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Avian *Salmonella enteritidis* serovar Enteritidis with *ClpP* deletion is attenuated and highly immunogenic in chickens

Xuhua RAN[1], Zhiyuan YANG[1], Guangxi SHAO[1,2], Xiaobo WEN[1], Siguo LIU[2], Hongbo NI[1,*]

[1]College of Animal Science & Veterinary Medicine, Heilongjiang Bayi Agricultural University, Daqing, P.R. China

[2]State Key Laboratory of Biotechnology, Division of Bacterial Disease,Harbin Veterinary Research Institute of the Chinese Academy of Agricultural Science, Harbin, P.R. China

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Abstract: *Salmonella enterica* subsp. *enterica* serovars are leading causative agents of gastroenteritis in humans and numerous animal species worldwide. The *ClpP* from *Salmonella enterica* is responsible for regulation under various stress conditions. We were interested in the effect of *ClpP* deletion mutation on attenuation of *Salmonella enterica* subsp. *enterica* serovar Enteritidis (SE) in chickens. Therefore, SM-6/Δ*ClpP*, deletion of the *ClpP* gene from a wild-type SE-6 (SM-6) strain, was generated using the phage λ Red homologous recombination. Compared with SM-6, SM-6/Δ*ClpP* represented a retarded growth rate and lower concentration in LB broth with and without 5% NaCl or lower pH values, indicating that it is sensitive to conventional disinfectants. SM-6/Δ*ClpP* represented reduced invasion into macrophages in vitro and an approximate 400-fold attenuation based on cell invasion assay and median lethal dose (LD_{50}) in specific pathogen-free (SPF) chickens. Furthermore, SM-6/Δ*ClpP* showed reduced colonization in the liver and spleen and faster clearance compared with SM-6, indicating a short discharge. The survival rate of 7-day-old SPF chickens immunized with SM-6/Δ*ClpP* against homologous challenge and antibody kinetics indicated that SM-6/Δ*ClpP* provided efficient protection from lethal challenge and induced robust antibody immune responses, demonstrating the potential of live, attenuated SE as a vaccine candidate.

Key words: *ClpP*, *Salmonella* Enteritidis, chickens, vaccine, immune responses

1. Introduction

Avian salmonellosis caused by *Salmonella enterica* subsp. *enterica* serovar Enteritidis is one of the leading diseases in the poultry industry and causes chicken diarrhea, reduces production performance, and can result in death. Moreover, poultry products are frequently contaminated with avian *Salmonella enterica* subsp. *enterica* serovars (1,2), which are a major cause of foodborne illnesses in humans, resulting in an estimated annual 155,000 deaths (3–5). It is thought that poultry is the major reservoir of *S.* Enteritidis and the other serovars for human populations (4,6). In addition to efficient hygiene regimes at all stages of production, antibiotic prophylaxis may be an alternative strategy to prevent and control avian salmonellosis. However, the use of antibiotics has been implicated in causing extensive multidrug resistance in *S.* Enteritidis (7,8). In addition, drug residues in edible poultry tissues and eggs are of increasing concern (7) and the use of antibiotics in the poultry industry has led to high levels of antibiotic residues in manure and litter, much of which is currently applied to agricultural land as fertilizer (9). Thus, the application of these fertilizers has created significant concern regarding public and environmental health. Based on these issues, immunization with either live or inactivated *S.* Enteritidis vaccines should be an effective and economical method to reduce salmonellosis incidence, contamination by avian *S.* Enteritidis strains in the food chain, and the incidence of human salmonellosis (10,11). Generally, it is presumed that attenuated vaccines have advantages over inactivated vaccines for the prevention of *S.* Enteritidis from intestinal colonization and systemic infection, because live *Salmonella* vaccines induce stronger local and systemic immunity (12,13).

