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RESEARCH ARTICLE

Development and Assessment of a Septuple-Deletion Mutant Live Attenuated Vaccine for *Actinobacillus pleuropneumoniae* in Swine

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ABSTRACT

Actinobacillus pleuropneumoniae (APP) is a respiratory pathogen which causes severe pleuropneumonia in swine, leading to substantial economic losses in the pig industry. Current vaccines have shown limited protection against APP. The present study aimed to develop and evaluate a septuple-deletion mutant strain of APP (SLW08; serovar 1) as a live attenuated vaccine candidate. The SLW08 was developed from the sextuple-deletion mutant APP WBY06 (ΔapxICΔapxIICΔorf1ΔcpxARΔarcAΔureA) by knocking out *ApxIVA* and inserting *ApxIIIA* into the *ApxIVA* locus. The virulence of mutant strain was assessed in mice. The immunogenicity, safety, and protective efficacy of SLW08 were evaluated in mice and piglets. Protection studies involved challenging immunized and control animals with a virulent APP strain (SLW01) and vaccine efficacy was determined using survival rates and lung pathology. The SLW08 strain exhibited slower growth rates as compared to the WBY06 and WT (SLW01) strains but showed significantly reduced virulence. Vaccinated pigs demonstrated strong immune responses without adverse effects. Upon challenge with virulent APP, the vaccinated pigs showed markedly reduced signs of infection compared to controls, indicating strong protective efficacy. SLW08, expressing *ApxIII*, provided effective protection to mice and piglets against APP, with less toxicity, resulting in higher survival rates and less lung pathology. The SLW08 mutant of APP with strong immune responses may represent a promising live attenuated vaccine candidate for protecting swine against APP infection.

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INTRODUCTION

Porcine contagious pleuropneumonia (PCP), caused by *Actinobacillus (A.) pleuropneumoniae* (APP), is an infectious respiratory disease that usually affects young pigs (Bao *et al*., 2020). There are two forms of PCP, including acute hemorrhagic fibrinous pleuropneumonia and chronic fibrinous necrotizing pleuropneumonia. APP has been classified into 19 serotypes with varying degrees of virulence (Bosse *et al*., 2002). The pathogen possesses several virulence factors which contribute to its pathogenicity, including Apx toxins, lipopolysaccharides, type IV pilus, outer membrane proteins, and adhesion molecules (Chiang *et al*., 2009; Bosse *et al*., 2015).

Although, the antibiotics are effective in controlling mortality and clinical disease during PCP outbreaks. However, increasing antibiotic resistance has been posing significant challenges in controlling this pathogen, making vaccination a more promising alternative for preventing APP infections (Gimenez-Lirola *et al*., 2014; Chuekwon and Cheng, 2023). Therefore, there is a dire need to design an effective vaccine against APP. Several subunit and inactivated vaccines for APP have been commercialized, however, these available APP vaccines provide limited protection (Hur *et al*., 2015). To find effective protection, novel vaccination strategies are being developed, including live attenuated vaccines (Soto Perezchica *et al*., 2023; He *et al*., 2024), DNA vaccines (Li *et al*., 2018; Chuekwon and Cheng, 2023), and subunit-based vaccines (Derevyanko and Ayshpur, 2023). Many researchers have focused on the development of live attenuated vaccines by constructing mutant strains of APP through knock down of virulence

genes. For example, Xie *et al.* (2016) demonstrated that the S-8ΔclpPΔapxIIC mutant strain could serve as a highly immunogenic and promising candidate for a live attenuated vaccine against APP. Additionally, a live attenuated strain of APP with a deleted znuA virulence factor had shown to induce effective cross-protection against different serovars, when administered via intratracheal immunization (Yuan *et al.*, 2014). Thus, live attenuated vaccines may act as promising alternatives for controlling APP infection.

Previously, we established a live vaccine candidate strain (SLW03) attenuated from the field-isolated APP strain (SLW01) that expressed non-toxic immunogenic *ApxI* and *ApxII* (Liu *et al*., 2015). Furthermore, we inactivated the possible *ApxIV* activating element ORF1, two-component system (TCS) coding sequences, *cpxAcpxR*, and *arcA*, and assessed the protective efficacy of these vaccine strains (Liu *et al*., 2009; Liu *et al*., 2015). To further reduce its virulence, we inactivated the *uraA* gene, resulting in the mutant strain designated as WBY06.

In this study, the coding sequence of *ApxIVA* gene was knocked out in an APP sextuple-deletion strain (WBY06: ΔapxICΔapxIICΔcpxARΔorf1ΔarcAΔureA) and the *ApxIIIA* gene sequence was inserted into the corresponding site, resulting in the development of a mutant strain (SLW08). Further, the biological properties of SLW08 and its protective efficacy against APP were assessed in mice and pigs.

MATERIALS AND METHODS

Experimental animals: In this experiment, 186 six-week-old female BALB/c mice were purchased from the Experimental Animal Center of Huazhong Agricultural University. Additionally, a total of 30 six-week-old piglets were purchased from Keqian Bio. Co., Ltd. All of experimental animals were tested negative for APP by PCR and ELISA (specifically, PCR-negative for nasal and tonsillar swabs and serologically negative in corresponding ELISA assays) and were reared under specific pathogen free environment for 3 weeks prior to vaccine trial (Kucerova *et al*., 2011).

