Protection of Piglets by a Haemophilus parasuis Ghost Vaccine against Homologous Challenge

Running title: Protective efficacy of Haemophilus parasuis ghost vaccine

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Abstract  Commercial bacterins for Glässers disease are widely used for prevention this disease caused by *Haemophilus parasuis*, however, the protective efficacy varied depending on the strains and serovars. Bacterial ghosts (BGs) are empty bacterial envelopes, unlike classical bacterins, suffer no denaturing steps during their production. These properties may lead to a superior protection. In this study, a BGs vaccine generated from the *Haemophilus parasuis* serovar 5 reference strain Nagasaki was prepared and used to inoculate piglets. The efficacy of the BGs vaccine was evaluated by clinical, bacteriological, serological, and post-mortem examinations. Inactivated bacterin (IB) and a placebo control (PC) were compared with the BGs vaccine in this study. The results showed that the piglets inoculated with the BGs vaccine developed a higher antibody activity and higher IFN-γ and IL-4 levels than those vaccinated with IB or the PC group after primary and secondary exposure to the antigens and challenge. The CD4⁺ T lymphocytes were observed to increase following secondary immunization in the BGs-vaccinated group more than in the IB (p < 0.05) and PC (p < 0.05) groups. The CD8⁺ T lymphocytes increased dramatically in all three groups after challenge, and the differences between each group all were significant (p < 0.05). There were fewer tissue lesions and lower bacterial load in the tissue homogenates observed in the BGs group after challenge. The results suggested that higher CD4⁺ T lymphocytes rate and both the CD4⁺ MHC-II restricted Th1 type and Th2 type immune responses in the BGs group appear relevant to protection.
Keywords: Bacterial ghosts; \textit{Haemophilus parasuis}; Humoral immune; Cellular immune
*Haemophilus parasuis* (*H. parasuis*) is a gram-negative, non-hemolytic, nicotinamide adenine dinucleotide (NAD)-dependent bacterium belonging to the Pasteurellaceae family. *H. parasuis* is a commensal organism of the upper respiratory tract of conventional pigs that can cause Glässers disease, which is characterized by a fibrinous polyserositis, polyarthritis, and meningitis (1-3). This disease causes significant losses in the swine industry worldwide, due to its high mortality and morbidity (1, 3). Vaccination is generally considered to be the most effective means to control Glässers disease; however, attempts to develop effective vaccines against *H. parasuis* have been hampered by a lack of overall knowledge of the virulence factors and protective antigens of this bacterium (1). Inactivated bacterin for *H. parasuis* is a traditional vaccine used around the world that can elicit efficient protection against homologous challenges; however, due to the serovar diversity of *H. parasuis*, inactivated bacterin is limited in cross-protection (2-4). In a previous study, subunit vaccines comprising the recombinant transferrin-binding protein (Tbp) B or outer membrane protein (OMP) formulations enriched with TbpB only conferred partial protection (5). OMPs PalA, Omp2, D15 and HPS-06257 have been shown to induced a protective response against *H. parasuis* (SH0165) in pigs (3), and OMPs SmpA, YgiW, and FOG have been shown to be protective both individually and synergistically against infection with the highly virulent *H. parasuis* in mice (6). However, as mentioned above, the major virulence factors and protective antigens of *H. parasuis* are still largely unknown and need to be further identified and characterized.
In contrast to the traditional inactivated bacterin, which lacks cross-protection and fails to prevent subclinical or chronic infection and pathogen colonization, a genetically inactivated vaccine — the ghost vaccine — that has emerged in recent years has been considered to be an alternative to traditional inactivated bacterin (7-9). Bacterial ghosts (BGs) are empty cell envelopes from gram-negative bacteria that can be produced by the controlled expression of the cloned lysis gene E from bacteriophage ΦX174, the plasmid-encoded protein E leads to the formation of a transmembrane tunnel structure through the cell envelope of gram-negative bacteria through which the cytoplasmic contents are expelled (10, 11). The resulting ghosts share the function and antigenic determinants of the envelope with their replicating counterparts because protein E does not cause any physical or chemical denaturation to the bacteria surfaces during the lysis processes (10-13). Owing to the particulate nature of the BGs and the fact that they contain many well-known immune-stimulating compounds, the intrinsic adjuvant properties of BGs enhance T-cell activation and systemic, mucosal and cell-mediated immune response to the envelope structures (12-15). This useful property makes BGs a promising versatile delivery vehicle for heterologous antigens, drugs and other biologically active substances (14-19). Another attractive character of the BGs vaccine is the easy-to-produce platform technology, requiring merely expression of the lysis gene E within the bacteria cells without the need for the information in the genome (12, 16), which is especially useful for those bacteria that have not been thoroughly studied regarding functional genes, e.g., H. parasuis.
The objective of the present study was to assess the efficacy of intramuscular immunization with *H. parasuis* ghosts to confer protection against homologous challenge in an intraperitoneal-infection model. The protective effect was compared to those induced by a formalin-inactivated bacterin. In this study, we describe for the first time the protective efficacy of *H. parasuis* ghosts.