Similar to many Enterobacteria, the infection rate of *S.* Enteritidis depends on various virulence factors that contribute to invasion and survival in host tissues (14,15). Thus, investigating virulence factors and identifying the effect of virulence-related genes on attenuation may facilitate the development of attenuated vaccines against avian salmonellosis (16,17). Of note, Methner et al. reported that generated *S.* Enteritidis with a double deletion mutation in *phoP* and *fliC* induced a robust

* Correspondence: nihongbo@sina.com

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adaptive immune response and protection against *Salmonella* exposure in older chickens, suggesting that it might be a promising live *Salmonella* vaccine candidate (18). Previous studies demonstrated that a lack of *ClpP* resulted in reduced virulence in mice (19,20). Furthermore, the Δ*ClpP* mutant had downregulated transcription of the *Salmonella* pathogenicity island 1 (*SPI1*) and *SPI4* virulence genes and upregulated RpoS-dependent genes and the *fliC* gene (21). This suggests that *ClpP* suppresses csrA expression, which attenuates *S.* Typhimurium in mice (21). However, whether the attenuation mechanism by the Δ*ClpP* mutant of *S.* Typhimurium can also apply to that of *S.* Enteritidis and be a potential vaccine candidate remains to be explored.

Homologous recombination mediated by λ Red recombinase is frequently used for the generation of deletion mutants in multiple microbial organisms (22,23). This study examined the effect of a Δ*ClpP* mutant on the attenuation of avian *S.* Enteritidis using the λ Red recombination strategy and analyzed the efficacy of the mutant in chickens with the aim of developing a vaccine against avian *S.* Enteritidis.

2. Materials and methods

2.1. Animal supplies and housing

Seven-day-old specific pathogen-free (SPF) chickens were purchased from the Experimental Animal Center of the Harbin Veterinary Research Institute (HVRI, China). All animal experiments were conducted in accordance with the regulations for the administration of affairs concerning experimental animals approved by the State Science and Technology Commission of the People's Republic of China.

2.2. Plasmids and bacterial strains

pKD46, pKD3, and pCP20 plasmids and *S.* Enteritidis serovar Enteritidis-6 (SM-6) were provided by Professor Siguo Liu, State Key Laboratory of Biotechnology, Harbin Veterinary Research Institute (24). *S.* Enteritidis and its mutants were cultured in Luria–Bertani (LB) broth.

2.3. Construction of S. Enteritidis with a *ClpP* deletion mutation

The phage λ Red homologous recombination system was used to generate a *ClpP* deletion mutation of *S.* Enteritidis SM-6 (25). In brief, primers P1 (5′-ttgttatgcttgaattatggcg atgccgtacccattacagagggactggctgataatAGCGATTGTGTA GGCTGGAG-3′), P2 (5′-tcaattacgatgggtcaaaattgagtcaa ccaaaccgtactctaccgcttcaggcgcTTAACGGCTGACATGG GAATTAG-3′), and pKD3 as a PCR template were used to amplify a 1166-bp linear PCR product. The two 57-nt homology arms targeting the *ClpP* gene are indicated by underlined, lowercase letters and sequences to amplify the chloramphenicol-resistance gene of the pKD3 plasmid are indicated by uppercase letters (the chloramphenicol-resistance gene's GenBank accession number: AY048742). The resulting PCR product was purified and residual pKD3 in the PCR product was removed by digestion with *Dpn* I endonuclease (New England Biolabs, USA), followed by purification with a gel extraction kit (Promega, USA). Electrocompetent *S.* Enteritidis SM-6 was prepared and a pKD46 plasmid was transformed into *S.* Enteritidis SM-6 by electroporation under conditions of 200 Ω, 25 µF, and 1800 V (Eporator 4309, Eppendorf, USA). *S.* Enteritidis SM-6 cells were added to 800 µL of LB broth and incubated at 30 °C for 1 h, added to an LB agar plate containing 25 µg/mL of ampicillin, and incubated at 30 °C overnight. Recombinant bacteria were identified by PCR with primers P3 (5′-TAGCGGATCCTACCTGAC-3′) and P4 (5′-ATCAGTTCCTGTGGGTCG-3′) according to the nucleotide sequence of pKD46 available in GenBank (AY048746.1). Recombinant *S.* Enteritidis SM-6 harboring the pKD46 plasmid, SM-6/pKD46, was grown. Then electrocompetent cells were prepared, followed by transformation with approximately 100 ng of purified PCR product. The cells were plated on an LB agar (Oxoid, UK) plate supplemented with 25 µg/mL chloramphenicol and incubated at 30 °C overnight. Recombinants with the chloramphenicol-resistance gene insertion and deletion mutation of the *ClpP* gene, termed SM-6/ΔClpP/pKD46, were identified by PCR using primers P5 (5′-GCTTGAATTATGGCGATGC-3′) and P6 (5′-ACGATGGGTCAAAATTGAGTC-3′) according to the nucleotide sequence of *S. enterica* available in GenBank (CP002487). The positive recombinant, SM-6/Δ*ClpP*/pKD46, was inoculated in LB broth at 37 °C for 16 h to remove the pKD46 plasmid. It was confirmed using PCR with primers P3 and P4. The recombinant cells, SM-6/Δ*ClpP* without the pKD46 plasmid, were isolated and electrocompetent cells were generated. To abrogate the chloramphenicol-resistance gene in recombinant SM-6/Δ*ClpP S.* Enteritidis, the pCP20 plasmid was electrotransformed into SM-6/Δ*ClpP* electrocompetent cells. The cells were dispensed on an LB agar plate containing 50 µg/mL ampicillin and incubated at 30 °C overnight. A single colony was inoculated in LB broth, followed by incubation at 30 °C for 6 h. Then the inoculum was maintained at 42 °C overnight. The abrogation of the chloramphenicol-resistance gene was confirmed by PCR with primers P5 and P6. The resulting PCR amplicon was subcloned into a pMD-19T vector and sequenced to verify construction of the *ClpP* gene deletion mutation.