The animal experimentation procedures used in this study were approved by the Animal Experiment Committee of Huazhong Agricultural University. The ethics approval numbers are HZAUMO-2024-0073 for mice and HZAUSW-2024-0031 for piglets.

Plasmids, primers, bacterial strains, and growth conditions: The primers, plasmids, and bacterial strains used in this investigation are listed in Tables 1 and 2. Briefly, APP strains were grown in tryptic soy broth (TSB) or tryptic soy agar (TSA), supplemented with 5% bovine serum albumin (BSA) and 10μg/mL nicotinamide adenine dinucleotide (NAD). *E. coli* DH5α, used for cloning, was cultured in Luria-Bertani (LB) media, with ampicillin added at 100μg/mL as needed. *E. coli* β2155, used for transconjugation, was cultured in LB, supplemented with 5μg/mL chloramphenicol and 50μg/mL diaminopimelic acid (DAP).

Construction of APP deletion mutant: A mutant strain with *ApxIVA* deletion was developed based on WBY06 (ΔapxICΔapxIICΔcpxARΔorf1ΔarcAΔureA) using two sequential homologous recombinant crossovers, aiming to obtain a live attenuated vaccine candidate for APP. The *ApxIIIA* gene from JL03 was then inserted into the missing *ApxIVA* gene locus of WBY06.

APP mutants were obtained as previously described by Lopez-Bermudez *et al*. (2014). The primers ApxIVA-F1/ApxIVA-R1 were used to amplify upstream fragments of *ApxIVA* from SLW01, while the primers ApxIVA-F3/ApxIVA-R3 were used to amplify downstream fragments of *ApxIVA* of serovar 7 (Bei *et al*., 2005). The primers ApxIIIA-F2/ApxIIIA-R2 were employed to amplify the *ApxIIIA* fragment of APP strain JL03 (GenBank No.: CP000687). The first fusion fragment linking *ApxIIIA* to the downstream fragment of *ApxIVA* was generated using overlap extension PCR with the primers ApxIIIA-F2/ApxIVA-R3. The second fusion fragment was then obtained by amplifying upstream fragments of *ApxIVA* from SLW01 and the first fusion fragment with the primers ApxIVA-F1/ApxIVA-R3. The PCR products were cloned into the pEMOC2 vector to construct the plasmid pEMΔApxIVA. This plasmid was then introduced into the strain WBY06 using *E. coli* β2155. The septuple-deletion mutant strain was selected using TSA containing chloramphenicol, BSA, and NAD. The APP septuple-deletion mutant SLW08 (ΔapxICΔapxIIC ΔcpxARΔorf1ΔarcAΔureAΔapxIVA) was verified by PCR.

Growth characteristics and genetic stability: Single colonies of the mutant strains SLW01, WBY06, and SLW08 were selected, inoculated into TSB, and incubated at 37℃ with shaking at 200rpm for 12h. They were then transferred to a new pre-warmed TSB medium at a dilution ratio of 1:1000. Samples were taken every hour to measure the OD_{600nm} value. The bacterial samples were serially diluted ten-fold, and three appropriate dilutions were selected. A total of 100μL of each dilution was spread onto TSA plates and incubated at 37℃ overnight in a shaking incubator. Each dilution was repeated three times, and the number of colonies was recorded. The average values were taken to plot the growth curve.

The obtained septuple-deletion mutant strain SLW08 was passaged on TSA for 14 consecutive generations, and the samples were taken after every two generations. Finally, the mutant strains were tested by PCR using appropriate primers as mentioned in Table 2.

Vaccine safety evaluation: To assess the pathogenicity of the APP strains, 96 mice were randomly selected and divided into 12 groups. Groups I, II, III, and IV were infected with the wild-type (WT) strain at doses of 1.0×10^7, 2.5×10^6, 6.0×10^5, and 1.5×10^5CFU, respectively. Groups V, VI, VII, and VIII were infected with WBY06 at doses of 5.0×10^9, 1.0×10^9, 2.0×10^8 and 4.0×10^7CFU, respectively. Groups IX, X, XI, and XII were infected with SLW08 at doses of 5.0×10^{10}, 1.0×10^{10}, 2.0×10^9 and 4.0×10^8CFU, respectively. All mice were observed for seven days to calculate the survival rates.

Protective efficacy of the mutants: The protective efficacy of mutants was evaluated by following the method described by Maas *et al.* (2006). Briefly, the remaining 90 mice were randomly divided into 9 groups, each containing

Table 1: Plasmids and bacterial strains used in the study.