**Material and methods**

**Bacterial strains, plasmids and media.** The *H. parasuis* serovar 5 reference strain Nagasaki was cultured in tryptic soy broth (TSB, Difco) medium or on tryptic soy agar (TSA) supplemented with 10 μg/mL nicotinamide adenine dinucleotide (NAD) and 5% fetal calf serum (Gibco) and incubated at 37 °C in a 5% CO2 incubator. The *E. coli* strain DH5α (Novagen) was grown in Luria-Bertan (LB) broth at 37 °C in an orbital shaker incubator; for cultivation of *E. coli* β2155, (dapA) diaminopimelic acid (1mM; Sigma Chemical Company) was added. The medium was supplemented with the appropriate antibiotics (ampicillin, 100 μg/mL) when needed for the selection or the stabilization of the plasmids. The bacterial strains, plasmids and primers used in this study are listed in Table1.

**Construction of bacteriolytic plasmid pMD-mE and electroporation of E. coli β2155.** A mutant lysis gene E that has a single base mutant at the 5’ terminal start codon ATG (A/C) and a truncated 201-bp lysis gene was inserted into plasmid pBV220, which harbored a thermo-sensitive regulator system Pr/Pl-cI857 from a Lambda bacteriophage (9). The lysis cassette gene Pr/Pl-cI857-mE was amplified by PCR and cloned into
plasmid pMD-18T (TaKaRa) to create the bacteriolytic plasmid pMD-mE. To construct
the recombinant *E. coli* β2155 that harbors a bacteriolytic plasmid, pMD-mE was
electroporated into *E. coli* β2155 competent cells with an electroporator (MicroPulser,
Bio-Rad) set at 12.5kV/cm, 200Ω, 25μF (20). The positive clone was identified by clone
PCR and restriction enzyme digestion, and the *E. coli* β2155 strain, which contains the
bacteriolytic plasmid pMD-mE, was designated as rEC-mE.

**Transconjugation rE. coli-mE with *H. parasuis* strain Nagasaki.** The bacteriolytic
plasmid pMD-mE was mobilized from rEC-mE to *H. parasuis* serovar 5 reference strain
Nagasaki using a filter mating technique, as described previously (20). Briefly, donor and
acceptor strains were grown on an appropriate solid medium overnight and resuspended
in TSB medium, and the OD_{600} was determined thereafter. Aliquots corresponding to 1
mL of donor and 0.1 mL of acceptor (each at OD_{600}=0.4) were mixed and filtered onto a
nitrocellulose disc (0.45 μm pore size, 2.5 cm diameter, Millipore). The filters were
placed onto solid medium to support the growth of the donor and acceptor (TSB
supplemented as above, containing 1mM dapA and 10 μg/mL NAD) and incubated for 24
h. Next, the bacteria were washed off the filters with supplemented TSB, plated onto
selective medium (supplemented TSB with 100 μg/mL ampicillin, 10 μg/mL NAD) and
incubated for 36 h. The positive *H. parasuis* clone that harboring bacteriolytic plasmid
pMD-mE was identified by clone PCR and was designated as rHps-mE.

**Preparation of *H. parasuis* ghosts vaccine and inactivated bacterin.** The rHps-mE
was inoculated into supplemented TSB (100 μg/mL ampicillin, 10 μg/mL NAD), and the
culture was incubated at 28 °C to an OD_{600} of 0.4~0.5. The temperature was subsequently raised to 42 °C to induce mE-mediated lysis (9-11). The OD_{600} was measured every 30 min until no further decrease in OD_{600} was detectable. After completion of the lysis, the BGs were harvested by centrifugation (4,000 g for 10 min at 4 °C), washed with PBS (pH 7.2), resuspended in sterile distilled water, lyophilized and stored at -20 °C. Loss of viability was assessed by plating the BGs samples onto supplemented TSA agar and into supplemented TSB broth (10 μg/mL NAD). The incubations were performed at 28 °C for 36 h. The lyophilized BGs were dissolved with PBS (pH 7.2) to an appropriate concentration (equivalent to 1×10^{11} CFU) and used directly as a BGs vaccine to inoculate the piglets. To prepare the inactivated bacterin, the *H. parasuis* serovar 5 reference strain Nagasaki was cultured, and eight to ten single colonies were selected and spread onto TSA agar plates (10 μg/mL NAD). After incubation at 37 °C for 16 to 18 h, the bacteria were harvested into sterile 0.01 mol/l PBS, diluted to 2×10^{11} CFU/mL, inactivated with 0.3 % formalin for 24 h, washed three times with PBS (pH 7.2), and finally resuspended in PBS to the original volume at 4 °C. Two hundred microliters of the prepared inactivated cultures were randomly sampled and cultured on LB plates overnight to determine bacterial growth. The sterile cultures were emulsified with equivalent amounts of complete Freund's adjuvant (Sigma-Aldrich, F5881) for the first injection or incomplete Freund’s adjuvant (Sigma-Aldrich, F5506) for the booster injection.

