–6-week-old female BALB/c mice were randomly assigned to two groups of 10 each. The purified rGbpA (100 μg), which was emulsified with Marcol 52 (ESSO)-based adjuvant, was tested in this experiment. The mice in group 1 were subcutaneously immunized with emulsified rGbpA. Subsequent booster injections with the same antigen were given on the 14th day. Mice in group 2 were mock-injected with phosphate-buffered saline (PBS) emulsified with the same adjuvant, as negative controls. On the 10th day after the booster immunization, serum was obtained from each mouse by tail vein bleeding. On the same day, all mice were intraperitoneally challenged with a lethal dose ($5LD_{50}$) of 7.3×10^9 colony-forming units (CFU) of log-phase *H. parasuis* strain Nagasaki in 0.5 m*l* of PBS. After challenge, all mice were observed for 5 days for morbidity and mortality.

Determination of specific antibody: Microtiter plates were coated with 200 *n*g per 100 μl of purified rGbpA (diluted in sodium carbonate buffer, pH 9.6), overnight at 4°C. Thereafter, the plates were saturated with 200 μl of PBS containing 0.5% BSA and 0.05% Tween-20 for 30 min at 37°C. Then, 100 μl of serially diluted mice sera was added to each well, and the plates were incubated for 30 min at 37°C. After washing three times with washing buffer (PBS containing 0.05% Tween-20), 100 μl of IgG-HRP (1:5,000 dilution) was added to each well, and the plates were incubated for 30 min at 37°C. Subsequently, the plates were washed three times with washing buffer. The reactions were developed by adding 100 μl of the activated substrate solution (sodium citrate buffer containing 1 mg/m*l* 3,3′,5,5′-tetramethylbenzidine and 0.03% H_2O_2), and the plates were incubated in the dark for 10 min. Finally, the reactions were stopped by adding 50 μl of 2 M sulfuric acid to each well. The plates were read by an enzyme-linked immunosorbent assay microplate reader at 450 nm.

Determination of viable bacteria in organs: On the 5th day after challenge, the surviving mice from the immunized group were euthanized with CO_2, and the hearts, livers, spleens, lungs and kidneys were obtained aseptically. For the control group, the organs of each mouse were obtained immediately after the mice had died. Samples of the organs (0.05 g/organ) were placed in 500 μl of PBS (pH 7.3) and homogenized with a vortexer. Then, 50 μl of serial dilutions of the homogenate in PBS was plated onto TSA plates containing 10% bovine serum and 0.01% NAD. The plates were incubated overnight at 37°C for 24 hr in a humidified 5% CO_2 incubator. Colonies were counted and expressed as CFU/0.05 g of organ samples.

Statistical analysis: Data were analyzed by Student's *t*-test. A value of $P<0.05$ was considered to indicate statistically significant differences.

GbpA is an immunogenic protein: Upon SDS-PAGE, the purified rGbpA showed a protein band of approximately 60 kDa, which corresponded to its predicted size (Fig. 1A). Western blotting analysis revealed this protein to have good immunoreactivity to convalescent sera, which is against *H. parasuis* (Fig. 1B).

rGbpA can elicit a significant humoral antibody response: To monitor the antigen-specific response after immunization with rGbpA, sera were collected from the immunized mice. Antibody titers against rGbpA were determined in the sera. Levels of specific IgG against rGbpA were significantly higher in the immunized group ($P<0.001$) than in the negative control group (Fig. 2).

Vaccinated mice survived challenge with virulent H. parasuis: On the 10th day after the booster immunization, all mice were challenged with a lethal dose of *H. parasuis* serotype 5 strain Nagasaki. In the control group (Fig. 3), all of the mice died within the first 2 days after infection. However, 90% of the mice that received rGbpA vaccination survived on day 1. After day 2, 50% of mice in the immunized group had survived and gradually recovered from the disease until the end of this study.

H. parasuis can be fully eliminated in the immunized mice: To further test the protective efficacy of rGbpA, organs of the dead mice in each group were cultured on TSA plates to recover *H. parasuis* cells. As mentioned above, all of the mice in the control group had died within the first 2 days. *H. parasuis* cells could be isolated from all of the tested organs of these control mice, with high levels of bacterial load found in the spleen and liver. In the immunized group, the 50% of mice that had survived and recovered from the infection were also euthanized to determine the bacterial load in different organs. Interestingly, no *H. parasuis*

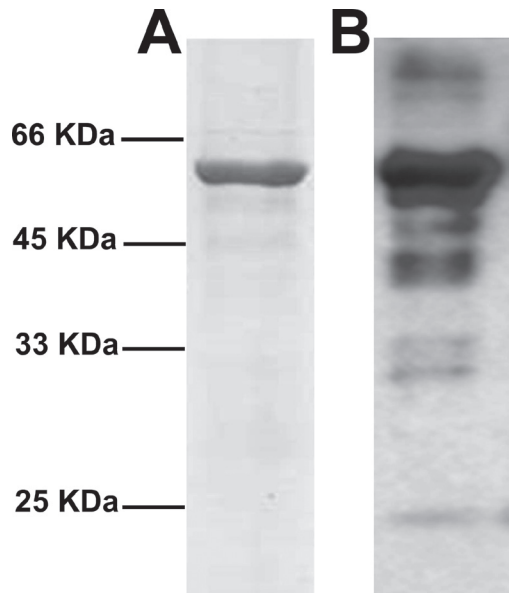

Fig. 1. Representative results from SDS-PAGE (A) and western blot (B) analyses of rGbpA. Molecular size markers are indicated in kDa on the left.