Strains and plasmids	Related characteristics	Sources
APP SLW01	wild-type (WT), serovar 1 strain isolated from the diseased pig's lung	(Liu *et al*., 2015)
APP JL03	serovar 3	(Loera-Muro and Angulo, 2018)
APP WBY06	sextuple-deleted mutant (Δ*apxIC*Δ*apxIIC*Δ*orf1*Δ*cpxAR*Δ*arcA*Δ*ureA*) based on SLW01	Laboratory
APP SLW08	*sexttuple-deleted mutant (ΔapxICΔapxIICΔcpxARΔorf1ΔarcAΔureAΔapxIVA) based on SLW01*	This work
E. coli DH5a	Cloning vehicle: *supE44 ΔlacU169 (ϕ80 lacZ*ΔM15) *hsd*R17 recA1 endA1 *gyrA96 thi-1 relA1*	Takara, Dalian, China
E. coli β2155	Transconjugation donor: *thrB1004 pro thi strA hsdS lacZ ΔM15 (F'lacZ ΔM15lacI^q traD36 proA$^+$proB$^+$) dap:: erm recA:: RP4-2-tet:: Mu-km λpir*,Erm^r Tet^r Kan^r	Provided by Prof. Gerald-F. Gerlach
pEMOC2	Transconjugation vector: ColE1 ori mob RP4 *sacB*, Amp^r Cm^r	Provided by Prof. Gerald-F. Gerlach

Table 2: List of primers used in this study

Primers	Sequences (5′–3′)	Uses
ApxIVA-F1	TTTGTCGACATCGATATGCCGCCGGGT	*ApxIVA* upstream
ApxIVA-R1	CATGCTTGACCAAGTACTCATTTTGTTTCCTTTCAAATTAATAAAAATAAT	homology arms
ApxIIIA-F2	ATTATTTTTATTAATTTGAAAGGAAACAAAATGAGTACTTGGTCAAGCATG	*ApxIIIA*
ApxIIIA-R2	CATATTATTCACTTTATCAGTAATATACATTTGTTGCTCCTAATTAAGCTGCTCTAGCTAGGTTAC	
ApxIVA-F3	GTAACCTAGCTAGAGCAGCTTAATTAGGAGCAACAAATGTATATTACTGATAAAGTGAATAATATG	*ApxIVA*
ApxIVA-R3	TTTGCGGCCGCACTTCCGCATAATTCACATCA	downstream homology arms
ApxIC-F	TGGTCGCACTACAGTAAATC	detection exterior
ApxIC-R	CGTTCCGAGAAACCTAATAG	of *ApxIC* mutants
ApxIIC-F	GTGAAGAGCCATTACCCAAC	detection exterior
ApxIIC-R	GAACTCGATTTCCTTGACCC	of *ApxIIC* mutants
ΔApxIV-orf1-F	GGACTGCTATTTGATTTGGTTTGTC	detection exterior
ΔApxIV-orf1-R	TCTTCTATAACAAAAAAGGCATTGT	of *ApxIV-orf1* mutants
CpxA/R-F	GCTCGCAACAAAATCACCTTGTTCA	detection exterior
CpxA/R-R	AAATGGCGCAATACCCTCAAAGCG	of *cpxAR* mutants
ArcA-F	ATCGGCTTTCGTAGTTTAGC	detection exterior
ArcA-R	TTGTCACCGTAATCCATACC	of *arcA* mutants
UreA-F	ATCAGTTGGCTGCCGAATA	detection exterior
UreA-R	GAAACCAATCACGCCCTAA	of *ureA* mutants
ApxIIIAin-F	GAATGGCTATGATGCTCGTC	detection interior
ApxIIIAin-R	CCCTTGTTCAGTCGCTTT	of *ApxIIIA* mutants
SLW08ApxIVAin-F	TCGCTGTTGGACTGCTATTT	detection interior
SLW08ApxIVAin-R	GGTTACCCATTTCCCTTCG	of SLW08 mutants
SLW08ApxIVAout-F	ATTAGTACGATTCGCTGTTGG	detection exterior
SLW08ApxIVAout-R	ATAGCCCTTCTTTCCATTCC	of SLW08 mutants

10 mice. The mice were reared in a controlled environment with a 12-hour light/ 12-hour dark cycle and were provided with sterile water and SPF mouse growth and reproduction compound feed (Henan Huanyu Hekang Biotechnology Co., Ltd) ad libitum. The log-phase live vaccine candidates (WBY06 and SLW08) were diluted in TSB to a concentration of 10^8 CFU/mL. Groups 1 and 2 received intraperitoneal injections of 0.2mL of SLW08 (2.0×10^7 CFU). Groups 3 and 4 were administered intraperitoneally with 0.2mL of WBY06. Groups 5 and 6 received subcutaneous injections of 0.2mL of a commercial APP-inactivated vaccine (CV, China). Groups 7 and 8 were injected intraperitoneally with 0.2mL of TSB. A booster immunization was performed two weeks after the first vaccination. Following another two weeks, groups 1, 3, 5, and 7 were challenged with a high dose (1.0×10^8CFU) of SLW01, while groups 2, 4, 6, and 8 were challenged with a low dose (1.0×10^7CFU) of SLW01. Group 9 received 0.2mL TSB as a negative control. Mouse survival was monitored and recorded daily for up to 7 days.

