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Use of a modified bacterial ghost lysis system for the construction of an inactivated avian pathogenic *Escherichia coli* vaccine candidate

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ABSTRACT

Vaccination is an effective strategy to prevent avian colibacillosis. Bacterial ghosts (BGs) are prepared by the controlled expression of the phiX174 gene *E*, which mediates the lysis of Gram-negative bacteria. Staphylococcal nuclease A may be used to produce BGs for further inactivation of host bacteria and elimination of residual genetic material. In this study, the double promoter lysis plasmid (pUC19-ΔcI857-E-rrnB-pL-SN) was successfully constructed and BGs were prepared at 37 °C. The cleavage efficiency of *Escherichia coli* BGs was 99.9%. Furthermore, to evaluate the immunological effects of the BG vaccines in chickens, a BG vaccine was prepared using the serotype O2 avian pathogenic *Escherichia coli* deletion strain (DE17Δ*luxS*Δ*aroA*). The results showed that the BG vaccine was able to achieve over 90% immune protection against virulent challenge using the same serotype O2 strain (DE17 or CE35), while it showed poor cross-protection against serotypes O1 and O78 (data not shown). The enzyme-linked immunosorbent assay results showed that the antibody levels in the immunized groups were higher than in the control group ($p < 0.05$), with the BG group being the highest. The cytokine tests showed that the levels of interferon-γ in the BG immune group were higher than in the phosphate-buffered saline (PBS) control group (non-immune) ($p < 0.01$) and the formalin-inactivated vaccine immune group ($p < 0.05$), and the levels of tumor necrosis factor-α in the BG group were higher than in the formalin-inactivated vaccine ($p > 0.05$) and the PBS control groups ($p < 0.05$). In addition, pathological analysis revealed that the PBS control group showed typical fibrinous pericarditis and perihepatitis, whereas the immune group showed no obvious pathological changes. In summary, our findings provide a new strategy for the prevention and control of avian colibacillosis.

1. Introduction

Avian pathogenic *Escherichia coli* (APEC) continues to threaten the poultry industry. It can cause severe respiratory and systemic diseases, mainly characterized by air sacculitis, perihepatitis and pericarditis, in poultry (Dho-Moulin and Fairbrother, 1999). It causes severe economic losses and restricts the development of the poultry industry. APEC strains constitute a pathotype on account of their virulence-associated traits. For example, some genes are associated with pathogenicity, including *cvaC*, *iroN*, *iss*, *iutA*, *sitA*, *tsh*, *fyuA* and *irp2* (Logue et al., 2012; Rodriguez-Siek et al., 2005). Furthermore, some studies showed that the most commonly occurring serogroups among APEC were O1, O2 and O78 (Ewers et al., 2003; Rodriguez-Siek et al., 2005). Immunoprophylaxis has become the preferred method for the prevention and control of this disease. However, there are no effective drugs or vaccines available for the control of APEC due to drug resistance and low cross-protection against different serotypes (Ghunaim et al., 2014; Rodriguez-Siek et al., 2005). The development of an attenuated mutant strain for use as a live attenuated vaccine is desirable (Salehi et al., 2012), however, the biosafety of such vaccines must be ensured.

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Table 1
Bacterial strains, plasmids and primers used in this study.

Strain/plasmid/primer	Relevant genotype and property	Source or reference
***Escherichia coli* strains**		
DH5α	F'*endA1 supE44 thi-1hsdR17*(rK$^-$ mK$^+$) *recA1gyrA relA1 Δ(lacIZYAargF) U169 deoR* [*φ80dlacΔ(lacZ)M15*]	Invitrogen
staphylococcus aureus		
ATCC 25923		ATCC
Avian pathogenic *Escherichia coli* strains		
DE17	Wild type	Han et al. (2015)
DE17△luxS△aroA	*luxS* and *aroA* double genes deletion APEC mutant	Han et al. (2015)
CE35	Wild type	This study
Plasmids		
pUC19	Amp^R Cloning vector	TaKaRa
pBV220	Containing the pL promoter	Hu et al. (2017)
pUC19-ΔcI857-E-rrnB	Containing the E gene, the λpR promoter is mutated from T to C	Dong et al. (2012)
pUC19-ΔcI857-E-rrnB-pL-SN	Containing the E gene, pL promoter and SN gene, the λpR promoter is mutated from T to C	This study