2.4. Growth experiments

The growth kinetics of the Δ*ClpP* mutant at 37 °C in conventional LB broth or with 5% NaCl or pH 4.5 was determined. The optical density was measured using GeneQuant pro (GE Amersham Biosciences, USA) at 600 nm (OD_{600}) with three replicates and the WT strain SM-6 was set as the control.

2.5. Cell invasion assay

Δ*ClpP* mutant bacteria in the exponential phase were harvested, centrifuged, washed with PBS 3 times, and resuspended with Dulbecco's modified Eagle's medium (DMEM, HyClone). Murine mononuclear macrophage cells, J774.1a (CELLBIO, Shanghai, China), were grown in DMEM supplemented with 10% fetal calf serum (FBS, GIBCO, USA) and seeded into 6-well cell culture plates at a density of 10^7 cells/well. The cells were challenged with the Δ*ClpP* mutant strain at a multiplicity of infection of 10 and the WT SM-6 bacteria were set as the control. After incubation at 37 °C for 2 h, the cells were washed 6 times with PBS and incubated for 3 h with DMEM containing 100 μg/mL gentamicin (Solarbio Life Science, Beijing, China). The cells were lysed with 0.025% Trion X-100 for 2 h and 10-fold dilutions were plated on LB agar plates and incubated at 37 °C overnight. Colonies on an LB agar plate were counted and the experiment was performed with three replicates.

2.6. Colonization in chickens

Comparison of the colonization time between strains SM-6/Δ*ClpP* and SM-6 WT was performed as follows: sixty 7-day-old SPF chickens were randomly divided into 2 groups. Each group of 30 chickens was inoculated intraperitoneally (i.p.) with 5×10^6 CFU of SM-6/Δ*ClpP* or SM-6 bacteria in 100 μL of PBS (pH 7.4). An additional 10 SPF chickens were inoculated with 100 μL of sterile PBS (pH 7.4) as controls. Three of 30 chickens injected with SM-6/Δ*ClpP* or SM-6 WT bacteria were randomly chosen and their livers and spleens were removed under sterile conditions at 3-day intervals. The bacterial colonies on an LB agar plate were counted, and the colony number was compared between the two strains.