A total of 30 six-week-old piglets were randomly divided into 5 groups and reared in a controlled environment with a 12-hour light/12-hour dark cycle. They were provided with sterile water and Nursery pig compound feed (Guangxi Yangxiang Group Co., Ltd). Log-phase live vaccine candidates (WBY06 and SLW08) were diluted to a concentration of 10^7 CFU/mL in TSB. Group 1 received an intramuscular injection of 2mL of SLW08, Group 2 received 2mL of WBY06, Group 3 was given 2mL of a commercial APP inactivated vaccine (CV), and Group 4 was administered 2mL of TSB. A booster immunization was performed three weeks after the first vaccination. Two weeks post-booster, Groups 1, 2, 3, and 4 were challenged with 1.0×10^8CFU of SLW01 via tracheal injection. Group 5 received 2mL of TSB as a negative control. Piglet survival was monitored and recorded daily for up to 7 days.

Detection of *ApxIII* and *ApxIV*: The expression of *ApxIII* in WBY06 and SLW8 strains was detected through western blotting using monoclonal antibody specific to *ApxIII* as previously described (Nahar *et al*., 2021). While the antibody levels against *ApxIV* were measured from serum of piglets in WT strain-infected (SLW01) and mutant strain-immunized groups (SLW08) using the indirect ApxIV-ELISA kit (Keqian Bio. Co. Ltd., Wuhan, China) by following the manufacturer's instructions. Briefly, blood samples from piglets in group 4 (received only TSB) after one day of challenge with wild type strain (SLW01) were collected. To examine the antibody level of *ApxIV* in mutant immunized group, the blood samples of piglets from group 1 were collected 7 days post-booster immunization.

Histopathological studies: For histological examination, lung necropsy samples from each group of piglets and mice were collected and fixed in 10% formalin. Then paraffin embedded tissue sections were prepared, stained with hematoxylin and eosin (H&E) and examined under a light microscope.

Statistical analysis: The obtained data were analyzed using GraphPad Prism 6.0 software and expressed as mean±standard deviation (SD). Analysis of variance (ANOVA) was performed, and the student's *t*-test was used for pairwise comparison. A P-value of <0.05 was considered significant for all tests.

RESULTS

Development and verification of septuple-deletion mutant(ΔapxICΔapxIICΔcpxARΔorf1ΔarcAΔureAΔapxIVA): The deletion mutant strain developed in the present study was designated as SLW08 (ΔapxICΔapxIICΔcpxARΔorf1ΔarcAΔureAΔapxIVA). These modifications were confirmed by PCR using the primer pairs SLW08ApxIVAin, SLW08ApxIVAout, and ApxIIIAin (Fig. 1). The amplified *ApxIVA* gene product with the primer pairs SLW08ApxIVAin was 505bp in WBY06, while no product was detected in SLW08, indicating that a portion of the *ApxIVA* gene was deleted from the SLW08 genome. In contrast, the amplified *ApxIIIA* gene product with the primer pair ApxIIIAin was 676bp in SLW08, whereas no product was detected in WBY06, confirming that the *ApxIIIA* gene was successfully inserted from the JL03 genome into SLW08. The amplified *ApxIVA* gene product with primer pair SLW08ApxIVAout was 3171bp in WBY06, while the product from SLW08 was 3447bp, indicating that the *ApxIIIA* gene from the JL03 genome was inserted into the missing *ApxIVA* gene site of WBY06.

Growth characteristics and genetic stability: The SLW08 strain exhibited slower growth rates compared to the WBY06 and WT (SLW01) strains (Fig. 2a). To assess the stability of the seven in-frame deleted genes in SLW08, PCR analysis was performed on the genomes of the mutant after 14 passages. The PCR results confirmed the presence of a stable in-frame deletion in the SLW08 genome (Fig. 2b).

Attenuated virulence in mice: To evaluate the safety of SLW08, its virulence in mice was assessed compared to the WT (SLW01) and WBY06 (sextuple-deletion) strains. As shown in Table 3, the LD_{50} of SLW08 (4.47×10^{9}CFU) was significantly higher (P<0.05) than that of WT (8.62×10^{5}CFU) and WBY06 (5.47×10^{8}CFU). This indicated that the virulence of SLW08 was attenuated by 5000-fold compared to SLW01 and by 8-fold compared to WBY06. These results suggested that SLW08 had reduced virulence in APP and was safer for immunization in mice.

Protective efficacy of SLW08 in mice: To determine the immune-protective efficacy of SLW08, immunized mice were challenged intraperitoneally with virulent SLW01 serovar 1. The survival rates were 90% (high dose) and 100% (low dose) in the SLW08-immunized group, 60% (high dose) and 80% (low dose) in the CV-immunized group, and 90% (high dose) and 100% (low dose) in the WBY06-immunized group. All mice in the control group challenged with APP died within 2 days (Fig. 3A). These results suggested that the SLW08 immunization provided stronger protection against APP as compared to WBY06 and CV immunization.

