Immunogenicity of live *phoP* gene deletion strain of *Riemerella anatipestifer* serotype 1

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ABSTRACT Duck infectious serositis is an acute and infectious disease caused by *Riemerella anatipestifer* (***R. anatipestifer***) that leads to perihepatitis, pericarditis, meningitis, and airbag inflammation in ducks, which causes serious economic losses to the global duck industry. The *phoP/phoR* is a novel 2-component signal transduction system first reported in gram-negative bacteria, of which phoP acts as a global regulator and virulence factor. In this study, the *phoP* gene from the *R. anatipestifer* YM strain was knocked out using homologous recombination technology and replaced with the spectinomycin resistance gene (*Spec*). The virulence of the *R. anatipestifer* YMΔ*phoP* strain was reduced by approximately 47,000 times compared to that of the wild-type *R. anatipestifer* YM strain. Ducks were immunized with live *R. anatipestifer* YMΔ*phoP* strain by subcutaneous inoculation at a dose of 10^6 to 10^7 CFU (0.2 mL per duck) and challenged with the wild-type *R. anatipestifer* YM strain 14 days later. The protection rate in the immunized group was 100%. The growth characteristics of ducks in the immunized and negative control groups were normal, and the research demonstrated *R. anatipestifer* YMΔ*phoP* strain have suitable immunogenicity and protective effects. Thus, the study findings suggest that the novel *R. anatipestifer* YMΔ*phoP* strain may provide a candidate for the development of a gene deletion activated vaccine against duck infectious serositis.

Key words: *Riemerella anatipestifer*, *R. anatipestifer* YMΔ*phoP*, Immunity

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INTRODUCTION

Riemerella anatipestifer (formerly *Pasteurella anatipestifer*) is a gram-negative rod-shaped bacterium that mainly infects ducks at 1-8 weeks of age, with particularly high infection rates at 2 to 3 wk of age (Segers et al., 1993; Pathanasophon et al., 1995; Birkey et al., 1998; Hu et al., 2011). Once *R. anatipestifer* invades the host body, the bacterium multiplies rapidly and reaches various tissues and organs through the blood, causing systemic serositis. The main clinical anatomical manifestations of *R. anatipestifer* infection are fibrinous pericarditis, perihepatitis, airsacculitis, and meningitis (Liu et al., 2015; Fernandez et al., 2018; Wang et al., 2019a; Yang et al., 2019). Duck infectious serositis was first reported in Long Island, New York in 1932 and subsequently spread throughout Australia, the United Kingdom, the former Soviet Union, and other countries (Hendrickson and Hilbert, 1932). Currently, duck infectious serositis occurs worldwide with high morbidity and mortality rates, and causes considerable economic losses to the duck industry (Cobb and Smith, 2015; Fernandez et al., 2016). Because long-term dependence on antibiotics may lead to drug resistance in pathogenic bacteria and drug residues in meat products (Gao et al., 2014; Tang et al., 2018; Yang et al., 2020), the development and application of safe and efficient vaccines are needed to prevent and control this infectious disease (Kang et al., 2018).

Many *R. anatipestifer* serotypes have been identified, displaying dynamic distribution in different countries and regions. Serotypes were reported with no cross-protective effects. Sandhu and Harry (1981) reported that serotypes 1, 2, and 5 were the main serotypes in the United States. Many studies have evaluated the use of inactivated vaccines against *R. anatipestifer*, revealing

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that their immune effects are directly related to locally circulating serotypes. However, producing inactivated vaccines is expensive, the immunization period is short, and the appropriate bacterial count and adjuvant type must be evaluated in depth. Indeed, a *P. anatipestifer* vaccine comprising a combination of inactivated *Escherichia coli* serotype O78 and *P. anatipestifer* bacterin showed high protection rates in ducks at 10 and 17 d of age but the protective effect only lasted for 2 wk, and research demonstrated that vaccination with aluminum-hydroxide-gel-adsorbed bacterin did not improve immunity compared to that achieved with the nonadjuvanted vaccine. Moreover, inoculation with oil-emulsion bacterin conferred adequate protection up to market age, but it caused local damage at the inoculation site (Sandhu and Layton, 1985). In contrast, live vaccines can induce humoral, cellular, and mucosal immune responses in the body for an extended period of time.

phoP/phoR is a novel 2-component signal transduction first reported in gram-negative bacteria (Wang et al., 2017), and also exists in many gram-positive bacteria, including *Bacillus subtilis* (Guo et al., 2010) and *Mycobacterium tuberculosis* (Cimino et al., 2012). The *phoP* gene is a member of the *pho* regulator family and is subject to autoregulation via promoter binding (Liu and Hulett, 1997; Sieberi et al., 2020; Singh et al., 2020). PhoP acts as a global regulator and virulence factor, playing important roles in the regulation of upstream and downstream gene regulation. Therefore, the objective of this study was to use homologous recombination technology (Lu et al., 2013; Luo et al., 2015) to replace *phoP* with the spectinomycin-resistance gene (*Spec*) in the *R. anatipestifer* YM strain (serotype 1), evaluate its virulence, and analyze the effects of immunization with different doses of live *R. anatipestifer* YMΔ*phoP* strain in ducks. Our findings elucidate the safety and efficacy of live *R. anatipestifer* YMΔ*phoP* strain and demonstrate its potential clinical application for the protection of ducks against *R. anatipestifer* infection.

