

# The Live Attenuated *Actinobacillus pleuropneumoniae* Triple-Deletion Mutant $\Delta$ *apxIC* $\Delta$ *apxIIC* $\Delta$ *apxIV-ORF1* Strain, SLW05, Immunizes Pigs against Lethal Challenge with *Haemophilus parasuis*

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*Haemophilus parasuis* and *Actinobacillus pleuropneumoniae* both belong to the family *Pasteurellaceae* and are major respiratory pathogens that cause large economic losses in the pig industry worldwide. We previously constructed an attenuated *A. pleuropneumoniae* serovar 1 live vaccine prototype, SLW05 ( $\Delta$ *apxIC*  $\Delta$ *apxIIC*  $\Delta$ *apxIV-ORF1*), which is able to produce nontoxic but immunogenic ApxIA, ApxIIA, and ApxIVA. This triple-deletion mutant strain was shown to elicit protective immunity against virulent *A. pleuropneumoniae*. In the present study, we investigated whether immunization with SLW05 could also protect against lethal challenge with virulent *H. parasuis* SH0165 (serovar 5) or MD0322 (serovar 4). The SLW05 strain was found to elicit a strong humoral antibody response in pigs and to confer significant protection against challenge with a lethal dose of *H. parasuis* SH0165 or MD0322. IgG subtype analysis revealed that SLW05 induces a bias toward a Th1-type immune response and stimulates interleukin 2 (IL-2) and gamma interferon (IFN- $\gamma$ ) production. Moreover, antisera from SLW05-vaccinated pigs efficiently inhibited both *A. pleuropneumoniae* and *H. parasuis* growth in a whole-blood assay. This is the first report that a live attenuated *A. pleuropneumoniae* vaccine with SLW05 can protect against lethal *H. parasuis* infection, which provides a novel approach for developing an attenuated *H. parasuis* vaccine.

*Actinobacillus pleuropneumoniae* is the etiologic agent of porcine pleuropneumonia and has a serious impact upon animal welfare and economics in the pig rearing industry (1). Several factors have been identified that are involved in the pathogenicity of *A. pleuropneumoniae*, of which the pore-forming exotoxins are probably the most important (2). The 15 serovars of *A. pleuropneumoniae* secrete different combinations of four exotoxins (ApxI, ApxII, ApxIII, and ApxIV) belonging to the RTX toxin family (1). ApxI and ApxIII are encoded on classical RTX operons in a *CABD* manner, and the ApxII operon in all *A. pleuropneumoniae* serovars is truncated, having only *CA* genes and missing the secretion genes *BD* (1). The ApxIV gene is not a classical RTX toxin gene and is expressed only in infected pigs (3). There is a correlation between virulence and the pattern of Apx toxin production, and *A. pleuropneumoniae* serovars 1, 5, 9, and 11, which produce ApxI and ApxII, are regarded as the most virulent (4).

Live attenuated vaccines hold much promise because protective antigens are produced in a natural context and because live vaccines have a greater ability to stimulate the production of cytokines, including interleukins, tumor necrosis factor, and interferons, which are known to play an important role as immune modulators (5). Previous studies have confirmed the feasibility of engineering mutant strains of *A. pleuropneumoniae* via the use of selectable antibiotic resistance determinants (6), but such strains are unsuitable as vaccines owing to biosafety concerns (7). We have therefore developed methods for the construction of *A. pleuropneumoniae* vaccine strains that avoid the use of antibiotic resistance genes. We previously developed a double-deletion  $\Delta$ *apxIC*  $\Delta$ *apxIIC* mutant strain, SLW03, of *A. pleuropneumoniae* serovar 1. Upon homologous or heterologous challenge, there was no overt clinical disease or mortality in pigs vaccinated with

SLW03. These results, combined with the fact that the strain contains no foreign DNA, emphasize the potential of SLW03 as a live attenuated vaccine (8).