Lung sections infected with APP were prepared and analyzed (Fig. 3B-F). The lung tissue of the immunized mice did not exhibit significant damage. In contrast, severe acute hemorrhagic pneumonia was observed in negative controls, characterized by thickening of the alveolar walls and filling of the alveoli with exudate. These findings indicated that SLW08 vaccination produced a strong protective effect against infection with APP.

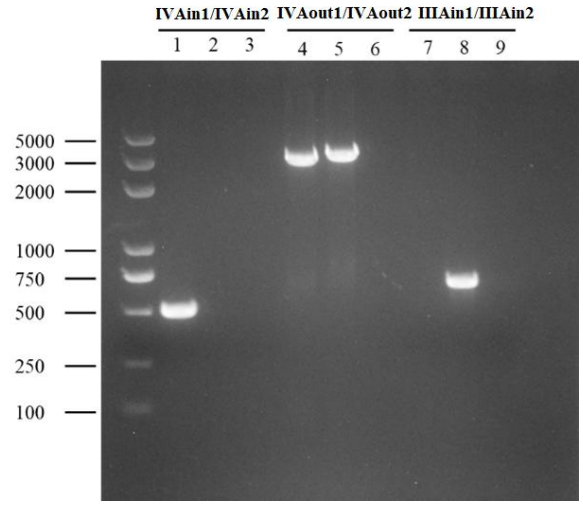

Fig. 1: Identification of mutant strains. Lanes 1 and 2 display the amplification of the *ApxIVA* gene from WBY06 (505bp) and SLW08 (Absent), respectively, using the primer pair SLW08ApxIVAin. Lanes 4 and 5 show the *ApxIVA* gene amplified from WBY06 (3171bp) and SLW08 (3447bp) with the primer pair SLW08ApxIVAout, respectively. Lanes 7 and 8 show the amplification of *ApxIIIA* gene from WBY06 (Absent) and SLW08 (676bp), respectively using the primer pair ApxIIIAin. Lanes 3, 6, and 9 serve as negative controls. Lane M contains a DNA marker.

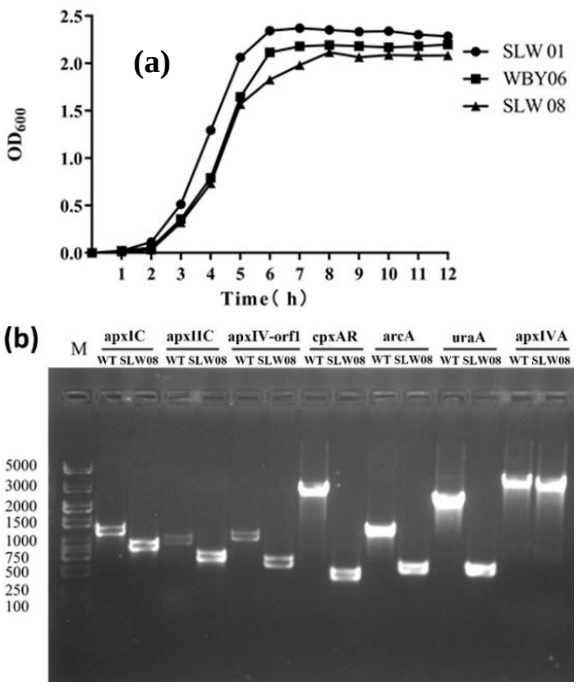

Fig. 2: Growth characteristics and genetic stability of SLW08. (a) Growth curves of SLW01, WBY06 and SLW08. (b) Identification of each deleted gene in the mutant strain SLW08 after 14 continuous passages on TSA.