MATERIALS AND METHODS

Animals

Five-day-old healthy Cherry Valley ducks were purchased from Yongsheng Duck Company (Wuhan, China) and housed in isolated animal rooms at 28 to 30° C. The ducks were housed in cages with a 12-h light/ duck per day with free access to food and water. All animal experiments and procedures were approved by the Research Ethics Committee of Huazhong Agricultural University (approval no. HZAUSW-2018-011).

Bacterial Strains, Plasmids, and Culture Conditions

Both the wild-type *R. anatipestifer* YM (serotype 1) and *R. anatipestifer* YMΔ*phoP* strains were grown in tryptic soy broth (TSB; Becton Dickinson, Sparks, MD), whereas *E. coli* X7213 cells were grown in Luria Broth. The incubation temperature was 37°C. The following antibiotics were added as needed: spectinomycin (Sigma-Aldrich, St. Louis, MO; 100 μg/mL), ampicillin (Sigma-Aldrich; 100 μg/mL), and chloramphenicol (Sigma-Aldrich; 50 μg/mL). For the cultivation of *E. coli* X7213, 50 μg/mL diaminopimelic acid (Sigma-Aldrich) was added to the medium. The pRE112 suicide plasmid was used to construct the gene deletion strain. The pIC333 plasmid was used to amplify the spectinomycin resistance gene (*Spec*). All strains and plasmids were retrieved from the repository of our laboratory. The primers used in this study are listed in Table 1.

Construction and Identification of the R. anatipestifer YMΔphoP Strain

The left and right homologous arms (L, R) were amplified using PCR with primer pairs *phoP*L-F/ *phoP*L-R and *phoP*R-F/*phoP*R-R. *Spec* was amplified using primers *phoP Spec*-F and *phoP Spec*-R. The 3

Table 1. Primers used in this study.

Name	Sequence (5′-3′)	Use	Reference or Designed
*phoP*L-F	CTGGTACCCACTATTGCTGATGAGGTTTACCTTGAAAA TCAACTAAAG	Amplification of the left arm of *phoP*	Designed
*phoP*L-R	AACGTGAGTTTTCGTTCCACTGCTTTTGGTCATCTTCT ACTAATAATATCCTGTTGCTCAT		
phoP-F	ATGAGCAACAGGATATTATTAGTAGAAGATGACCAAAG	Identification of mutant *phoP*	Designed
phoP-R	ATTTTTAACTAGAAGCCTAAACCCTTCCCCGTGTAC ATT		
*phoP*R-F	CAGGTGCTTACTTTTAAAACTACTGTTCGGGGAAGGG TTTAGGCTTCTAGTTAAAAATTAA	Amplification of the right arm of *phoP*	Designed
*phoP*R-R	AGAGCTCGCACCACTCATTATGATTTTCTTTTGTATTT ATTGTTAGAG		
phoP Spec-F	ATGAGCAACAGGATATTATTAGTAGAAGATGACCAAAG CAGTGGAACGAAAACTCACGTT	Amplification of *Spec*	Designed
phoP Spec-R	TTAATTTTTAACTAGAAGCCTAAACCCTTCCCCGCAGT AGTTTTAAAAGTAAGCACCTG		
Spec-F	AAGAAAAAAATAAAATCATGAGTAGAGCAGTGGAACG AA AACTCACGTT	Identification mutant of *phoP*	Designed
Spec-R	GCCCTTAAACCACATTTATAAAAGCCAGTAGTTTTAAA AGTAAGCACCTG		

gene fragments were concatenated using overlap extension PCR and digested with KpnI and SacI. The LSR fragment (spectinomycin resistance marker flanked by *phoP* homologous arms) was ligated into the pRE112 plasmid, resulting in the successful construction of the suicide plasmid pRE112-LSR (Zhao et al., 2016; Guo et al., 2017a). Transformation with *E. coli* X7213 as the donor strain and wild-type *R. anatipestifer* YM as the recipient strain was conducted to construct the *R. anatipestifer* YMΔ*phoP* strain (Figure 1A). The combined and transferred bacterial solution was spread on a TSA plate containing spectinomycin, and a single colony was selected after 5 consecutive passages for identification (Guo et al., 2017b; Gong et al., 2020), using primers *Spec*-F *Spec*-R and *phoP*-F *phoP*-R.