However, previous studies showed that ApxIVA retains weak hemolytic activity in the presence of ORF1, a protein encoded immediately upstream of *apxIVA* (3), although it remains to be determined whether ORF1 acts in the same way as the ApxC post-translational activators of other Apx toxins. We therefore constructed a live mutant by introducing an *apxIVA-ORF1* deletion into the double-mutant  $\Delta$ *apxIC*  $\Delta$ *apxIIC* *A. pleuropneumoniae* strain SLW03 (9). This triple-deletion  $\Delta$ *apxIC*  $\Delta$ *apxIIC*  $\Delta$ *apxIV-ORF1* mutant strain, named SLW05, was found to have reduced virulence in both mice and pigs compared to that of SLW03 and could elicit protection against *A. pleuropneumoniae* homologous and heterologous serovar lethal challenge (9).

*Haemophilus parasuis* is the causative agent of Glässer's disease, and it has become one of the most important bacterial pathogens of livestock worldwide (10). So far, 15 *H. parasuis* serovars have been described, but up to 25% of isolates in some countries could not be allocated to any known serovar (11, 12). Although vaccination is commonly considered to be the most effective way to control and eradicate infectious disease (13), currently, there is

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not one vaccine known to induce protection against all pathogenic *H. parasuis* serovars (14).

It was therefore unexpected that preliminary findings from pig farms employing immunization with *A. pleuropneumoniae* strain SLW05 suggested that the prevalence of Glässer's disease was markedly diminished in vaccinated herds. Both *H. parasuis* and *A. pleuropneumoniae* belong to the family *Pasteurellaceae*, and this raised the possibility that the *A. pleuropneumoniae* vaccine strain SLW05 might elicit a degree of cross-protection against *H. parasuis*. Therefore, the goal of the present study was to evaluate the immune response to and the efficacy of SLW05 as an attenuated vaccine to protect pigs against lethal challenge with *H. parasuis* serovars 4 and 5.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** *A. pleuropneumoniae* (serovar 1, strain 4074), the live attenuated triple mutant ( $\Delta$ apxIC  $\Delta$ apxIIC  $\Delta$ apxIV-ORF1) strain of *A. pleuropneumoniae*, SLW05, and *H. parasuis* (serovar 5, strain SH0165; serovar 4, strain MD0322) (15) were grown in tryptic soy broth (TSB) (Difco) or tryptic soy agar (TSA) (Difco) supplemented with 10  $\mu$ g/ml NAD (Sigma, St. Louis, MO) and 10% newborn calf serum (Gibco). All bacterial strains were grown at 37°C.

**Live-vaccine preparation.** SLW05 was inoculated into 100 ml TSB supplemented with 10% newborn calf serum and NAD (10  $\mu$ g/ml) and propagated overnight at 37°C. The bacterial suspension was then diluted 10-fold into fresh prewarmed TSB and was cultured for 16 h at 37°C to obtain the log-phase bacteria; then, the log-phase bacteria were washed once in phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 2.8 mM KH<sub>2</sub>PO<sub>4</sub> [pH 7.2]) and mixed with gelatin to yield a concentration of 1.5%, and then the mixture was lyophilized.

For use in pig immunizations, the mixture was diluted in PBS and adjusted with PBS to approximately  $5 \times 10^7$  CFU/ml.

**Protection studies with pigs.** This study was carried out in strict accordance with the recommendations in the *China Regulations for the Administration of Affairs Concerning Experimental Animals* 1988 and the *Hubei Regulations for the Administration of Affairs Concerning Experimental Animals* 2005. The protocol was approved by China Hubei Province Science and Technology Department [permit number SYXK(ER) 2010-0029]. The animals were euthanized at the end of the experiments or when they reached a particular clinical score during the experiment.

Forty 30-day-old naturally farrowed early-weaned (NFEW) piglets which were negative for detection of antibody against *H. parasuis* by INGEZIM *Haemophilus* 11.HPS.K1 (INGEZIM, Spain), also negative for the isolation of *H. parasuis* from a nasal cavity swab, and also negative for detection of antibody against *A. pleuropneumoniae* by the *A. pleuropneumoniae* ApxI detection kit (Keqian, China) were used. Optical densities (OD) of antibody against *A. pleuropneumoniae* and *H. parasuis* were determined (data not shown). The piglets were randomly divided into four experimental groups each of 10 animals. Groups 1 and 3 were vaccinated twice intramuscularly (i.m.) with  $1.0 \times 10^8$  CFU of SLW05 in 2 ml PBS, whereas groups 2 and 4 (negative-control groups) were injected intramuscularly with 2 ml PBS. A second identical immunization was performed 14 days after the primary vaccination.