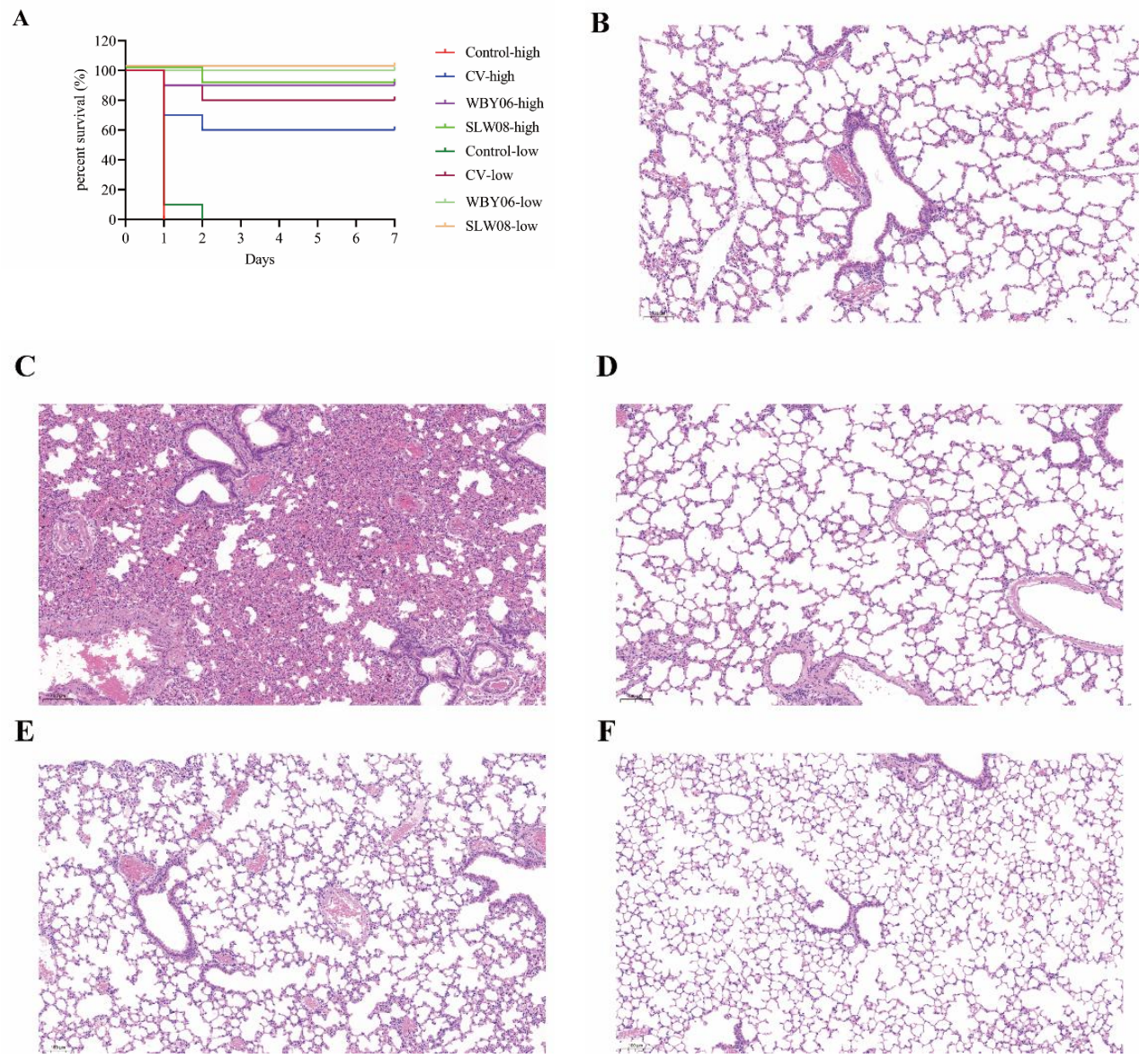

Fig. 3: Protection against virulent challenge in mice vaccinated with different vaccines. (A) Survival rates of mice. (B-F) Lung sections of mice vaccinated with TSB, TSB, WBY06, CV, and SLW08, (B) negative control, (C-F) challenged with SLW01. Scale bars = 100μm.

Table 3: Assessment of SLW08 Safety in Mice.

Strains	Infective dose (CFU)	Mortality	LD_{50}（CFU）
SLW01	1.0×10^{7}	8/8	8.62×10^{5}
	2.5×10^{6}	7/8	
	6.0×10^{5}	3/8	
	1.5×10^{5}	0/8	
WBY06	5.0×10^{9}	8/8	5.47×10^{8}
	1.0×10^{9}	5/8	
	2.0×10^{8}	2/8	
	4.0×10^{7}	0/8	
SLW08	5.0×10^{10}	8/8	4.47×10^{9}
	1.0×10^{10}	6/8	
	2.0×10^{9}	2/8	
	4.0×10^{8}	0/8	

LD_{50} was calculated using the Karber's method (Nielsen, 1979).

Protection efficacy of SLW08 in piglets: To determine the protective efficacy of SLW08, the piglets in immunized and control groups were challenged intra- peritoneally with virulent SLW01 serovar 1. The results were similar to those observed in the mouse experiments. No obvious damage to lung tissue was observed in the immunized piglets. The mortality rate for piglets immunized with SLW08 was 0%, which was lower ($P<0.05$) than that of TSB (100%), WBY06 (16.67%), and CV (33.33%) (Fig. 4). These findings indicated that vaccination with attenuated SLW08 mutant strain in piglets conferred strong protection against APP infection.

Detection of *ApxIII* and *ApxIV*: The insertion and successful expression of *ApxIII* in SLW08 was confirmed through western blotting. As shown in Fig. 5b, *ApxIII* was detected in SLW08 while it was not detected in the WT strain, indicating that SLW08 successfully expressed the *ApxIII* protein.

Further, to differentiate between piglets infected with the WT strain and those immunized with the live attenuated SLW08 vaccine, the antibody levels against *ApxIVA* in the serum of two groups were determined through ELISA. As shown in Fig. 5a, *ApxIV* was not detected in the SLW08-immunized group, whereas it was detected in the WT-infected group. This indicated that SLW08 could be effectively used to differentiate between WT infection and live vaccine immunization.