LD_{50} Determination of Wild-Type *R. anatipestifer* YM and *R. anatipestifer* YMΔphoP Strains

The wild-type *R. anatipestifer* YM and *R. anatipestifer* YMΔ*phoP* strains were centrifuged at 1,600 × *g* for 3 min and resuspended in PBS. The bacterial solution for each strain was diluted to five different concentrations, including 10^9, 10^8, 10^7, 10^6, and 10^5 CFU/mL. Seven-day-old Cherry Valley ducks were divided into 11 groups (10 ducks per group), with ducks in each group receiving 1 of the 5 concentrations of wild-type *R. anatipestifer* YM strain, *R. anatipestifer* YMΔ*phoP* strain, or no treatment (control). Duck feet were injected with 0.2 mL bacterial solution. Phenotypic changes were noted daily after injection, deaths were recorded, and LD_{50} values were calculated (Hu et al., 2010). The ratio of the LD_{50} of the deletion strain to that of the wild strain is the fold reduction of the virulence of the deletion strain.

Recovery, Culture, and Preparation of *R. anatipestifer* YMΔphoP Strain

The *R. anatipestifer* YMΔ*phoP* strain was inoculated on TSA plates and placed in an incubator at 37°C and 5% CO_2 for 48 to 72 h. A single colony of the *R. anatipestifer* YMΔ*phoP* strain was selected, inoculated into TSB medium, and cultured at 37°C with shaking (180 r/min) until the logarithmic growth phase was reached [optical density at 600 nm (OD_{600}) = 0.8]. Subsequently, the bacterial suspension was centrifuged at 4,200 × *g* for 10 min at 18 to 25°C, and the pellet was washed 3 times with PBS. The OD_{600} value of the bacterial solution was then adjusted to 1.0 using PBS.