2.7. Analysis of virulence of SM-6/ΔClpP

Numbers of SM-6/Δ*ClpP* or WT SM-6 cultures were counted and then diluted from 2.5×10^{11} to 2.5×10^7 CFU/mL. Sixty 7-week-old SPF chickens were randomly divided into 6 groups and each group of 10 chickens was challenged i.p. with 0.5 mL of various doses of SM-6/Δ*ClpP* or SM-6 bacteria. The death rate of chickens was recorded for 5 days following the challenge and median lethal dose (LD_{50}) for SM-6/Δ*ClpP* or SM-6 was calculated according to the modified Karber formula.

2.8. Chicken immunization and protection against homologous challenge

Thirty 7-week-old SPF chickens were i.p. immunized with 5×10^6 CFU of SM-6/Δ*ClpP* or SM-6 twice at 2-week intervals. In addition, 10 chickens were injected with PBS as a control. The growth of chickens in all groups was monitored and recorded following the immunization at 3-day intervals, and blood samples of 500 μL were harvested by bleeding of the wing vein at 7-day intervals. Three chickens immunized with strain SM-6/Δ*ClpP* or SM-6 were randomly sacrificed at 3-day intervals; their whole livers and spleens were weighed and homogenized and 10-fold serial dilutions of homogenates were plated on *Salmonella* chromogenic agar. The colonies were recorded and the bacterial load in the liver or spleen was calculated. Three weeks after immunization, the remaining chickens from the SM-6/Δ*ClpP* or control groups were challenged i.p. with 5×10^9 CFU of SM-6. Chickens that succumbed to infection were autopsied to examine tissue lesions.

2.9. Sera experiments

Titers of antibodies were measured by an indirect enzyme-linked immunosorbent assay (ELISA) as described by Si et al. (24). In brief, a 96-well ELISA plate (Corning, USA) was coated with 50 μL of 20 μg/mL LPS isolated from SM-6 in 0.05 M carbonate buffer solution (CBS, pH 9.6) at 4 °C overnight under optimized parameters, followed by washing 3 times with 0.01 M PBS containing 0.05% Tween (PBST; Beyotime, Beijing, China). The plate was blocked with 5% fat-free milk in PBST at 37 °C for 1 h and washed with PBST 3 times. The plate was incubated at 37 °C for 1 h with 50 μL of 100-fold diluted sera of chickens immunized with SM-6/Δ*ClpP* or WT SM-6. The plate was washed and incubated at 37 °C for 1 h with 50 μL of 10,000-fold diluted HRP-conjugated goat antichicken Ig Y (Sigma, USA). The plate was developed with DAB substrate and read at 450 nm (OD_{450}). The *S.* Enteritidis negative sera from SPF chicken were used as a control. In addition, the cytokines IL-2, IL-6, and IFN-γ in the peripheral blood were measured by a capture ELISA according to the manufacturer's instructions (BioLegend, USA)

2.10. Statistical analysis

Statistical analysis was conducted using the analysis of variance (ANOVA) program of SAS version 8.0 software (SAS Institute, USA) to determine whether the difference between multiple experimental groups was significant. Data are presented as the mean ± standard deviation, and the probability level for significance was $P < 0.05$.

3. Results

3.1. Construction of S. Enteritidis with *ClpP* deletion mutation

The chloramphenicol-resistance gene flanked by both 57-bp homology arms targeting the *ClpP* gene was generated by PCR using primers P1 and P2 and the pKD3 plasmid as a template. A fragment of 1166 bp was amplified, which harbored the FRT sites required for recombination (Figure 1A). The recombinant SM-6 strain harboring the pKD46 plasmid, SM-6/pKD46, was transformed with the purified 1166-bp PCR product and colonies on agar plates were identified by PCR using the P5 and P6 primers. A fragment of approximately 1153 bp was

visualized as the positive recombinant, indicating that the chloramphenicol-resistance gene was integrated into the SM-6/pKD46 bacteria. A fragment of approximately 728 bp was amplified from the parental strain (Figure 1B). To abrogate the chloramphenicol-resistance gene in the SM-6/Δ*ClpP* strain, a temperature-sensitive plasmid, pCP20, was transformed into the recombinants and FLP recombinase encoded by pCP20 was expressed under incubation at 42 °C. FLP recombinase recognized the FRT recombination sites flanking the 5′ and 3′ termini of the chloramphenicol-resistance gene, and the fragment between the two FRT sites was removed. This was confirmed by PCR using primers P5 and P6 (Figure 1C) and gene sequencing (data not shown).