On day 14 following the booster immunization, all pigs in groups 1 and 2 were challenged intranasally with  $1.5 \times 10^{10}$  CFU of SH0165 strain; groups 3 and 4 were challenged intranasally with  $1.5 \times 10^{10}$  CFU of MD0322 strain. Pigs were monitored regularly for 14 days following challenge, and clinical signs were scored according to the *A. pleuropneumoniae* scoring method (16) and *H. parasuis* scoring method (17). At 14 days postchallenge, all surviving pigs were euthanized and lung lesion scores were recorded as previously described (18).

**Antibody measurements.** Sera were isolated from blood samples collected from pigs by anterior vena cava venipuncture on day 14 after the second immunization prior to challenge. Serum antibodies against ApxI

of *A. pleuropneumoniae* or the mixture of 121°C high-pressure *H. parasuis* serovar 4 and 5 heat-stable antigens for 2 h were determined by enzyme-linked immunosorbent assay (ELISA) as described previously (19, 20), with some modifications. Briefly, 100  $\mu$ l coating buffer (50 mM sodium carbonate buffer, pH 9.6) containing appropriate ApxI or the mixture of 121°C high-pressure *H. parasuis* serovar 4 and 5 heat-stable antigens was used at 4°C overnight to coat 96-well ELISA plates. The plates were then washed three times with PBST (PBS supplemented with 0.05% Tween 20) and blocked at 37°C for 1 h with blocking buffer (PBS with 5% bovine serum albumin [BSA]); they were then washed three times with PBST. Serum samples diluted in PBST were added to each well and incubated at 37°C for 1 h. After three washes, horseradish peroxidase (HRP)-conjugated goat anti-porcine IgG diluted 1:5,000 in PBST was added to each well. For determining the IgG isotypes, the sera were added to the coated plates and incubated with mouse anti-pig IgG1-HRP (AbD Serotec, United Kingdom) or IgG2a-HRP (AbD Serotec). After a washing, substrate solution 3,3',5,5'-tetramethylbenzidine (TMB) and H<sub>2</sub>O<sub>2</sub> were added to each well, the plates were incubated at room temperature for ~20 min, and 1% SDS was added to stop the reaction. The plates were read at an absorbance of 630 nm, and serum antibody IgG titer was calculated as the reciprocal of the serum dilution which gave an OD at 630 nm of 0.3 above that of the preimmune sera.

**Determination of cytokines by ELISA.** Sera isolated from blood samples as described above were analyzed for interleukin 2 (IL-2), IL-4, and gamma interferon (IFN- $\gamma$ ) using ELISA kits (R&D systems) according to the manufacturer's instructions. Briefly, sera were added to the coated plates, incubated for 3 h, washed with PBST, and blocked with blocking buffer (PBS with 5% BSA). Cytokine concentrations were determined using an ELISA reader.

**Blood bactericidal assay.** The bactericidal assay was performed as described previously (21), with some modifications. Briefly, live bacteria of *H. parasuis* strain SH0165 or MD0322, or *A. pleuropneumoniae* strain 4074, were washed and diluted in sterile physiological saline. Samples of diluted bacteria (20  $\mu$ l;  $10^6$  cells) were mixed with 180  $\mu$ l inactivated test sera (the test sera were inactivated in a 56°C water bath for half an hour) diluted 2-fold with physiological saline, or diluted bacteria were mixed with saline alone. After incubation at 30 min at 37°C and then 30 min on ice, nonimmune whole heparinized (10 U/ml) pig blood (100  $\mu$ l) was added, and the mixture was incubated at 37°C with shaking for 1 h. Bacteria were then plated on TSA containing 10% newborn calf serum and 1 mM/ml NAD (Sigma). Bacterial colony counts (CFU) were recorded after 24 h. Results were expressed as percent killing according to the following formula: (CFU after 1 h of growth with control sera – CFU after 1 h of growth with immune sera)/CFU after 1 h of growth with control sera  $\times$  100. Data presented are the means of three independent assays.