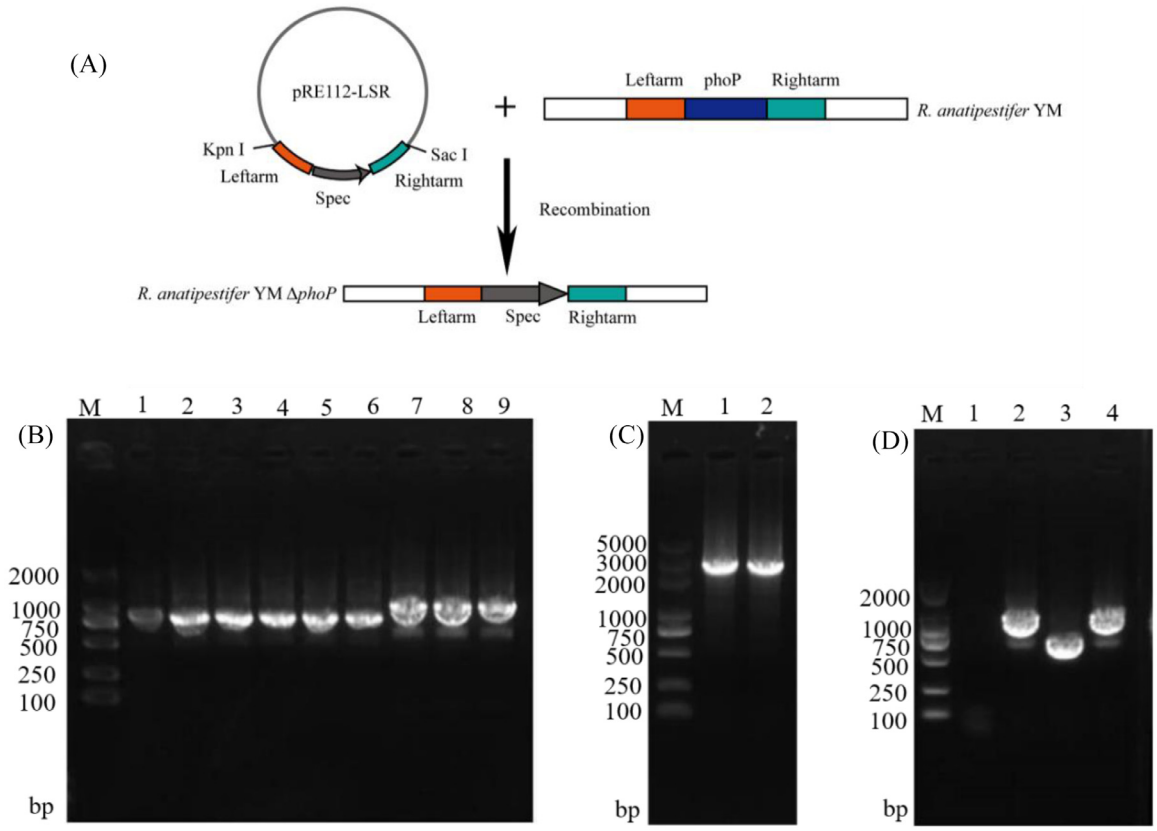

Figure 1. Construction of the *R. anatipestifer* YMΔ*phoP* strain. (**A**) Diagram of the designed *R. anatipestifer* YMΔ*phoP* mutant strain. (**B**) PCR amplification of the left and right homologous arms of the *phoP* and *Spec* genes. Lanes 1–3: PCR products of the left homologous arm. Lanes 4–6: PCR products of the right homologous arm. Lanes 7–9: PCR products of the *Spec* gene. (**C**) Overlap extension PCR amplification of the left and right homologous arms of the *phoP* and *Spec* genes. Lanes 1 and 2: PCR amplification using the left arm, right arm, and *Spec* gene as templates. (**D**) Identification of the *R. anatipestifer* YMΔ*phoP* strain. Lane 1: Identification of deletion strain with *phoP* primers. Lane 2: Identification of deletion strain with *Spec* primers. Lane 3: Identification of deletion strain with *phoP* primers. Lane 4: Identification of deletion strain with *Spec* primers. RA, *Riemerella anatipestifer*.

Assessment of Safety of R. anatipestifer YMΔphoP Strain

Seven-day-old Cherry Valley ducks were randomly divided into 2 groups (10 ducks per group), including a negative control group and an immunized group subcutaneously inoculated with the *R. anatipestifer* YMΔ*phoP* strain (10^8 CFU per duck). The growth, behavior, diet intake, and water consumption of the ducks were observed for 7 consecutive days.

Assessment of the Protective Effects of R. anatipestifer YMΔphoP Strain

Seven-day-old Cherry Valley ducks were randomly divided into the following 4 groups (10 ducks per group): groups 1 and 2 were subcutaneously inoculated with the *R. anatipestifer* YMΔ*phoP* strain at a dose of 10^6 to 10^7 CFU; group 3 was the *R. anatipestifer* YM challenge control group; and group 4 was the negative control group. Blood was collected on d 7 and 14 after immunization, the serum was separated, and the antibody titer was determined by IgY ELISA. Fourteen days after immunization, ducks in groups 1, 2, and 3 were infected with the wild-type *R. anatipestifer* YM strain at an infection dose of 10 LD_{50} and observed for 7 consecutive days to determine the protection rate (Chu et al., 2015).

Determination of Serum IgY Antibody Levels in Immunized Ducks

The *R. anatipestifer* YMΔ*phoP* strain was collected, washed 3 times with PBS, and sonicated. Microplates were then coated to prepare detection plates, and the sera from 14-day-old ducks were added as the primary antibody for 1 h at 37°C. Horseradish peroxidase-labeled goat anti-duck IgY (Luoyang Bai Aotong Experimental Materials Center, Henan, China) was used as a secondary antibody to measure serum IgY antibody levels in immunized ducks. Incubation with the secondary antibody was performed for 1 h at 37°C (Huang et al., 2002; Lobbedey and Schlatterer, 2003; Jia et al., 2009; Huang et al., 2011).

Detection of Serum Cytokine Levels in Immunized Ducks

Blood was collected 72 h after immunization, left to stand at 37°C for 30 min, and centrifuged at 1,600 × *g* for 10 min to obtain the serum. The expression levels of duck IL-2, IL-4, IL-10, and interferon (IFN)-γ were assessed using specific ELISA kits (BIOSAMITE, Shanghai, China). Serum cytokine levels were calculated from standard curves constructed using the concentration of the standard product as the abscissa and corresponding OD value as the ordinate.

Observation and Pathological Examinations

Ducks inoculated with *R. anatipestifer* YMΔ*phoP* and wild-type *R. anatipestifer* YM strains were autopsied to observe pathological changes focusing on changes detected in the heart, liver, spleen, and brain. Additionally, the presence of bleeding, necrosis, and surface cellulose coverage was noted. The corresponding tissues were fixed in formalin, embedded in paraffin, and cut into 4-μm-thick sections for hematoxylin and eosin staining. The sections were observed under a microscope (Gao et al., 2021).

Statistical Analysis

Data are presented as the mean ± SEM. Differences among groups were determined using one-way analysis of variance with GraphPad Prism software (GraphPad Software Inc., San Diego, CA). Results with P-values < 0.05 were considered statistically significant.