**Animals and design.** A total of 18 piglets (5 to 6 weeks of age) from *H. parasuis* and other respiratory pathogens-free farm (PCR or RT-PCR negative with the nasal and
tonsillar swabs, no serological responses in relevant enzyme-linked immunosorbent
assays (ELISAs)) were selected from two litters of the same age and weights (10 piglets
from one litter, and 8 piglets from the other), and were blocked by different sex. The 18
piglets were randomly allotted into three groups of equal size according to sex, and
housed separately with identical feeding conditions. Thus, in each group, there was an
equal proportion of each sex to avoid any sex-based effects. Selecting and allotting
piglets was a blinded study. All of the experimental works involving animals described in
this study were conducted with approval from Harbin Veterinary Research Institute
Animal Ethics Committee in accordance with the guidelines for the use of animals in
research based on considerations of animal welfare and ethics.

Vaccination and challenge of piglets. The piglets in groups I and II were injected with
BGs (*H. parasuis* ghosts, $1 \times 10^{11}$ CFU/mL/piglet) and IB (inactivated bacterin, $1 \times 10^{11}$
CFU/mL/piglet), respectively. The placebo control group (PC) was injected with the same
dose of PBS. Over a two-week interval, the vaccines were administered twice by deep
intramuscular injection into the pig’s neck. Fourteen days after the second immunization,
all of the piglets received an intraperitoneal challenge with a dose of $5 \times 10^9$ CFU/piglet of
*H. parasuis* strain Nagasaki. The challenged pigs which showed severe clinical signs of
Glässers disease, e.g., dyspnea, prostration, and incoordination were subjected to
euthanasia, the surviving pigs in all of the experimental groups were euthanized on day 7
post-infection. Rectal temperatures and clinical signs were recorded twice a day during
the first 3 days post-infection (dpi), and after that once a day until the end of the study. A
clinical scoring system was used to assess the clinical signs of each individual animal as follows. A score of 1 was given for each case of coughing, anorexia, and lameness, resulting in a minimum clinical score of 0 and a maximum score of 3 per day; pigs euthanized from the disease were assigned a score of 4. The added daily clinical scores of days 1 to 7 after challenge for each piglet were designated the total clinical score, the arithmetic mean and standard deviation were determined in each group. The average temperature value for each piglet during the observation period was calculated, and the mean temperature value for each group was determined. All animals were subjected to necropsy, and gross lesions were recorded. The severity of the pathological changes was scored blindly as follows, a score of 1 was given for each case of pleuritis, peritonitis, arthritis, meningitis, and pericarditis, resulting in a minimum pathological score of 0 and a maximum score of 5 for each piglet. The arithmetic mean and standard deviation were determined in each group.

**Serological analysis.** Blood and sera were collected prior to and following the primary and secondary immunizations and challenge. Serum antibody activities were measured using an indirect ELISA as described previously based on inactivated whole-cell antigens (8). Briefly, 96-microwell plates (Costar, eBioscience) were coated with $5 \times 10^7$ bacteria per well in carbonate buffer, pH 9.6, overnight at rt (room temperature). Next, the plates were blocked overnight at 4 °C. After blocking, the plates were incubated with the sera at
37 °C for 1 h. The sera of the immunized pigs, diluted (1:100, 50 μL/well) with PBST (PBS with 0.1% Tween 20), were used as the primary antibodies, and HRP-conjugated goat anti-pig IgG (H+L) (Sigma-Aldrich) (1:5000 dilution, 50 μL/well) was used as the secondary antibody. The convalescent sera from \( H. \ parasuis \) infection and the pre-immune sera were used as the positive and negative control, respectively. The enzymatic reaction was developed with chromogen containing 20 μL 1% H₂O₂ (vol/vol) in 10 mL 0.1M citrate buffer, pH 5.0, and 0.1 mg/mL TMB (3, 3’, 5, 5’-tetramethylbenzidine) for 10 min and was stopped by the addition of 2 M sulfuric acid. Between each step, the plates were washed 3 times with PBST. The results were read on a Dynatech MR 7000 ELISA reader (Bio-Rad model 680, Bio-Rad Laboratories). The OD₄₅₀ was recorded to determine the serum antibody activities in the piglets. All of the reaction mixtures were prepared in triplicate, and the average values were used for recording and calculation. The mean values of 6 pigs within each group were reported. The amounts of IFN-γ and IL-4 were determined using interferon-γ and interleukin-4 ELISA test kits (Dalian Pan-State Biotechnology Company). These two cytokine detection kits were based on a solid phase sandwich ELISA using paired cytokine-specific monoclonal antibodies (mAbs). Reactions were detected with TMB substrate and read at a wave length of 450 nm using an ELISA reader. The concentrations of IFN-γ or IL-4 in the samples can be read from the standard curve plotted based on the values of the serial diluted standard. The detection limit for IFN-γ was <2.0 pg/mL and the standard curve range was 0-500 pg/ml; the detection limit for IL-4 was 2.0 pg/mL and
the standard curve range was 0-1000 pg/ml. Each serum sample was tested in triplicate, and the mean value in each group was calculated.