**Histopathological analysis.** Lung and brain tissues were fixed by immersion in 10% neutral buffered formalin and embedded in paraffin; 4- $\mu$ m tissue sections were then cut and stained with hematoxylin and eosin according to a standard protocol and examined under light microscopy.

**Statistical analysis.** The experimental data were expressed as means  $\pm$  standard deviations (SD). The difference between two groups was analyzed using the two-tailed Student *t* test, and survival analysis was done with the log rank test. A *P* value of <0.05 was considered to indicate a statistically significant result.

## RESULTS

**Immune response.** Two weeks after booster immunization with the triple-mutant strain SLW05, blood samples from all pigs were collected via anterior vena cava venipuncture. The results showed that all pigs immunized with SLW05 had significant antibody titers, whereas no antibody was detected in the negative-control groups (*H. parasuis* serovar 4 and 5 heat-stable antigens, *P* < 0.05; ApxI, *P* < 0.001) (Fig. 1A). There were no significant differences in the antibody titers between the different animals immunized

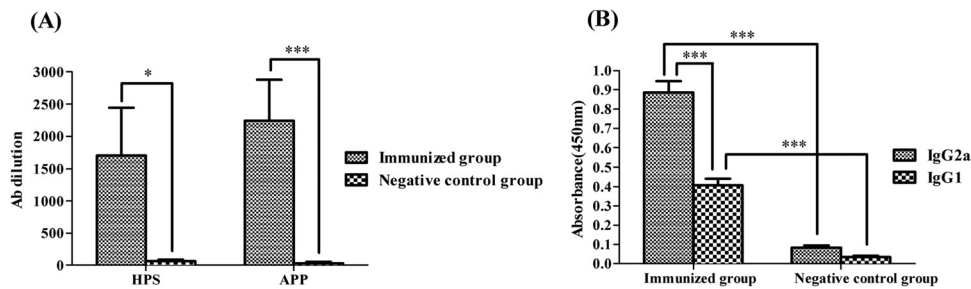


FIG 1 (A) Antibody titers in pigs immunized with SLW05 against the mixture of *H. parasuis* serovar 4 and 5 heat-stable antigens (HPS) or Apx1 (APP). Groups of pigs were immunized with SLW05 and boosted after 14 days. Negative controls received PBS. Blood samples were collected 2 weeks after the booster injection, and the antibody response was determined by ELISA; optical density readings of  $\geq 0.3$  were scored as positive. \*, significance at a  $P$  value of  $<0.05$ ; \*\*\*, significance at a  $P$  value of  $<0.001$ . (B) IgG1 and IgG2a levels in pigs immunized with SLW05 of the antibodies against the *H. parasuis* serovar 4 and 5 heat-stable antigens (ELISA). Results are expressed as means  $\pm$  SD; \*\*\*, significance at a  $P$  value of  $<0.001$ .

with SLW05 ( $P > 0.05$ ). We then performed isotyping to determine the specific antibody types against the mixture of *H. parasuis* serovar 4 and 5 heat-stable antigens of *H. parasuis* induced by vaccination. Levels of isotypes IgG1 and IgG2a were significantly higher in the immunized group than in the negative-control group ( $P < 0.001$ ) (Fig. 1B). IgG2a titers predominated over IgG1 titers, suggesting a bias toward a Th1-type immune response ( $P < 0.001$ ) (Fig. 1B).

We also determined levels of cytokines induced by immunization. Blood serum samples from day 14 after the last immunization were analyzed by ELISA. Levels of IFN- $\gamma$  were significantly higher in sera from immunized animals than in sera from negative controls ( $P < 0.001$ ) (Fig. 2). Levels of IL-2 production were also significantly higher in immunized animals ( $P < 0.001$ ) (Fig. 2). However, there were no significant increases in IL-4 levels in vaccinated animals versus negative controls (data not shown).