3.2. Growth experiments

To determine the growth kinetics of the SM-6/Δ*ClpP* mutant, compared to WT SM-6 under selected conditions, SM-6/Δ*ClpP* was inoculated in conventional LB broth (pH 7.0) or LB broth with 5% NaCl or LB broth with pH 4.5. SM-6/Δ*ClpP* showed comparatively slow growth and low quantities under all selected inoculation conditions compared with WT SM-6 (Figures 2A–2C). Furthermore, SM-6/Δ*ClpP* in conventional LB broth reached the recession stage at 12 h after incubation, while WT SM-6 bacteria were still in the plateau phase (Figure 2A). In LB broth with 5% NaCl, SM-6/Δ*ClpP* also had a slower growth rate compared with WT bacteria. The OD_{600} of SM-6/Δ*ClpP* 12 h after incubation was five times lower than that of WT SM-6 (Figure 2B). The growth of SM-6, and especially of SM-6/Δ*ClpP*, was reduced by 5% NaCl. Similarly, SM-6/Δ*ClpP* was highly sensitive to low pH compared with SM-6 (Figure 2C). Incubation in LB broth at pH 4.5 slightly affected the growth of SM-6, reaching the plateau phase 2 h later than when cultured in LB broth at pH 7.0. Scanning electron microscopy demonstrated that the size of SM-6/Δ*ClpP* was slightly smaller and both ends were not as smooth and round as those of SM-6 (Figure 3).

3.3. Cell invasion assay

Survival of bacteria in J774.1a macrophage cells was determined to analyze the in vitro virulence of Δ*ClpP* mutant bacteria. When equal amounts of bacteria were used to challenge J774.1a cells, 4.32×10^7 of SM-6 were counted compared with 9×10^5 SM-6/Δ*ClpP* bacteria. The ability of SM-6/Δ*ClpP* bacteria to invade macrophage cells was decreased 48-fold compared with WT SM-6 (Figure 4A).

3.4. Colonization of SM-6/Δ*ClpP* in chickens

Colonization of SM-6/Δ*ClpP* or WT SM-6 bacteria in the liver and spleen from 3 to 12 days after injection is shown in Figures 4B and 4C. The bacterial load in the liver peaked at 6 days and in the spleen at 9 days in the two groups. Bacteria in the spleen of chickens injected with SM-6 peaked after injection. The disappearance of SM-6/Δ*ClpP* in the liver and spleen was confirmed at 12 days after the initial injection; however, SM-6 was still detectable in the liver and spleen 12 days after injection ($P < 0.05$). The growth of chickens in the SM-6/Δ*ClpP* and control groups was not different during the first 4 weeks; however, chickens immunized with SM-6 bacteria showed a slightly slower growth compared with the SM-6/Δ*ClpP* group (data not shown).

3.5. Virulence attenuation of the Δ*ClpP* mutant in chickens

To validate the safety of the mutant as a live attenuated vaccine candidate, the virulence of the Δ*ClpP* mutant was determine by the LD_{50} in 7-day-old SPF chickens. Deletion of the *ClpP* gene induced a 400-fold attenuation (SM-6/