**CD4^+CD8^+ T cell subset counts.** The blood samples were collected by aseptic venipuncture from the jugular vein into commercial tubes containing heparin sodium as anticoagulant. The blood samples were analyzed within 8 h. A three-color staining method was used in this study. One μL each of Fluorescein isothiocyanate (FITC)-conjugated CD4 mAb, PE (phycoerythrin)-conjugated CD8 mAb and SPRD (Spectral Red)-conjugated CD3 mAb (SouthernBiotech) were added to 0.1 mL of a blood suspension and incubated in the dark at rt for 20 min. Next, 2 mL of FACS Lysing Solution (BD Biosciences), pre-warmed to 37 °C, was added, and the resulting mixture was incubated in the dark at rt for 15 min. The cell suspension was centrifuged at 200 g for 5 min, and the supernatant was discarded. The pellets were washed with PBS twice at 200 g for 5 min, and the cells were subsequently resuspended in 400 μL of PBS. The FACS analysis was performed using a flow cytometer (BD Biosciences) equipped with the CELLQuest software (BD Biosciences). Before analysis, the unstained cells sample and FITC-, PE-, and SPRD-labeled cells were run to set proper compensation and to define quadrants. The lymphocytes were selected for analysis by gating to exclude both macrophages and cellular debris based on size and granularity. A total of 10000 events were measured per sample, the percent of each lymphocyte subpopulation was recorded.

**Bacteriological analysis.** At necropsy immediately after euthanasia, tissues from the lungs, spleen, and inguinal lymph nodes were aseptically collected from each animal,
weighed and homogenized with aseptic glass dismembyators. The tissue homogenates were serially diluted with PBS, and 100 μL each of $10^3$, $10^4$, and $10^5$-fold dilutions were plated onto selective medium and incubated at 37 °C for 36 h. Confirmation of *H. parasuis* was carried out by colony PCR, the CFUs per mg of homogenized tissue were determined.

**Histopathological examination.** At necropsy, the liver, spleen, lung, kidney, and small intestine were collected and processed into samples of 0.5–1.0 cm³ size and fixed in 10 % formol saline for 6 to 24 h. The tissue blocks were then dehydrated with ethanol, treated with xylene and embedded in paraffin. Sections measuring 5–6 μm in thickness each were prepared using a microtome, placed onto slides and dried. After washing with xylene and dehydration with ethanol, the slides were stained with hematoxylin and eosin and were observed under a microscope.

**Statistical analysis.** A statistical analysis was conducted using the SAS v. 9.0 software. An analysis of variance (ANOVA) with post hoc Bonferroni adjustments was used to determine the significance of the differences in the means between multiple experimental groups. The data were expressed as the mean +/- standard deviation, and values of $P<0.05$ were considered to be significant.

**Results**

**Preparation of *H. parasuis* ghosts and lysis efficacy.** As shown in the lysis efficacy curve (Fig.1), the lysis plasmid, pMD-mE, demonstrated high efficiency in the *H.*
*parasuis* strain Nagasaki. When the culture temperature was shifted from 28 °C to 42 °C, the OD$_{600}$ value of the recombinant *H. parasuis* (rHps-mE) that harbored the lysis plasmid pMD-mE declined after 30 minutes. The OD$_{600}$ value declined continuously until the lysis was completed after induction for 180 minutes, and the lysis efficiency as high as 99.99% was subsequently obtained. In comparison, the OD$_{600}$ value of the parent strain of *H. parasuis* Nagasaki increased continuously during the observation period. Though there were few viable cells after lysis, no viable cells were observed from the lyophilized ghosts. The transmission electron microscopy photos of the *H. parasuis* parent strain and its ghost are shown in Fig. 2A and 2B.