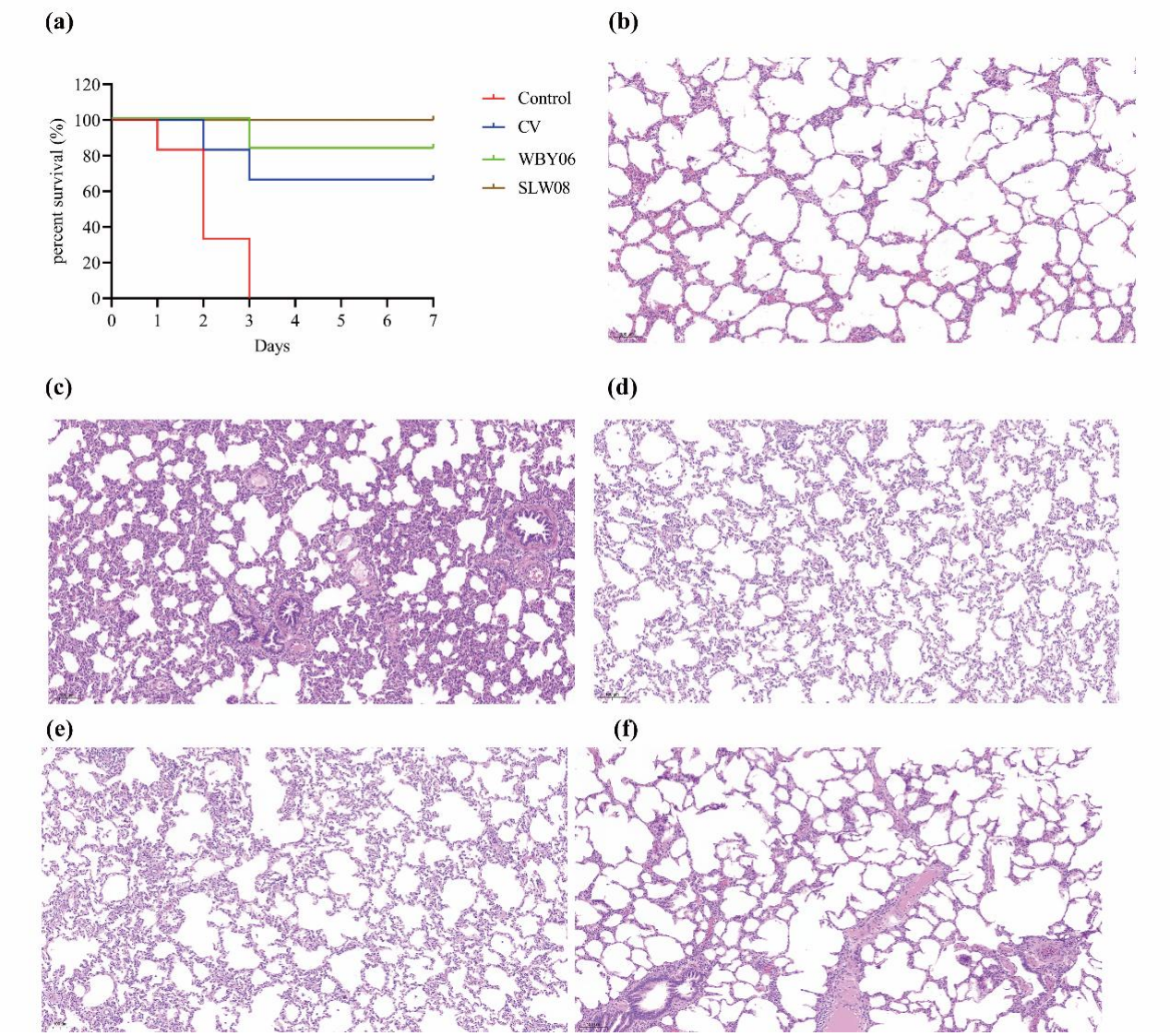

Fig. 4: Protection against virulent challenge in piglets vaccinated with the live vaccines. (a) Survival rates of piglets. (b-f) Lung sections of piglets vaccinated with TSB, TSB, WBY06, CV, and SLW08, (b) negative control, (c-f) challenged with SLW01. Scale bars = 100μm.

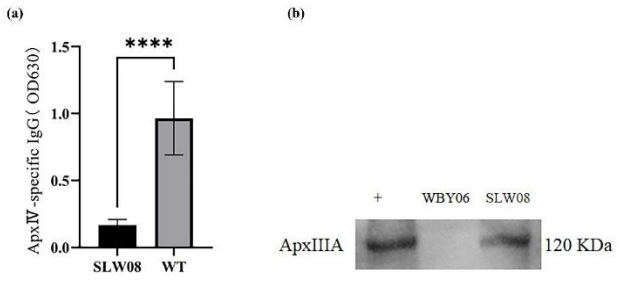

Fig. 5: Detection of *ApxIII* and *ApxIV*. (a) ELISA for serum *ApxIV* antibody levels in WT strain-infected and SLW08 strain-immunized groups (P<0.0001). Data are expressed as mean±SD (N=6). (b) Detection of *ApxIII* expression in WBY06 and SLW08 through western blotting.

DISCUSSION

Live vaccines emerged as the most promising option for achieving effective protection against APP (Sassu *et al*., 2018; Mortensen *et al*., 2022). This study demonstrated that the septuple-deletion mutant strain (SLW08) attenuated from the field-isolated strain (SLW01) exhibited key features desirable for an effective vaccine, such as genetic stability, attenuated virulence, and strong immunogenicity, resulting in a strong protection against APP infection. To the best of our knowledge, this is the first attenuated mutant candidate for immunization against APP that could be effectively utilized for differential diagnosis between WT infection and vaccine immunization.