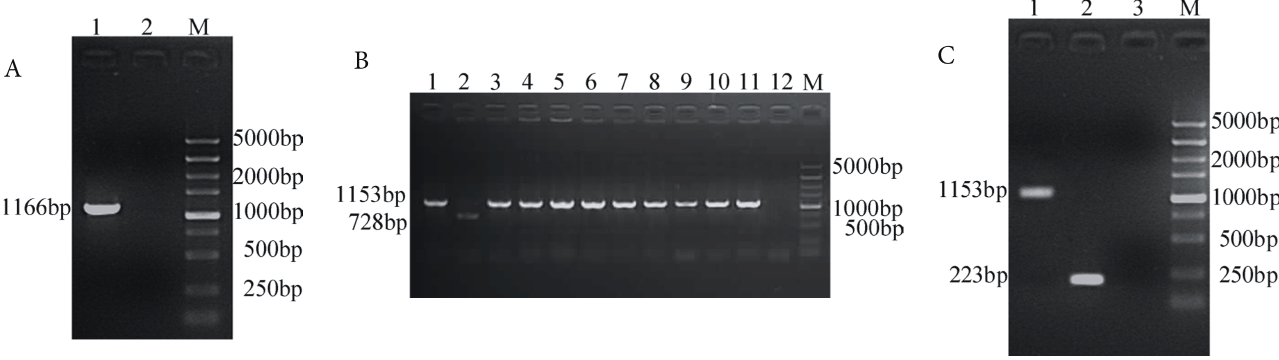

Figure 1. Construction of *S.* Enteritidis with a ClpP deletion mutation. A) PCR amplification of the chloramphenicol-resistance gene with homology arms targeting the ClpP gene. Lane 1: Targeting DNA fragment; Lane 2: negative control; Lane M: DM 5000 DNA molecular marker. B) Agar electrophoresis analysis of PCR products to identify positive recombinant clones containing the insert for chloramphenicol-resistance gene. Lanes 1, 3–11: Putative positive recombinant clones; Lane 2: parental strain; Lane 12: negative control; Lane M: DM 5000 DNA molecular marker. C) Identification of removal of the chloramphenicol-resistance gene by PCR. Lane 1: *S.* Enteritidis strain with the chloramphenicol-resistance gene; Lane 2: *S.* Enteritidis strain without chloramphenicol-resistance gene; Lane 3: negative control; Lane M: DM 5000 DNA molecular marker.

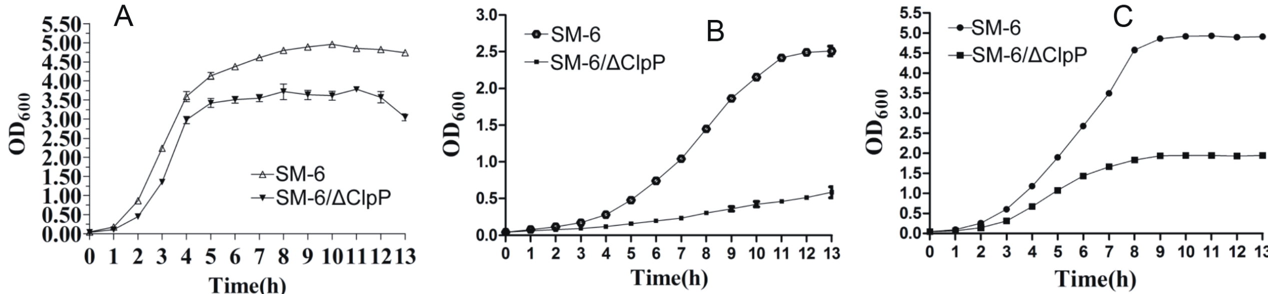

Figure 2. Growth curves of SM-6/Δ*ClpP* and SM-6 under selected conditions. Bacterial strains were cultured with LB broth (A), LB containing 5% NaCl (B), and LB at pH 4.5 (C).