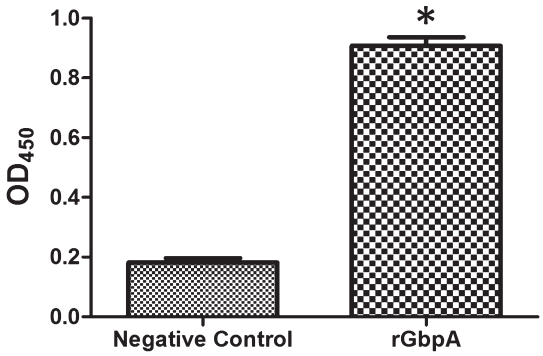

Fig. 2. Immune response induced by rGbpA in mice. The levels of specific IgG against rGbpA were significantly higher in the immunized group than in the negative control group. "*" represents $P<0.05$ for rGbpA versus the negative control.

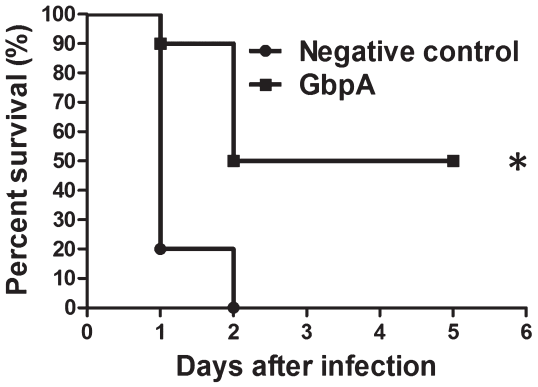

Fig. 3. Survival of mice, immunized either with rGbpA or an adjuvant (negative control), following challenge with *Haemophilus parasuis*. "*" represents $P<0.05$ for rGbpA versus the negative control.

Table 1. Bacterial load (colony-forming units) in major organs after challenge with *Haemophilus parasuis* strain Nagasaki

Organ	GbpA group	Control group
Heart	0	$1.5 \pm 0.2 \times 10^6$
Liver	0	$1.1 \pm 0.4 \times 10^8$
Spleen	0	$2.3 \pm 0.6 \times 10^8$
Lung	0	$3.2 \pm 0.4 \times 10^7$
Kidney	0	$5.5 \pm 0.1 \times 10^6$

cells could be found in the organs of these mice (Table 1).

H. parasuis, which is responsible for Glässer's disease, has attracted increasing attention in the past decade owing to the significant economic losses it causes in the pig industry worldwide. The development of a subunit vaccine of *H. parasuis* is therefore considered useful for the livestock industry, and identification of more novel protective antigens is of interest to researchers in this field. Therefore, the present study evaluated the protective efficacy of rGbpA.

Glutathione is a vital intracellular cysteine-containing tripeptide across all kingdoms of life and assumes a plethora of cellular roles. In unicellular yeasts and bacteria, imported glutathione is a supply of organic sulfur [5, 13–16]. Genetic inactivation of the *yliABCD* operon, which encodes a putative ABC superfamily importer in *E. coli*, resulted in severely retarded growth of the bacterium on minimal medium plates supplemented with glutathione as a sole sulfur source [13]. During infection, the GbpA of *H. parasuis* may help these bacteria to obtain the necessary sulfur from their host.

In this paper, we cloned and overexpressed a recombinant GbpA in *E. coli*. The rGbpA showed high immunoreactivity to convalescent serum, indicating that the protein had the potential to be a candidate vaccine. Therefore, to evaluate the protective efficacy of rGbpA, we employed a mouse challenge model that had recently been used [7, 17, 18]. rGbpA was able to induce a high level of antibodies and confer high protection against challenge with a lethal dose of a highly virulent *H. parasuis* strain. Moreover, compared with the non-immunized mice, the immunized mice could fully eliminate the invasive bacteria in a few days. Although

only part of the genome data of *H. parasuis* serotypes have been released, the *gbpA* gene was found to be highly conserved among serotypes 2, 4, 9, 11, 13 and 14 (data not shown). This suggests that rGbpA may have immunoprotective potential against different serotypes. All of these data strongly indicate that rGbpA can serve as an effective component of a *H. parasuis* vaccine. A further study will optimize the immunization strategy and explore the efficacy of rGbpA as a vaccine candidate to protect pigs against *H. parasuis* infection.

CONFLICTS OF INTEREST. The authors declare no conflicts of interest.

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