In APP, RTX exotoxins were considered important virulence factors, each with distinct properties (Tang *et al*., 2023). *ApxI* was highly hemolytic and cytotoxic, *ApxII* was moderately cytotoxic and weakly hemolytic, while *ApxIII* was intensely cytotoxic (Schaller *et al*., 2001). Previous reports indicated that the expression of both *ApxI* and *ApxII* were required by APP to become fully pathogenic (Schaller *et al*., 1999). The *ApxCABD* operon from different serovar strains encoded various RTX exotoxins, which were released by components B and D after being activated by activator C (Schaller *et al*., 2000). Deletion of *ApxIC* and *ApxIIC* limited the ability of APP to survive *in vivo*, resulting in the expression and secretion of inactive *ApxI* and *ApxII* (Liu *et al*., 2015). Deletion of *cpxAR* significantly reduced APP colonization in the lungs (Shao *et al*., 2010), while *arcA* deletion mutants exhibited reduced colonization and lung damage (Liu *et al*., 2015). Although *ApxIV* exhibited weak hemolytic toxicity, it was

found necessary for the full virulence of APP, which was reduced when ApxIV was absent (Sjölund and Wallgren, 2010). In this study, the *ApxIVA* gene of WBY06 was deleted to further reduce its virulence. The safety assessment results revealed that SLW08 had a lower virulence compared to the WBY06 and WT (SLW01) strains.

The seven-gene attenuated vaccine mutant strain SLW08 effectively protected mice and pigs against APP serovar 1 infection, as demonstrated by immunization experiments. Pathological changes in the lung tissues of immunized animals were significantly reduced compared to the control group. The live vaccine SLW08 proved to be more efficient than both WBY06 and CV.

The *ApxIV* protein was identified as a marker for the differential diagnosis of APP infection, as it was expressed only in pigs infected with APP and not expressed under *in vitro* conditions (Wang *et al*., 2010; Stringer *et al*., 2021). While *ApxI*, *ApxII*, and *ApxIII* could also be detected in pigs infected with other less pathogenic *Actinobacillus* spp. (e.g., *A. rossii*, *A. suis*) or those infected with *E. coli* or *Pasteurella* spp. (Xie *et al*., 2016). Since SLW08, with the *ApxIVA* gene knocked out, showed negative results for *ApxIV* antibody using the ApxIV-ELISA kit after immunization. This feature allowed for distinguishing between vaccine-immunized and naturally infected pigs, which is crucial for vaccine monitoring and accurate disease surveillance.

Currently, commercial vaccines used to prevent APP infections include bacterin and subunit vaccines; however, these vaccines typically provide limited cross-protection (Hur *et al*., 2015; Augustyniak and Pomorska-Mól, 2023). Most APP vaccines did not prevent infection or transmission but only mitigated clinical symptoms (Soto Perezchica *et al*., 2023). Inactivated APP vaccines failed to fully activate the immune system, primarily eliciting humoral immunity without replicating the respiratory colonization that occurs in natural infections (Mortensen *et al*., 2022). In addition, several studies demonstrated that subunit vaccines containing multiple recombinant APX toxin components provided superior protection and excellent cross-protective capabilities (Schaller *et al*., 2000; Yuan *et al*., 2018; Zhang *et al*., 2022). Therefore, *ApxIIIA* was inserted into the gene locus of *ApxIVA* to enable the SLW08 strain to secrete inactivated *ApxI*, *ApxII*, and *ApxIII*, enhancing cross-protection against APP infections of different serovars. Although WB confirmed the expression of *ApxIII* protein, challenge protection experiments with other serovars were not conducted in this study, leaving its specific cross-protective efficacy to be further evaluated.

This study on the septuple-deletion mutant strain SLW08 of APP serovar 1 had several limitations. The focus on a single serovar restricted the generalizability of the findings to other APP serovars. Small sample sizes and short-term assessments necessitate larger, long-term studies to confirm the efficacy and duration of immunity.

Conclusions: In conclusion, the septuple-deletion mutant SLW08 has shown significant potential as a live attenuated vaccine for *Actinobacillus pleuropneumoniae* (APP). This mutant strain effectively protected mice and piglets from APP infection. The successful integration of the *ApxIII* gene into the corresponding site of *ApxIV* suggests that the vaccination of this mutant strain may provide cross-protection against various APP serovars. The *ApxIV* deletion in mutant strain may also aid in differentiation between vaccinated and naturally infected animals. However, further studies are necessary to assess its long-term safety and efficacy in diverse pig populations, paving the way for better control of APP in the swine industry.

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Authors contribution: Cao Weiwei designed the experiments. Wei Zudan, Wei Xinchen, and Chen Yunpeng performed data analysis. He Qiyun and Liu Jiahui assisted in writing the manuscript. Chen Huanchun and Bei Weicheng were responsible for editing and proofreading of the manuscript.

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