RESULTS

Construction and Identification of R. anatipestifer YMΔphoP Strain

The genome of the wild-type *R. anatipestifer* YM strain was used as the template to amplify the left and right homologous arms of the *phoP* gene, and agarose gel electrophoresis showed that both bands were detected at approximately 800 bp (Figure 1B). The pIC333 plasmid was used as the template to amplify the *Spec* gene, and agarose gel electrophoresis revealed a band of approximately 1,100 bp (Figure 1B), which was consistent with our expectations. The *Spec* gene flanked by the left and right homologous arms of *phoP* was connected by overlap extension PCR, yielding a band size of 3,000 bp via agarose gel electrophoresis (Figure 1C), as expected. The overlapping fragment was ligated to the pRE112 plasmid for deletion of the *phoP* gene. The putative *phoP* deletion and wild-type *R. anatipestifer* YM strain DNA was amplified with *phoP* and *Spec* primers. Electrophoresis of the PCR products demonstrated no amplification of the *phoP* fragments for the putative *phoP* deletion strain, but *Spec* amplification was detected. Conversely, *phoP* amplification was demonstrated in the wild-type *R. anatipestifer* YM strain, whereas *Spec* amplification was not detected. These results demonstrated that the *R. anatipestifer* YMΔ*phoP* strain was successfully generated (Figure 1D).

LD_{50} Determination Using Wild-Type R. anatipestifer YM and R. anatipestifer YMΔphoP Strains

The ducks began to die on day 1 after inoculation with the wild-type *R. anatipestifer* YM strain. The number of deaths peaked on d 2 to 3 and decreased on d 4 to 5, with no deaths observed on d 6 to 7. The surviving ducks

Table 2. LD_{50} values for wild-type *R. anatipestifer* YM and *R. anatipestifer* YMΔ*phoP* strains.

Strains	Dose	Survived/Total	LD_{50} (CFU)
Wild-type *R. anatipestifer*	1.0×10^5	2/10	3.98×10^4
	1.0×10^6	1/10	
	1.0×10^7	0/10	
	1.0×10^8	0/10	
	1.0×10^9	0/10	
R. anatipestifer YMΔphoP	1.0×10^5	10/10	1.88×10^9
	1.0×10^6	10/10	
	1.0×10^7	10/10	
	1.0×10^8	9/10	
	1.0×10^9	7/10	
Negative control group	n/a	10/10	n/a

CFU, colony-forming units; LD_{50}, 50% lethal dose; n/a, not applicable; R. anatipestifer, Riemerella anatipestifer.

did not move, preferring instead to rest in their nests. In contrast, ducks inoculated with the *R. anatipestifer* YMΔp*hoP* strain showed normal behavior after inoculation, with no changes in diet or water consumption. Deaths were observed only on d 3 and 4. The LD_{50} of the wild-type *R. anatipestifer* YM strain was 3.98×10^4 CFU, compared to 1.88×10^9 CFU for the *R. anatipestifer* YMΔ*phoP* strain. The virulence of the *phoP* deleted strain was reduced by approximately 47,000 times compared to that of the wild-type *R. anatipestifer* YM strain (Table 2).

Safety of R. anatipestifer YMΔphoP Strain

Ducks inoculated with *R. anatipestifer* YMΔ*phoP* bacterial suspension were observed for 7 consecutive days, revealing no abnormalities in their appearance, behavior, or inoculation site, and no changes in the amount of water or food consumed.

Protective Effects of R. anatipestifer YMΔphoP Strain

All ducks in the immunized groups survived, regardless of the dose, whereas those in the *R. anatipestifer* YM challenge group died within 1 to 7 d (Table 3, Figure 2). The ducks grew normally and had no clinical symptoms caused by *R. anatipestifer* YM inoculation. The indirect ELISA results revealed that the serum from the immunized groups contained higher levels of antibodies than that from the negative control and *R. anatipestifer* YM challenge groups. Additionally, serum levels of all tested cytokines in the immunized groups were significantly increased at 72 h compared to those in the negative control and *R. anatipestifer* YM challenge groups (Figure 3).

Observation and Pathological Examination

Ducks in the immunized and negative control groups exhibited normal tissues (Figure 4A, B), whereas those in the *R. anatipestifer* YM challenge group showed enlarged liver tissue, symptoms of fibrinous pericarditis, perihepatitis, and meningitis, as well as cerebral hemorrhage. Pathological examinations revealed large amounts of inflammatory substances in the epicardium, extensive fatty degeneration of liver cells, necrosis of some spleen cells, and small amounts of a red homochromatic substance in the splenic nodules. Additionally, some neurons exhibited pyknotic pyknosis, deep staining, and appeared neurotropic (Figure 4C).