**Protective efficacy against lethal challenge with *H. parasuis* serovars 4 and 5.** The protective efficacy of SLW05 against lethal challenge with *H. parasuis* serovar 4 or serovar 5 was evaluated in terms of survival and clinical signs in pigs, including appetite, dyspnea, lethargy, lameness, and neurological signs. Separate groups of pigs were challenged intranasally with a lethal dose of  $1.5 \times 10^{10}$  CFU of *H. parasuis* strain SH0165 or strain MD0322 2 weeks after the booster immunization; animals were monitored after challenge, and survival, clinical scores, and lung lesion scores were recorded (Table 1). All pigs in the nonimmunized control groups developed clinical disease, with an elevated average rectal temperature ( $41.5^\circ\text{C}$ ), and were inappetent and depressed (Table 1). However, pigs in the immunized groups displayed little or no

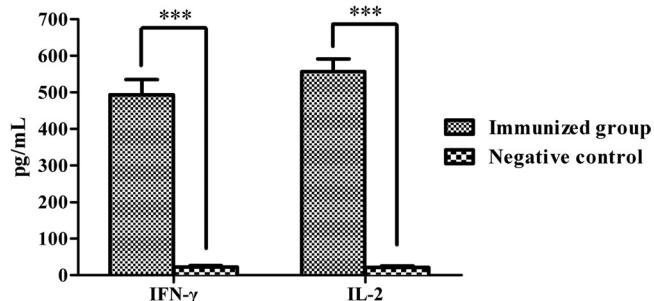


FIG 2 Serum levels of IFN- $\gamma$  and IL-2 production in serum of pigs immunized with SLW05. Results are means  $\pm$  SD; \*\*\*, significance at a  $P$  value of  $<0.001$ .

depression, anorexia, dyspnea, lameness, or neurological signs, and lung lesions assessed by tissue analysis following necropsy 14 days postchallenge were significantly diminished ( $P < 0.05$ ) (Table 1). In addition, there was no pathological change at the injection site. Overall survival rates were markedly and significantly ( $P < 0.05$ ) increased in animals immunized with SLW05 compared to those in control groups (Fig. 3). The majority of immunized animals survived lethal challenge with either SH0165 (90% survival) or MD0322 (80% survival), although the difference in the survival rates between the two challenge groups was not statistically significant ( $P > 0.05$ ) (Fig. 3). These results demonstrated that SLW05 could provide significant immunoprotection against *H. parasuis* infection.

**Blood bactericidal assay.** We then evaluated whether immunization with SLW05 induces the production of antibody that is bactericidal and/or opsonic against live *H. parasuis* and *A. pleuropneumoniae* *in vitro*. The results showed that sera from nonimmunized animals failed to inhibit the growth of either *H. parasuis* or *A. pleuropneumoniae*. In contrast, sera from immunized animals caused a marked reduction in the viable titers of both *H. parasuis* SH0165 and MD0322, as well as *A. pleuropneumoniae* 4074 ( $P < 0.001$ ) (Fig. 4). There was a 55% reduction the viability of SH0165 following incubation with immune sera and a 51% reduction in MD0322 viability, demonstrating that immunization with SLW05 elicits the production of bactericidal antibodies against *H. parasuis*. In addition, there was a 61% reduction the viability of *A. pleuropneumoniae* 4074 following incubation with immune sera.

**Histopathological analysis.** To evaluate the protective efficacy of SLW05 against the development of disease induced by challenge with *H. parasuis* strains SH0165 and MD0322, brain and lung samples of the surviving animals from the vaccine-immunized groups 14 days postchallenge and the animals from the negative-control groups postchallenge were euthanized when the clinical disease became severe and then were examined for tissue pathology. In the negative-control groups challenged with either SH0165 or MD0322, all animals developed severe disease (Fig. 5A, C, E, and G). As shown in Fig. 5A and C, lung tissue showed extensive edema, with massive proliferation of fibroblasts and the formation of connective tissue. Bronchioles were filled with cellular exudate containing large numbers of neutrophils. Portions of the parenchyma were completely necrotic or collapsed. Congestion of brain blood vessels was prominently observed in histological specimens from negative-control groups (Fig. 5E and G). In