**Clinical monitoring.** All of the BGs-vaccinated pigs and four of those vaccinated with IB gave the best clinical performance. A slight and transient rise in rectal temperature (< 40.5 °C) in several of the piglets was recorded within 24 hpi (hours post-infection), and moderate clinical signs, such as cough and anorexia were observed in individual pigs. However, all of the pigs in the PC group and two pigs in the IB group were euthanized due to severe clinical signs suspicious of Glässers disease from 6 hpi to 5 dpi. All of the euthanized pigs with severe clinical signs had shown elevated temperatures from 40.5 to 42.1 °C, and the clinical signs including prostration, incoordination, and severe dyspnea. Summaries of the clinical signs are listed in Table 2.

**Histopathological examination.** The pigs that had been euthanized on day 7 post-challenge had no or moderate lesions when examined, and focal pneumonia and mild peritonitis were observed in individual pigs. However, in the pigs that had been
euthanized due to the severe clinical signs, the lesions were severe, with at least one of the following fibrinous inflammations being observed in each of the pigs: fibrinosuppurative pneumonia, pericarditis, pleuritis, peritonitis, arthritis and meningitis, characterized by the presence of fibrin strands or layers on serosal surfaces. The microscopic lesions showed a comprehensive fibrinous exudative inflammation in the organ samples examined, with a mixed inflammatory exudate being composed of variable amounts of fibrin, macrophages, neutrophils and lymphocytes. Vascular alterations, such as edema, congestion, hemorrhages, and thrombosis, were observed in most of the organs (heart, lungs, lymph nodes, kidneys, liver, spleen, brain) and blood vessels. Summaries of the histopathological findings are listed in Table 2.

**Serological analysis.** The sera antibody against the whole-cell antigen of *H. parasuis* was observed in both immunized groups following primary and second immunization, and the antibody activity increased over time (Fig. 3A). On days 7 and 14 after the initial injection, IB group showed significant differences in antibody activity from those in the non-vaccinated PC group (*P*<0.05); the difference between the BGs group and the PC group became significant on day 7 (*P*<0.05) and on day 14 (*P*<0.01). On days 21 and 28, the piglets in the BGs group showed significant differences in antibody activity from those in the IB group (*P*<0.05), and the differences between the BGs group and the PC group were significant (*P*=0.0008). The differences between the IB group and the PC group were also significant (*P*<0.01). On day 35, there were significant differences in the antibody activity between groups BGs and IB (*P*<0.05) and between groups BGs and PC.
The difference between groups IB and PC was also significant \((P<0.05)\) on day 35.

The concentrations of IFN-\(\gamma\) in the different groups are summarized in Fig.3B. The IFN-\(\gamma\) concentration in the sera of the vaccinated piglets in the BGs group was significantly higher than those in groups IB and PC on day 14 \((P<0.05)\), and on day 28 \((P<0.01)\). On day 35, the IFN-\(\gamma\) concentration increased in all of the experimental groups, and the difference between groups BGs and IB, groups BGs and PC, and groups IB and PC all were significant \((P<0.05)\).

The concentrations of IL-4 in the different groups are summarized in Fig. 3C. On day 14, the IL-4 concentration in the sera of the piglets in the BGs group showed a significant difference from those in the PC group \((P<0.01)\), and the differences between groups BGs and IB and between groups IB and PC were also significant \((P<0.05)\). From the results collected on days 28 and 35, the differences in IL-4 concentrations between groups BGs and PC \((P<0.001)\), groups IB and PC \((P<0.01)\) and groups BGs and IB \((P<0.05)\) all were significant.

**The relative proportion of CD4\(^+\) and CD8\(^+\) T cell subsets.** The relative proportions of CD4\(^+\) and CD8\(^+\) T cell subsets in the peripheral blood of the piglets in each group were analyzed using FACS. On day 14, the number of CD4\(^+\)T cells in each group showed no significant difference; however on day 28, the proportion of CD4\(^+\) T cells in the BGs group increased and was significantly higher than that observed in groups IB and PC \((P<0.05)\). On day 35, the percentage of CD4\(^+\) T cells in the BGs group were significantly
higher than those in the IB group (P<0.05) and the PC group (P<0.01). Though the CD4+ T cells in the IB group showed no significant difference compared to the PC group on day 14 and 28 (P>0.05), the difference was significant on day 35 (P<0.05, Fig. 4A). On day 14 and 28, there were no significant differences in the proportion of the CD8+ T cells subset among all the experimental groups. On day 35, one week after challenge, the percentage of CD8+ T cells in all three groups increased dramatically, there were significantly more CD8+ T cells in groups IB and PC than in the BGs group (P<0.05), and the difference between groups IB and PC was also significant (P<0.05) at this time point (Fig. 4B).

Bacteriological analysis of tissue homogenates. The number of recovered CFUs in the homogenized inguinal lymph nodes, spleen, and lung tissues was significantly lower in the BGs group compared to the IB group (P<0.05) and the PC group (P<0.01), and the differences in the bacterial load between the IB group and the PC group were also significant (P<0.05) (Fig. 5).