DISCUSSION

R. anatipestifer is a major bacterial pathogen that causes economic losses in duck farming worldwide. Drugs offer a highly effective means by which to control this disease; however, drug resistance can develop with long-term drug use. Thus, few effective drugs target *R. anatipestifer* in many geographic areas, resulting in increased healthcare costs, deficits in food safety, and increased risk to human health. Vaccination is an effective strategy for preventing and controlling infectious serositis in ducks (Rubbenstroth et al., 2009; Tomida et al., 2019). Higgins et al. (2000) studied the cellular immune response mechanism of formalin-inactivated and live attenuated serotype 2 *R. anatipestifer* vaccines, reporting that the isolated wild-type strain was less pathogenic after being passaged 62 times in liquid and solid medium. After immunization of young ducks using drinking water, aerosol, or subcutaneous inoculation, analysis of serum titers using ELISA demonstrated that the attenuated vaccine induced high levels of immune antibodies that persisted for a long time compared to inoculation with the inactivated vaccine. Lymphocyte transfer tests also indicated that the attenuated vaccine induced a strong cellular immune response (Dou et al., 2018). Compared with the above study, The *R. anatipestifer* YMΔ*phoP* strain was constructed by homologous recombination, which mainly has a protective effect on ducks infected with serotype 1. Since the experimental animals were vaccinated with the *R. anatipestifer* YMΔ*phoP* live vaccine, they were capable of exhibiting humoral immunity and cellular immunity,

Table 3. Protective effects of the *R. anatipestifer* YMΔ*phoP* gene deletion strain.

Group	Immune dose (CFU)	Infectious dose (CFU)	Survived/Total	Survival%
Low dose	1.0×10^6	1.0×10^6	10/10	100
High dose	1.0×10^7	1.0×10^6	10/10	100
YM challenge	n/a	1.0×10^6	0/10	0
Negative control	n/a	n/a	10/10	100

R. anatipestifer, Riemerella anatipestifer; cfu, colony forming units; n/a, not applicable.

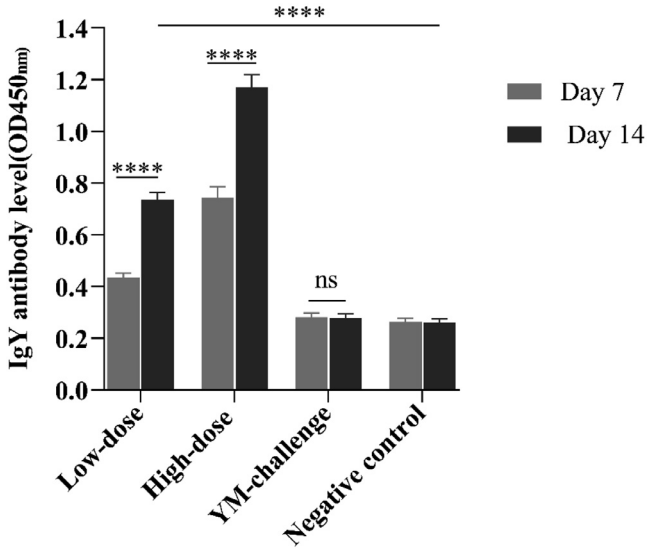

Figure 2. Protection and antibody levels for different groups. IgY antibody levels in ducks on days 7 and 14. All data are expressed as the mean ± SEM of 3 independent experiments. $^{****}P < 0.0001$.

which protected the ducks from *R. anatipestifer* infection. This is supported by our result of significantly increased serum levels of all tested cytokines. The cytokines produced after immunization include IL-2 and IFN-γ secreted by Th1 cells that mediate cellular immunity and IL-4 and IL-10 secreted by Th2 cells that mediate humoral immunity. The research indicates that the *R. anatipestifer* YMΔ*phoP* strain can effectively enhance humoral immunity and cellular immune response.

Researchers have developed a live vaccine for the prevention of *R. anatipestifer* serotypes 1 and 2 (Kang et al., 2018). However, the vaccine requires 2 oral doses for immunization, which is costly for farmers. Wang et al. (2014, 2016) used a strain with *pncA* gene deletion as a vaccine candidate and obtained 90% protection after 2 vaccinations. Liu et al. (2016, 2018) employed homologous recombination to produce a virulence gene deletion strain. After knocking out the *ΔB739_1343* gene, the virulence of the live vaccine candidate strain was decreased by approximately 10,000 times and the