**TABLE 1** Protective effect of the live mutant against *H. parasuis* SH0165 and MD0322 challenge in pigs

<i>H. parasuis</i> strain used for challenge	Group	Appetite <sup>a</sup>	Dyspnea <sup>a</sup>	Lethargy <sup>a</sup>	Lameness <sup>a</sup>	Neurological signs <sup>a</sup>	Lung lesion score <sup>b</sup>
SH0165	Immunized group	0.2 ± 0.4	0.3 ± 0.5	0.2 ± 0.4	0.1 ± 0.3	0.1 ± 0.3	1.9 ± 2.4
	PBS (negative control) <sup>c</sup>	2.6 ± 0.5	2.7 ± 0.5	2.8 ± 0.4	1.0 ± 1.0	0.6 ± 1.0	18.7 ± 4.8
MD0322	Immunized group	0.3 ± 0.5	0.4 ± 0.5	0.2 ± 0.4	0.1 ± 0.3	0	2.6 ± 3.1
	PBS (negative control) <sup>c</sup>	2.7 ± 0.5	2.6 ± 0.5	2.6 ± 0.5	0.9 ± 1.0	0.5 ± 1.0	21.1 ± 6.1

<sup>a</sup> Clinical signs were scored as described by Jolie et al. (16) and Blanco et al. (17). Appetite was scored as follows: 0, did eat, and 1, did not eat. Total score = number of 12-h periods of not eating over the 36-h observation period. Dyspnea was scored as follows: 0, normal; 1, slight; 2, moderate; and 3, severe. Lethargy was scored as follows: 0, normal; 1, slight inactivity; 2, moderate; and 3, severe. Lameness was scored as follows: 0, normal; 1, lameness in 1 limb; 2, lameness in 2 limbs; and 3, severe lameness. Neurological signs were scored as follows: 0, normal; 1, muscular rigidity or tremors; 2, convulsions; and 3, paralysis.

<sup>b</sup> The lung lesion score was determined as described by Hannan et al. (18).

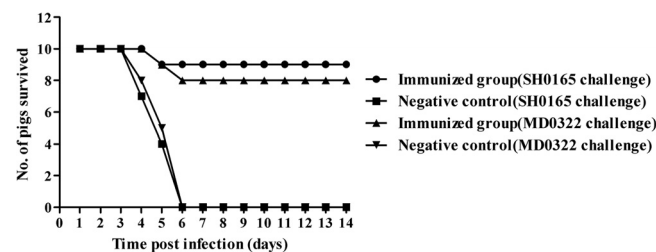
<sup>c</sup> The clinical signs of negative control groups were recorded for the animals prior to death. The final scores were obtained by the average of all the data within the observation time and are expressed as arithmetic means ± SD.

contrast, only minor signs of clinical disease were observed in tissues of pigs immunized with SLW05 vaccine (Fig. 5B, D, F, and H). These data demonstrate that immunization with SLW05 can prevent pathological changes following challenge infection with *H. parasuis* and confirm that the triple-deletion mutant strain of *A. pleuropneumoniae* can induce robust immunoprotection against *H. parasuis* infection in pigs.

## DISCUSSION

*Haemophilus parasuis* and *Actinobacillus pleuropneumoniae* are two of the most serious porcine respiratory diseases and cause large economic losses in the pig industry worldwide (22, 23). A live vaccine is considered to offer a better prospect of obtaining cross-serovar protection against *A. pleuropneumoniae* serovars or *H. parasuis*, and live attenuated deletion mutants have been shown to be effective in controlling infection by *A. pleuropneumoniae* (24, 25). Based on the observation of cross-protection of SLW03 against heterologous *A. pleuropneumoniae* infection in recovery pigs, we constructed SLW05. It was shown to induce broad-range protective immunity against *A. pleuropneumoniae* infection in vaccinated pigs (9).