Bacterial ghosts (BGs) are empty bacterial envelopes of Gram-negative bacteria produced by the controlled expression of the cloned phiX174 gene *E*, which leads to the formation of a transmembrane tunnel structure through the cell envelope and the release of cytoplasmic contents. The original cell morphology and antigenicity of the bacteria are maintained (Lubitz, 2001; Witte et al., 1990). BGs are popular in vaccine development because of the advantages they offer compared with the traditional formalin-inactivated vaccines of bacteria. For example, the inner and outer membrane structures and surface components of BGs remained intact. In addition, BGs possess several of the same properties as their living counterparts and they are able to induce both cellular and humoral responses (Langemann et al., 2010).

The generation of BGs has been reported in a range of Gram-negative bacteria. In addition to the earliest reports in *E. coli*, BGs have since been reported in *Salmonella*, *Helicobacter pylori*, *Haemophilus parasuis*, *Vibrio cholerae*, *Pasteurella multocida*, *Aeromonas hydrophila*, *Actinobacillus pleuropneumoniae*, *Mannheimia haemolytica* and *Flavobacterium columnarii* among others (Kudela et al., 2010; Langemann et al., 2016). Although the use of BG vaccines is widespread, there are few studies on the use of BG vaccines against APEC in poultry. At present, the preparation of BGs is primarily based on the temperature-controlled expression vector of the rightward phage λ pL/pR promoter and the corresponding temperature-sensitive repressor cI857. This is achieved by the strict expression regulation of the phiX174 cleavage gene *E* in Gram-negative host bacteria (Haslberger et al., 2000). However, there are a number of drawbacks in this system. First, the expression of gene *E* is usually severely inhibited at 28 °C. At temperatures greater than 30 °C, the expression of the heat-inactivating gene *E* of the repressor protein cI857 is induced, leading to bacterial lysis (Jechlinger et al., 1999). However, bacteria must be cultured at 28 °C before temperature induction. However, 28 °C is a sub-optimal temperature for the growth of many pathogenic bacteria, and it is also not conducive to the maintenance of important antigenic determinants on some bacterial surfaces (Porta et al., 2008). Second, the rapid increase from 28 °C to 42 °C during the preparation of BGs may induce thermal shock, which can affect the structure of the antigenic determinants and thus affect immunogenicity, as has been demonstrated for bacillary dysentery vaccine (Remaut et al., 1981). Third, the prepared BGs cannot be completely inactivated by the lysis gene *E*, especially in *E. coli*, and bacteria that have not been inactivated at the late stage of lysis induction often begin to proliferate (Haidinger et al., 2003).

Previous studies have shown that by site-directed mutagenesis, the 9th base of the 2nd operator (OR2) of the λpR promoter is mutated from T to C, and the mutated λpR system suppresses the expression of gene *E* at 37 °C, allowing for stable bacterial growth at 37 °C. After induction at 42 °C, bacteriolysis of bacteria can occur and produce BGs (Dong et al., 2012). Furthermore, another lethal protein, staphylococcal nuclease (SN), was designed to combine with protein E to prepare the BGs. The collaboration of protein E and SN not only killed the bacteria more efficiently, but also cleared the residual genetic materials, such as the genome of pathogenic bacteria and the antibiotic resistance genes of the vectors, therefore minimizing the potential risk of gene transfer (Fu and Lu, 2013; Kwon et al., 2009).

To overcome these limitations, the present study constructed a double promoter in the lysis plasmid (pUC19-ΔcI857-E-rmB-pL-SN) to control the expression of the *E* and *SN* genes, while the 9th base of OR2 of the λpR promoter was mutated from T to C. Furthermore, the *luxS-aroA* double gene deletion APEC mutant (strain DE17Δ*luxS*Δ*aroA*, O2 serotype) was used to prepare the DE17 BGs using the lysis plasmid (pUC19-cI857-E-rmB-pL-SN) and the vaccine immunization effect was evaluated. This study aimed to develop a new strategy for the prevention of avian colibacillosis.

2. Materials and methods

2.1. Bacterial strains, plasmid and culture media

APEC strain DE17, isolated from a duck in China, was selected for use in this study because it is amenable to DNA manipulation, and mutant strain DE17Δ*luxS*Δ*aroA* was shown in our previous study to be a safer candidate vaccine strain (Han et al., 2015). In addition, strain DE17 is a serotype 2 (O2) strain belonging to group B2, which is one of the most prevalent APEC serotypes in China (Dou et al., 2016; Han et al., 2018). The bacterial strains and plasmid used in this study are listed in Table 1.