Discussion

In this study, we have constructed a series of bacteriolytic plasmids based on different plasmids, including the Actinobacillus pleuropneumoniae-Escherichia coli shuttle vector pGZRS-18 (isolated from A. pleuropneumoniae), the broad-host-range cloning vector pBBR1MCS-4 (isolated from Bordetella bronchiseptica), the Pasteurella multocida-Escherichia coli shuttle vector pPBA1100 (isolated from P. multocida), and the TA-cloning vector pMD-18T (derived from plasmids isolated from E. coli). Because
A. pleuropneumoniae, B. bronchiseptica, and P. multocida have a closer relationship with H. parasuis than E. coli, we anticipate that the pGZRS-18-, pBBR1MCS-4-, and pPBA1100-based bacteriolytic plasmids should have better replicative ability than the pMD-18T-based bacteriolytic plasmid in H. parasuis and should express and yield more E protein, thereby leading to higher lysis efficiency. However, the lysis efficiency of the bacteriolytic plasmids pGZRS-E, pBBR-E, and pPBA-E were all lower than 90% when E protein expression in H. parasuis was induced (data not shown), whereas the lysis efficiency of pMD-E reached as high as 99.9966%. Thus, the bacteriolytic plasmid pMD-E was used to prepare the H. parasuis ghosts in this study.

Our study investigated the dynamic changes in CD4+ and CD8+ T lymphocyte subsets, the secretion of cytokines, and the humoral immune response to H. parasuis in BGs- and IB-vaccinated piglets. Our data showed that there were no significant differences in the CD4+ and CD8+ T cells populations between the treatment groups at early time points (days 0, 14 and 28), except for the CD4+ T cells in the BGs group that apparently increased on day 28 (P<0.05 compared to the IB group and the PC group). However, greater differences were observed following high-dose challenge; the CD4+ T cells increased significantly in both the BGs- and IB-vaccinated groups, the difference compared to the PC group was significant for the IB group (P<0.05) and for the BGs group (P<0.01), and the difference between these two vaccinated groups was also significant (P<0.05). Our result was similar to that observed in a previous study with another gram-negative organism, A. pleuropneumoniae, which also showed a greater
cell-mediated immune response after infection than after immunization (21). The CD8+ T cells subset was also observed to increase dramatically in all three experimental groups one week after challenge (day 35). The ranking of the numbers of CD8+ T cells in each group was PC, IB, and BGs, and the differences between each group all were significant (P<0.05). From our results, the better protective efficacy of the BGs vaccine appeared to be correlated with the increased numbers of CD4+ T cells and thus the increased CD4+:CD8+ ratios prior to and following challenge. In contrast, in the unprotected PC group which had the highest numbers of CD8+ T cells after challenge, the CD4+ T cells and the CD4+:CD8+ ratios were the lowest. In a previous study, the CD4+:CD8+ ratio was considered to be relevant to protection in an immunization and challenge study on Actinobacillus pleuropneumoniae (21). In this study, the CD4+:CD8+ ratio in the BGs-vaccinated group was 1.5-fold higher than that in the IB-vaccinated group and the PC group on day 28. On day 35, this ratio became 1.8-fold and 5.6-fold compared to that of the IB-vaccinated group and the PC group, respectively. Similarly, in the present study, the CD4+:CD8+ ratio in the BGs group was also 1.5-fold higher than that in the IB group and the PC group on day 28, and the ratio became 1.8-fold and 5.7-fold on day 35, respectively.

In combination with the results of cytokines IFN-γ and IL-4 in which the BGs group showed a much higher level than those samples in the IB group and the PC group throughout the whole experimental period, this data indicate that both the CD4+ major histocompatibility complex class II (MHC-II) restricted Th1 type cell-mediated immune
responses (produced IFN-γ) and the Th2 type humoral immune responses (produced IL-4) may play an important role in the BGs-vaccine-induced immune protection. Similarly, a previous study (22) reported that when using bacterial ghosts to vaccinate animals, significant activation of IL-12 had been observed. IFN-γ and IL-12 are known to be of special importance in the activation of cellular Th1 immune responses. Because of the unique structure of the BGs envelope with preserved pathogen-associated molecular patterns (PAMPs), including the outer membrane proteins, adhesins, lipopolysaccharide (LPS) and peptidoglycan, BGs can be recognized by pattern recognition receptors (PRRs) of the innate immune system and consequently effectively stimulate the antigen presenting cells (APCs) to trigger immune responses (15). Dendritic cells (DCs) are potent antigen presenting cells that are essential for the initiation of primary immune responses. A previous study found that DCs were highly effective in the uptake of A. pleuropneumoniae BGs, as well as in the activation of cellular immune responses (22). This study showed that APCs were not only able to internalize BGs but also to process and present them to T cells. The process of internalization was related to increased expression of MHC–II molecules on the cell surface that can be recognized by CD4+ T cells.