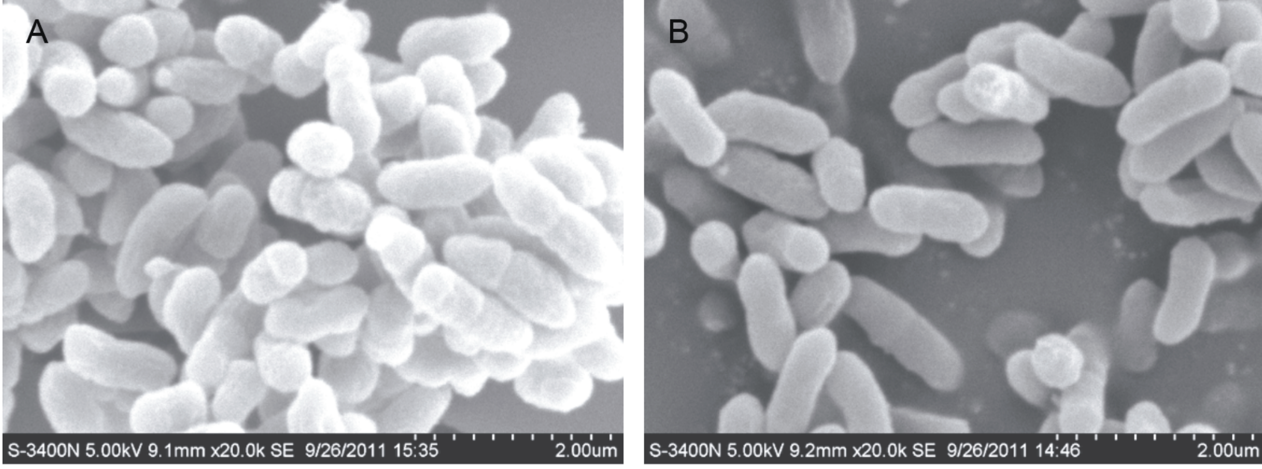

Figure 3. Scanning electron microscopy of SM-6/Δ*ClpP* (A) and SM-6 (B).

Δ*ClpP*, 1.33 × 10^{10}) of the LD_{50} compared with WT SM-6 (3.51 × 10^7 CFU).

3.6. Protection of chickens against homologous challenge

After challenge with WT SM-6, chickens in the control group showed fluffed feathers, dropped wings, drowsiness, refusal to walk, diminished feed consumption, and sticky diarrhea in some of the chickens was observed. In comparison, only a few chickens in the SM-6/Δ*ClpP* vaccinated group showed drowsiness or lassitude. Four days after the homologous challenge, chickens died in both groups. The survival rate in the control group was 10%, while it was 90% in the SM-6/Δ*ClpP* vaccinated group (Figure 4D). In addition, severe perihepatitis in the liver and gross lesions were observed in dead chickens from the control group. In the vaccinated group, 1 of 10 chickens was dead at 3 days after SM-6 challenge, and only mild pathological lesions were observed (data not shown).

3.7. Serological analysis

Anti-SM-6 antibodies were raised in the SM-6/Δ*ClpP* and SM-6 groups, and antibody titers increased over time (Figure 5). Antibodies induced by SM-6/Δ*ClpP* or SM-6 peaked at 3 weeks after the second immunization. The titer of sera induced by SM-6 was slightly higher than that of SM-6/Δ*ClpP*, but this difference was not significant ($P > 0.05$) (Figure 5). In addition, SM-6/Δ*ClpP* induced a similar level of IL-2, IL-6, and IFN-γ cytokines as WT SM-6 (data not shown).

4. Discussion

Either cellular or humoral immune responses provide necessary protection against salmonellosis. Live attenuated vaccines against *Salmonella* induce robust local and systemic immune responses. Thus, deletion of the specific gene associated with the virulence of *Salmonella* may help generate live attenuated vaccines against the diseases due to *Salmonella* infection. To evaluate the effect of a *ClpP* deletion mutation on the virulence of avian *S.* Enteritidis and validate its efficacy in chickens, the *ClpP* gene from the SM-6 strain was deleted by the Red homologous recombination system.

ClpP is involved in proteolysis of abnormal proteins that accumulate under stress and regulates several regulatory proteins, including the stationary phase sigma factor RpoS (26). In this study, we did not investigate the mechanism

Figure 4. In vitro and in vivo virulence analysis of SM-6/Δ*ClpP* in chickens. Cell invasion assay in the macrophage cell line J774 (A); colonization in the liver (B) and spleen (C) of chickens immunized with 5×10^6 CFU of SM-6/Δ*ClpP* or SM-6 12 days after vaccination. Survival curve after challenge with 5×10^9 CFU SM-6 in the vaccinated group and the control group (D). **: $P < 0.05$.