The bacteria were grown routinely in Luria–Bertani (LB) broth, with or without shaking, or on solid medium containing 1.5% agar at 37 °C. When necessary, the LB medium was supplemented with ampicillin at 100 μg/mL or chloramphenicol at 30 μg/mL. All chemicals used were of analytical grade and were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Animals

One hundred and twenty 1-day-old Sanhuang chickens were obtained from Songjiang Chicken Farm (Shanghai, China) and were housed in cages at a controlled temperature range of 28 °C – 30 °C and a 12 h light/dark cycle with free access to food and water during the study period. Care and maintenance of all animals were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences (CAAS). The Ethics Committee of CAAS approved the use of chicks for this study.

Table 2
Primers used in this study.

Primers	Oligonucleotide sequence (5′ to 3′)	Description	Product size
SN-F	5'-CGGGGTACCATGGCAACTTCAACTA-3' (*Kpn* I)	For amplification SN	450 bp
SN-R	5'-CGCGAGCTCTTATTGACCTGAATCAGCG-3'(*Sac* I)		
pL-F	5'-CGCGGATCCCTCTCACCTACCAAACAATGC-3' (*Bam* HI)	For amplification pL promoter	260 bp
pL-R	5'-CGGGGTACCCTCCTTAATTTTTAACCAAT-3' (*Kpn* I)		
cI857-F	5'-CAACACGCGCGGTGTTAGATA	For amplification cI857	869 bp
cI857-R	5'- TATCTAACACCGCGCGTGTTG		
E-F	5'-ATGGTACGCTGGACTTTG-3'	For amplification E	276 bp
E-R	5'-TCACTCCTTCCGCACGT-3'		
rrnB-F	5'-CTGTTTTGGCGGATGAGAG-3'	For amplification rrnB	417 bp
rrnB-R	5'-GTAGAAACGCAAAAAGG-3'		

2.3. Construction of plasmid pUC19-ΔcI857-E-rrnB-pL-SN

The primer pairs pL-F/pL-R containing restriction sites were designed to PCR amplify the pL promoter of the pBV220 plasmid to obtain the pL promoter fragment (Table 2). The pL promoter fragment and pUC19-ΔcI857-E-rrnB were then subjected to double enzyme digestion with *Bam*HI and *Kpn*I. The products were recovered and ligated with Ligation Mix ligase (TaKaRa Bio Inc., Dalian, China) and transformed into *E. coli* DH5α cells. Positive clones were identified and the newly constructed vector was termed pUC19-ΔcI857-E-rrnB-pL.

The primer pairs SN-F/SN-R were designed according to the *Staphylococcus aureus* nuclease A gene sequence (*SN*, GenBank number: DQ507381.1), with *Kpn*I and *Sac*I restriction sites added at the 5′ end to protect the bases. The *SN* gene was amplified from the *S. aureus* ATCC 25923 genome using PCR and digested with *Kpn*I and *Sac*I. Meanwhile, pUC19-ΔcI857-E-rrnB-pL was digested with *Kpn*I and *Sac*I, and the product was recovered, ligated with Ligation Mix ligase, transformed into *E. coli* DH5α cells, and positive clones were identified. Finally, a lysis plasmid termed pUC19-ΔcI857-E-rrnB-pL-SN was successfully constructed.

2.4. Induction, expression and detection of BGs

The plasmids pUC19 and pUC19-ΔcI857-E-rrnB-pL-SN were transformed into DH5α cells and the APEC mutant strain DE17Δ*luxS*Δ*aroA*, respectively. The bacteria were grown routinely in LB (plus ampicillin, 100 μg/mL) at 37 °C for 18 h. Single colonies were picked for identification, and the positive clones were screened. The strains were identified as DH5α (pUC19), DH5α (pUC19-ΔcI857-E-rrnB-pL-SN), DE17Δ*luxS*Δ*aroA* (pUC19) and DE17Δ*luxS*Δ*aroA* (pUC19-ΔcI857-E-rrnB-pL-SN).