In contrast to the BGs vaccine, the Th1 type cell-mediated immune responses and Th2 type humoral immune responses appeared induced in the inactivated bacterin (IB)-vaccinated group, however, both of these two immune responses in IB group were
inferior to those in the BGs group. The level of IFN-γ in the IB group were much lower than that in the BGs group on days 14 ($P<0.05$) and 28 ($P<0.01$); for IL-4, the difference was also significant on days 14 and 28 ($P<0.05$).

In accordance with the results of the T-cell phenotype and cytokines, the humoral antibody level also showed that the BGs vaccine is superior to the inactivated vaccine in promoting antibody production. Though there were no significant differences in the antibody levels between the BGs-vaccinated group and the IB-vaccinated group on days 7 and 14, the antibody level in the BGs-vaccinated group was continuously higher than that in the IB-vaccinated group after secondary exposure to the antigen (day 21 to 35). The superior protection effect of the BGs vaccine was also seen as the vaccinated animals exhibited better clinical performance and fewer pathological lesions. The effective protection imparted by the BGs vaccine indicates that this vaccine may act as a promising candidate for the prevention of Glässers disease caused by *H. parasuis*. However, as aforementioned, due to the serovar diversity and lacking of overall knowledge of the virulence factors and protective antigens for this germ, the difference in recognized antigens spectrum between the BGs vaccine and the inactivated bacterin and the cross-protective ability for BGs vaccine were not examined in this work and need to be further studied and clarified in the future.

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Figure legends

Fig. 1. Growth curve of Haemophilus parasuis strain Nagasaki and the combinant strain rHps-mE under induction condition. rHps-mE means the combinant strain of Haemophilus parasuis Nagasaki that harboring lysis plasmid pMD-mE, ps Hps means Haemophilus parasuis parent strain Nagasaki. When the culture was incubated at 28 °C to an OD_{600} of 0.4-0.5, the temperature was then raised to 42 °C to induce mE-mediated lysis. The OD_{600} value of rHps-mE declined after induction for 30 minutes, the OD_{600} value declined continuously until the lysis was completed after induction for 180 minutes. In comparison, the OD_{600} value of the parent strain Haemophilus parasuis Nagasaki
increased continuously during the observation period.

Fig. 2. The transmission electron microscopy photos of *Haemophilus parasuis* strain Nagasaki (A) and *Haemophilus parasuis* ghosts (B). Figure 2B showed that the cytoplasmic contents were expelled from *Haemophilus parasuis* cells, left the empty cells envelope. Arrow shows the trans-membrane lysis tunnel.

Fig. 3. The levels of IgG antibody (A), IFN-γ (B) and IL-4 (C) in the sera of piglets from each group. BGs group, vaccinated with *Haemophilus parasuis* ghosts; IB group, vaccinated with *Haemophilus parasuis* inactivated bacterin; PC group, placebo control group which was injected with PBS. * Difference significant compared to PC group (P<0.05). ** Difference significant compared to PC group (P<0.01). *** Difference significant compared to PC group (P<0.001). ※ Difference significant compared to IB group (P<0.05). ※※ Difference significant compared to IB group (P<0.01).

Fig. 4. FACS analysis of CD8⁺ and CD4⁺ T lymphocyte numbers (A) and the corresponding significance analysis (B and C). The peripheral blood of the piglets from each group were collected prior to and following immunization at day 0, 14, 28 and 35, the CD3⁺CD4⁺ and CD3⁺CD8⁺ T-lymphocyte populations were analyzed by Fluorescence Activated Cell Sorter (FACS). BGs group, vaccinated with *Haemophilus parasuis* ghosts; IB group, vaccinated with *Haemophilus parasuis* inactivated bacterin; PC group, placebo control group which was injected with PBS. * Difference significant compared to PC group (P<0.05). ** Difference significant compared to PC group (P<0.01). ※ Difference significant compared to IB group (P<0.05).
Fig. 5. Bacterial loads in inguinal lymph nodes (A), spleen (B), and lung (C) homogenates after *Haemophilus parasuis* strain Nagasaki challenge. The logarithm value (Lg) of CFU (colony forming units) in each milligram tissue sample was recorded. BGs group, vaccinated with *Haemophilus parasuis* ghosts; IB group, vaccinated with *Haemophilus parasuis* inactivated bacterin; PC group, placebo control group which was injected with PBS. * Difference significant compared to PC group (*P*<0.05). ** Difference significant compared to PC group (*P*<0.01). ※ Difference significant compared to IB group (*P*<0.05).
### Bacterial Strains, Plasmids, and Primers Used in This Study