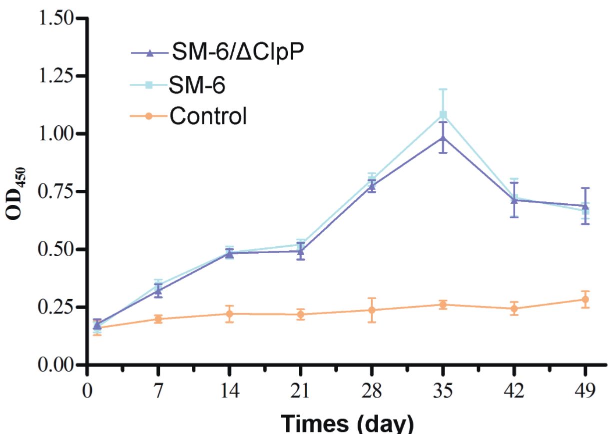

Figure 5. The dynamics of antibodies against LPS in chickens.

by which *ClpP* protease attenuated avian *S.* Enteritidis but focused on whether Δ*ClpP* mutation attenuated virulence in vitro and in vivo, and determined its efficacy compared with the wild-type strain. A previous study demonstrated that *S.* Typhimurium *ClpP* mutation showed significantly attenuated invasion of a human-derived epithelial cell line Int407 (21) and resulted in avirulence in mice (20). However, the Δ*ClpP* mutation strain showed no difference in uptake by the macrophage cell line J774 when compared with the WT strain (21). Of note, we demonstrated that the Δ*ClpP* mutation in avian *S.* Enteritidis showed significantly attenuated invasion of the J774 cell line (Figure 4A).

In vivo, data indicated that the virulence of SM-6/Δ*ClpP* was reduced by 400-fold compared with WT SM-6, suggesting that ClpP is associated with the virulence

of bacteria and that the combined deletion mutation of multiple virulence genes might have synergistic effects on the attenuation of *S*. Enteritidis. Si et al. demonstrated that triple deletions of *lon*, *cpxR*, and *cpdB* from avian *S*. Enteritidis induced a 1000-fold attenuation of virulence (24). The colonization of strain SM-6/Δ*ClpP* and SM-6 in the livers and spleens of 7-day-old chickens challenged with a low dose indicated that WT SM-6 was detected in the livers and spleens at 12 days after the injection, whereas SM-6/Δ*ClpP* was undetectable (Figures 4B and 4C). However, the triple deletion of *lon*, *cpxR*, and *cpdB* from avian *S*. Enteritidis indicated a longer colonization period in the liver and spleen compared with the WT strain (24). Our data, based on cell invasion and animal tests, demonstrated that the deletion mutation of *ClpP* from avian *S*. Enteritidis resulted in attenuation, similar to that reported for *S*. Typhimurium (21).

A previous study demonstrated that ClpP was important for *S*. Typhimurium growth under various stressful conditions and that the *ClpP* mutant was more sensitive to environmental stress (27). The data in our study confirmed that avian *S*. Enteritidis with a *ClpP* mutation was highly sensitive to high concentrations of salt and acidity, resulting in poor growth (Figure 2). Of note, chickens or laying hens vaccinated with such live attenuated *S*. Enteritidis vaccine candidates might shed vulnerable bacteria to the environment, which can be inactivated by conventional disinfectants to protect the environment. Moreover, the short colonization time of SM-6/Δ*ClpP* in challenged chickens suggested that bacterial shedding may cease after a short period of time. Therefore, in addition to the prevention of salmonellosis, the administration of the SM-6/Δ*ClpP* vaccine might reduce contamination of the food chain with *Salmonella* and reduce harm to the environment.

In this study, SM-6/Δ*ClpP* demonstrated an approximate 400-fold attenuation in virulence and meanwhile provided efficient protection against the homologous wild-type avian *S*. Enteritidis, indicating that it is a potential live vaccine candidate. Cross-protection against heterogeneous *S*. Enteritidis in chickens and laying hens should be examined, as well as virulence reversion testing for gene-deleting mutants. Further attenuation studies by the combined deletions of virulence genes to evaluate the potential of this live vaccine candidate are warranted.

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