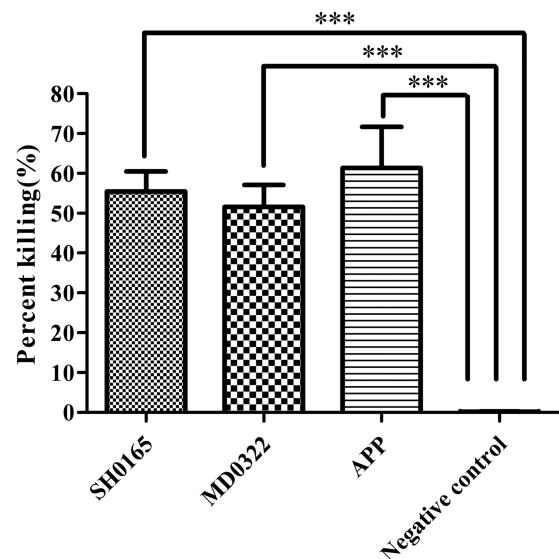
At present, commercial vaccines against *H. parasuis* are predominantly inactivated vaccines based on well-characterized strains, and there are commercially produced autogenous bacterins that are used some places. These vaccines usually induce strong immunoprotection against homologous serovar challenge (14, 26, 27), but some inconsistent results have been obtained for cross-protection, depending on serovars and strains of *H. parasuis* (26, 28). Although recombinant vaccines based on *H. parasuis* outer membrane proteins have been constructed, they only provide limited protective efficacy and fail to confer cross-protection



**FIG 3** Survival of pigs immunized with *A. pleuropneumoniae* SLW05 and negative-control pigs following intranasal challenge with  $1.5 \times 10^{10}$  CFU of *H. parasuis* strain SH0165 or MD0322.

(29). Attention has therefore focused on inoculation of animals with live attenuated vaccines because these present a wide spectrum of immunogenic antigens in a native form (25), so a live attenuated vaccine is considered to offer a better prospect of obtaining cross-serovar protection against *H. parasuis*. However, the development of attenuated vaccines for commercial use has been hindered by the lack of methods for modifying the *H. parasuis* genome without the concomitant insertion of an antibiotic resistance marker.

*H. parasuis* and *A. pleuropneumoniae* both belong to the family *Pasteurellaceae*, and it is therefore possible that they share antigenic sites on the cell wall and might afford a measure of cross-protection. Our results showed that immunization with SLW05 elicits the production of bactericidal antibodies against *H. parasuis*, and this is the first report that the antibodies against *A. pleuropneumoniae* have the characteristic of bactericidal activity. At present, some common antigens are being identified between



**FIG 4** Blood bactericidal activity of SLW05-vaccinated and negative-control animals. Results are expressed as the percent reduction of viable bacteria in the presence of immune blood serum. Results are determined from three independent assays. A >50% reduction in the viable titer in at least one test was considered to be a positive result. \*\*\*, significance at a *P* value of <0.001. APP, *A. pleuropneumoniae*.

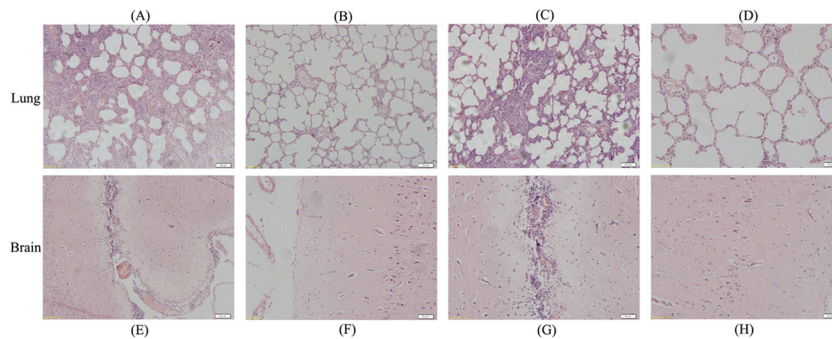


FIG 5 Representative lung and brain sections of pigs from different treatment groups. (A and E) Negative-control group challenged with strain *H. parasuis* SH0165; (B and F) *A. pleuropneumoniae* SLW05-immunized group challenged with *H. parasuis* strain SH0165; (C and G) negative-control group challenged with *H. parasuis* strain MD0322; (D and H) *A. pleuropneumoniae* SLW05-immunized group challenged with *H. parasuis* strain MD0322.