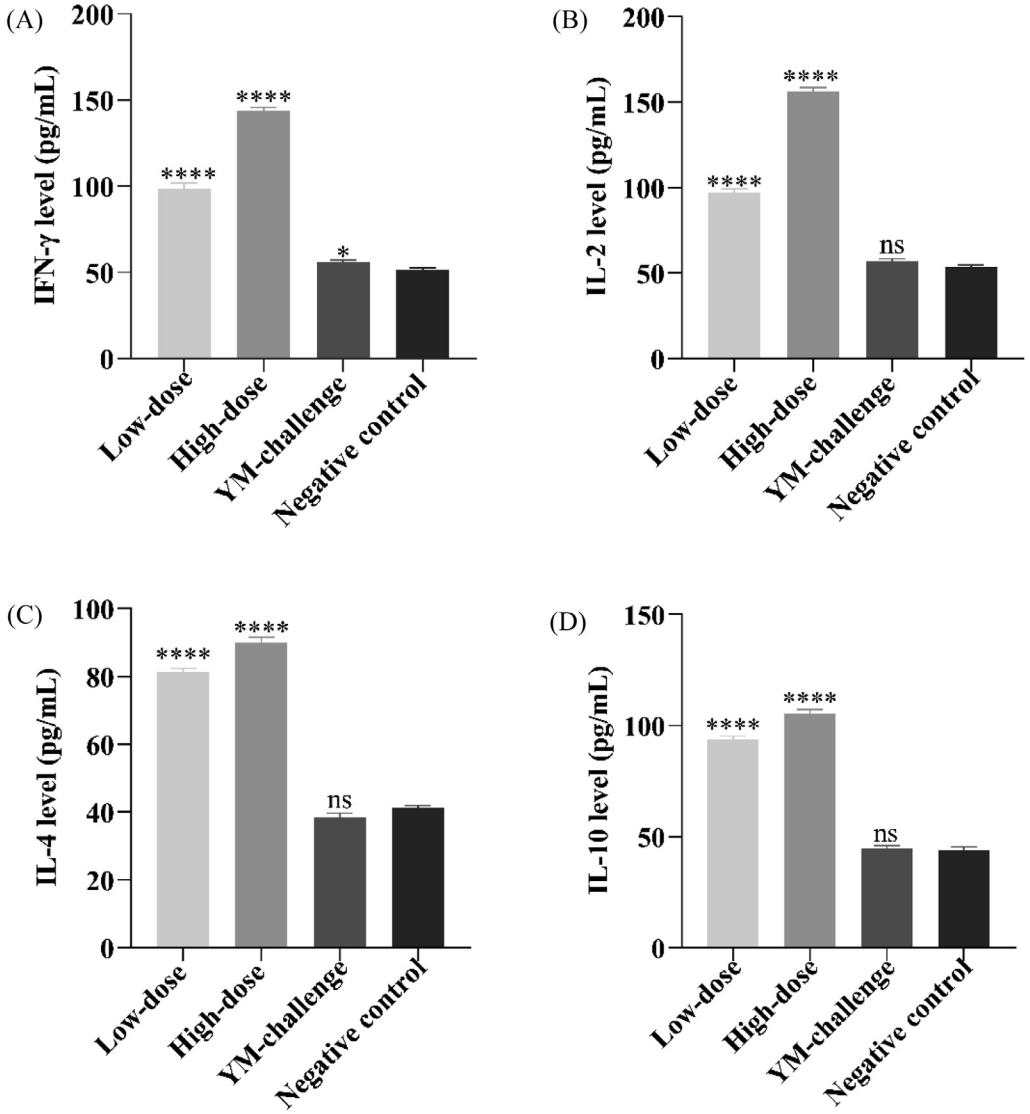

Figure 3. Determination of serum cytokine levels in different groups, as measured by ELISA. (**A**) IFN-γ, (**B**): IL-2, (**C**): IL-4, (**D**): IL-10. All data are expressed as the mean ± SEM of 3 independent experiments. $^{****}P < 0.0001$; $^{*}P < 0.05$.