<table>
<thead>
<tr>
<th>Strains</th>
<th>Characteristics</th>
<th>Source/Condition</th>
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</thead>
<tbody>
<tr>
<td><em>E. coli</em> DH5α</td>
<td>$F^{-}p80dlacZΔM15Δ(lacZYA-argF)U169 deoR recA1lendA1$</td>
<td>Novagen</td>
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<td></td>
<td>hsdR17 $mk^{	ext{+}}$ $phoA$ $supE44$ $λ^{	ext{-}}$ $thi-1$ $gyrA96$ $relA1$</td>
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<td><em>E. coli</em>β2155</td>
<td>$thrB1004$ $pro$ $thi$ $hsdS$ $lacZ$ $M15$ ($F^{-}lacZ-M15$ $lacI^{	ext{+}}$ $traD36$)</td>
<td>Gerald-E. Gerlach</td>
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<td>Bacteriophage PhiX174</td>
<td>$proA^{	ext{+}}$ $proB^{	ext{+}}$ $dap$: $erm$ ($Erm^{	ext{r}}$)</td>
<td>Novagen</td>
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<td><em>H. parasuis</em> Nagasaki</td>
<td><em>H. parasuis</em> serovar 5 reference strain</td>
<td>P. J. Blackall</td>
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### Plasmids

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<th>Plasmid</th>
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<td>pMD-18T</td>
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<td>TakaRa</td>
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<td>pMD-E</td>
<td>pMD-18T carrying gene mE</td>
<td>This study</td>
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<td>pBV220</td>
<td>expression vector carrying $λ$ bacteriophage $P_{λ/P_{λ}}$ promoter</td>
<td>Guihong Zhang</td>
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<td>pBV-220 carrying gene mE</td>
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<td>pGZRS-18</td>
<td><em>A. pleuropneumoniae-E. coli</em> shuttle vector</td>
<td>Susan E. H. West</td>
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<td>broad-host-range cloning vector</td>
<td>M.E. Kovach</td>
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<tr>
<td>pBBR-E</td>
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<td>pPBA1100</td>
<td><em>P. multocida-E. coli</em> shuttle vector</td>
<td>Ben Alder</td>
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<tr>
<td>pPBA-E</td>
<td>pPBA1100 carrying gene mE</td>
<td>This study</td>
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### Primers

| Lysis E-U        | 5’-AGGGGAATTCTGGTACGCTGGACCTTTTGTGG-3’ ($EcoR$ I site) | 95°C 5 min 30 cycles |
| Lysis E-L        | 5’-AGGGGATCCGAGCTTACTGCTCTTCCG-3’ ($BamH$ I site)    | 94°C 30 s 59°C 30s |

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Department of Microbiology and Immunology, Louisiana State University Medical Center.

Australian Research Council Center of Excellence in Structure and Functional Microbial Genomics,

Monash University.
<table>
<thead>
<tr>
<th>Group</th>
<th>No. of animals with</th>
<th>Clinical signs score</th>
<th>Temperature</th>
<th>No. of animals with</th>
<th>Lesions score</th>
<th>Survival</th>
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</thead>
<tbody>
<tr>
<td>BG-vaccinated</td>
<td>3</td>
<td>2.6**±2.3</td>
<td>39.6**±0.3</td>
<td>3</td>
<td>0.7**±0.5</td>
<td>7.0**±0.0</td>
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<tr>
<td>IB-vaccinated</td>
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<td>9.3±4.6</td>
<td>40.3±0.6</td>
<td>6</td>
<td>2.0±0.3</td>
<td>5.8±3.2</td>
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<tr>
<td>PC control</td>
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<td>23.8±3.2</td>
<td>41.3±0.8</td>
<td>6</td>
<td>3.0±0.1</td>
<td>1.1±0.6</td>
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</tbody>
</table>

* A total of 18 post-wean piglets from *Haemophilus parasuis* and other respiratory pathogens-free farm were randomly divided into three groups with 6 pigs in each group, the groups were designated as: BG-vaccinated, *Haemophilus parasuis* ghosts vaccinated; IB-vaccinated, inactivated bacterin vaccinated; PC control, placebo control.

b The assessed clinical signs include coughing, anorexia, and lameness.

c A score of 1 was given for each case of clinical sign for each individual piglet, the added daily clinical scores of days 1 to 7 after challenge for each piglet were designated the total clinical score, the arithmetic mean and standard deviation were determined in each group.

d The average temperature value for each piglet during the observation period was calculated, and the mean temperature value for each group was determined.

e The assessed histopathological lesions include pleuritis, peritonitis, arthritis, meningitis, and pericarditis.

f A score of 1 was given for each case of histopathological lesion for each individual piglet, the arithmetic mean and standard deviation were determined in each group.
Days of survival after challenge for each piglet was recorded, the arithmetic mean and standard deviation were determined in each group.

* Difference significant compared to PC control group (P<0.05).

** Difference significant compared to PC control group (P<0.01).

# Difference significant compared to IB-vaccinated group (P<0.05).