*A. pleuropneumoniae* and *H. parasuis* serovars 4 and 5 by a proteomics approach in our laboratory.

The route of immunization may also have an effect on raising the level of cross-immunoprotection. *A. pleuropneumoniae* is a respiratory pathogen, and the endotracheal or intranasal administration route is thought to be a better way to simulate the natural routes of infection (30). However, compared with other immunization routes such as endotracheal administration, the i.m. administration route is more convenient and used widely in practice. Furthermore, our laboratory and other researchers also proved that i.m. immunization could also elicit protection equal to that of the intranasal or tracheal route against challenge with homologous or heterologous serovars of a virulent *A. pleuropneumoniae* strain (31, 32), so in this study we used the i.m. administration route.

The predominant immunological effector response against extracellular bacteria is generally thought to operate via circulating antibodies that lead to inactivation of the pathogen either via complement activation or by opsonization and phagocytosis (33). Examination of sera from pigs inoculated with the *A. pleuropneumoniae* vaccine confirmed that SLW05 could induce a significant humoral immune response. It has been reported that the specific IgG subtype and the Th type are crucial determinants of protective immunity against a particular disease (34). Enhanced production of IgG1 indicates the induction of a Th2 immune response, whereas IgG2a is representative of a Th1 response. Although antibody subtype analysis of SLW05-immunized animals indicates that the vaccine induces the production of both IgG1 and IgG2a, the higher levels of IgG2a suggest a bias toward a Th1-type immune response; this was further confirmed by cytokine assay. But one study showed that anti-HLY (Apx) antibodies of *A. pleuropneumoniae* were consistently associated with protection and that IgG1 may be more efficacious than IgG2 at low-dose challenge (30); specific mechanisms need further study. Th1 cells mainly secrete IL-2 and IFN- $\gamma$ , and Th2 cells mainly secrete IL-4. In this study, SLW05 induced higher levels of IL-2 and IFN- $\gamma$  production, which may be related to the stimulation of the bacteria. Sera from immunized animals demonstrated pronounced bactericidal activity against both *H. parasuis* and *A. pleuropneumoniae*; we surmise that this is mediated by antibody binding followed by complement activation, but further experiments will be required to determine the mechanism of cell killing *in vitro*.

The majority of studies addressing immunoprotection against

*H. parasuis* have employed intraperitoneal challenge of mice; however, this does not mimic the natural route of infection (35). The present study therefore used intranasal challenge with virulent strains of *H. parasuis*. Ninety percent of immunized pigs survived lethal challenge with SH0165, whereas 80% of immunized pigs survived challenge with MD0322. This is the first report that the SLW05 strain can confer a high level of immunoprotection against heterogenous attack by *H. parasuis* of either serovar 5 or 4, so our next goal is the development of SLW05 as a commercial vaccine by exploring the more accurate immunization doses and routes.

In summary, the present study demonstrates that immunization of pigs with SLW05 induces a significant increase of antibody titers against *H. parasuis* compared with negative-control groups. The response was predominantly of the Th1 type, and sera from immunized animals showed *in vitro* bactericidal activity against live *H. parasuis*. Importantly, immunization with the triple-mutant SLW05 conferred marked resistance against lethal challenge with virulent heterologous *H. parasuis*. We conclude that the triple-deletion mutant SLW05 has significant potential in the development of an attenuated vaccine to prevent and control *H. parasuis* infection. In addition to being a potential vaccine against both *A. pleuropneumoniae* and *H. parasuis*, the mutant strain also holds promise as a delivery vehicle for protective antigens of other pathogens and has great potential in the development of new multivalent vaccines. The work is ongoing in our laboratory to standardize production of the SLW05 vaccine, and field trials are under way.

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