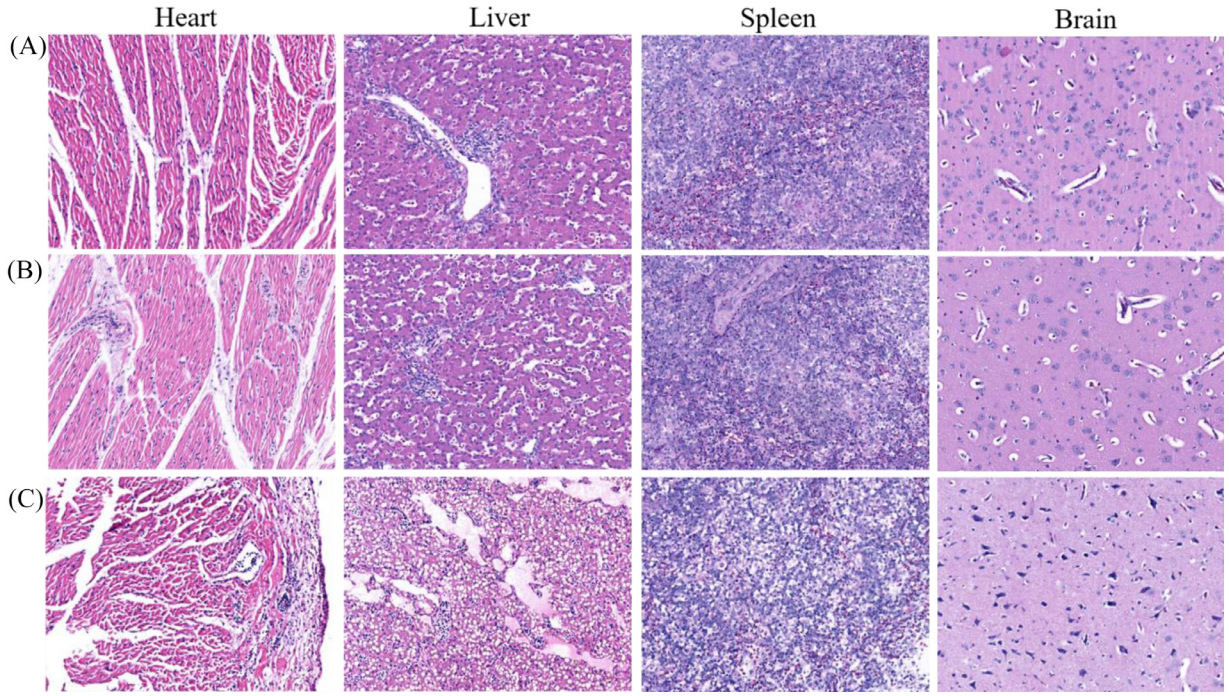

Figure 4. Staining results for histopathological sections from the ducks. (**A, B**) Left to right, Observations from tissue sections from the immunized and negative control groups. (**C**) Abnormal myocardial structure with large amounts of viscous material in the epicardial membrane. Abnormal liver tissue structures, with extensive steatosis of the liver cells. Some spleen cells were necrotic, and a small amount of red homogeneous material was detected in the splenic nodules. Some neurons were pyknotic and hyperchromatic, showing neurotropic effects. The tissues were stained with hematoxylin and eosin (200 ×).

protection rate was 83.3%. Moreover, we previously generated a *Cas9* gene deletion strain by homologous recombination that demonstrated protective effects and a 317-fold reduction in virulence. Although the protection rate of the *Cas9* gene deletion strain reached 80% through intranasal inoculation, subcutaneous inoculation only achieved a protection rate of 30% (Wang et al., 2019b). In the current study, the virulence of the *phoP* deletion strain was reduced by approximately 47,000 times, and ducks were immunized with the live *R. anatipestifer* YMΔphoP strain by subcutaneous inoculation. The live *R. anatipestifer* YMΔphoP strain only requires one dosage for immunization, and the rate of protection to ducks could reach 100%. Compared to the existing methods, the vaccine described herein provides better protection, requires fewer immunizations, and uses lower immunization dosages.

Several inactivated vaccine products are commercially available in China. The production process for inactivated vaccines utilizes inactivated bacteria, resulting in suitable safety profiles. Xu et al. (2020) reported that prokaryotic ompA protein, as a predominant immunogenic protein of *R. anatipestifer*, was an immunizing antigen but only provided partial protection against challenge with *R. anatipestifer*. Studies with deletion strains of *R. anatipestifer* have shown that the main biochemical characteristics of the Δ*phoP*/*phoR* double gene deletion strain are similar to those of the wild-type *R. anatipestifer* YM strain, albeit with significantly reduced virulence and host tissue invasion capacity. Indeed, the double gene deletion strain showed an LD_{50} > 10^{10} CFU, suggesting that the strain was avirulent. However, no effective protection was achieved after ducks were immunized with the Δ*phoP*/*phoR* double gene deletion strain, suggesting that the double-component deletion strain lost both virulence and immunogenicity (Lu. T, unpublished). The Δ*phoP* gene deletion strain obtained in this study showed 100% protection against serotype 1 *R. anatipestifer* infection in ducks, while the virulence was reduced by 47,000 times. The reason that the protection effect of the *phoP* deletion strain was 100% and Δ*phoP*/*phoR* deletion strain had less protection effect needs further study. The *phoP* gene of RA is a virulence gene that has been identified using in vivo antigen technology. In the current study, homologous recombination and combined transfer were used to replace the *phoP* target gene with the *Spec* gene, yielding the *R. anatipestifer* YMΔ*phoP* strain. This strain showed greatly reduced virulence and resulted in high antibody production after inoculation in ducks. Furthermore, ducks immunized with this strain were protected against infection by the wild-type strain. Overall, these findings suggest that *R. anatipestifer* YMΔ*phoP* could be used as a vaccine candidate strain, thereby establishing a basis for further studies on gene deletion live vaccines for duck infectious serositis. The *R. anatipestifer* YMΔ*phoP* strain exhibited a suitable level of safety and protective effects against the epidemic strain. Future studies will explore the protective effects of different vaccination doses on ducks. Additionally, the cross-protection effect of the *R. anatipestifer* YMΔ*phoP* strain against other *R. anatipestifer* serotypes needs to be further verified owing to the large number of pathogenic serotypes. Thus, future research should

employ proteomics to screen ectoproteins with cross-protection against various pathogenic RA serotypes and investigate aspects related to subunit vaccines.

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DISCLOSURES

The authors declare that they have no conflicts of interest.

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