These four strains were each inoculated into LB (plus ampicillin, 100 μg/mL) liquid medium and cultured at 37 °C until an optical density of 1 was reached at 600 nm (OD_{600}). Then, a sample was transferred into 100 mL of LB (plus ampicillin, 100 μg/mL) liquid medium and incubated at 37 °C until the OD_{600} value reached ~0.4. The temperature was then rapidly increased to 42 °C for induction. Simultaneously, $CaCl_2$ and $MgCl_2$ were added to the bacterial liquid giving final concentrations of 10 and 1 mM, respectively, to induce maximum SNA activity. Meanwhile, bacteriolytic changes were monitored by measuring the OD_{600} values and viable cell numbers at different lysis time periods (every 30 min between 0–360 min). The bacterial cultures at 0 h and 4 h were diluted with sterile phosphate-buffered saline (PBS) solution. Then, 20 μL of the diluted culture was inoculated onto LB solid medium at 37 °C overnight. The viable count of each fraction (colony-forming units, CFU) was determined, with three replicates at each dilution, and the bacteriolytic efficiency was calculated by the appropriate gradient CFU. The bacteriolysis efficiency of bacteria was calculated according to the following formula: Cleavage efficiency = (1 − CFU after induction/CFU before induction) × 100%.

2.5. Nuclease activity test

Nuclease activity was detected according to the method of Fu et al. (2016). First, methylaniline blue DNA agar was prepared and poured into a sterilization plate; then a hole was introduced using a perforator (5-mm diameter) to remove the agar. The induced bacterial liquid was then centrifuged (1800 × *g*, 10 min) at regular intervals, and the 20-μL supernatant was added to the wells to detect the nuclease activity in the supernatant. Meanwhile, the precipitate was resuspended with the same amount of sterilization LB liquid and boiled for 15 min. The mixture was re-centrifuged and 20 μL of supernatant was added to the other wells to determine the activity of the intracellular nuclease. Finally, the sample plates were cultured overnight at 28 °C A pink circle around the sample hole indicated a positive reaction, whereas no discoloration indicated a negative reaction. Furthermore, the diameter of the pink ring was representative of the activity of the nuclease. If the diameter was zero, this indicated a negative reaction.

At different time points post-induction, each 1 mL of the bacterial culture was sampled and total nucleic acids were isolated to confirm degradation by nuclease activity. Bacterial genomic DNA of *E. coli* was prepared using a Genomic DNA Kit (Tiangen, Beijing, China) following the manufacturer's instructions. Degradation of genomic DNA by nuclease activity was analyzed on a 1% agarose gel.

2.6. Electron microscopic analysis

Cultures of DE17 wild-type and the DE17Δ*luxS*Δ*aroA*-BG mutant strain were collected and washed with PBS (pH = 7.4) three times before electron microscopy. Pre-treated cells were placed in 2.5% glutaraldehyde solution and fixed at 4 °C for 2 h. The concentration was gradually increased from low to pure ethanol, and the dehydrating agent was gradually replaced with an embedding agent. The embedding agent uniformly soaks into all of the interstices of the cell structure and finally polymerizes into a hard solid that can be sectioned by an ultramicrotome. Ultrathin sections were examined on a Tecnai 12 microscope (Philips, Amsterdam, Netherlands). Pre-treated cells were further fixed with 2.5% glutaraldehyde, then post-fixed with 1% aqueous osmium tetroxide and dehydrated with 30%, 50%, 70%, 90%, and 100% ethanol. Subsequently, samples were critical-point dried and coated with gold-palladium alloy. Scanning electron microscope (SEM) imaging was performed using a Nova NanoSEM (FEI, Hillsboro, OR, USA).

2.7. Production of BGs of DE17ΔluxSΔaroA

As described above in Section 2.4, the pUC19-ΔcI857-E-rrnB-pL-SN plasmid was introduced into APEC DE17Δ*luxS*Δ*aroA* competent cells by electroporation using Gene Pulser II transfection apparatus (Bio-Rad, Hercules, CA, USA) for the preparation of BGs of DE17Δ*luxS*Δ*aroA*. Briefly, DE17Δ*luxS*Δ*aroA* containing plasmid pUC19-ΔcI857-E-rrnB-pL-SN was inoculated into 50 mL of LB broth containing ampicillin

(100 μg/mL), and was cultured with shaking at 28 °C. When the OD_{600} value of the culture reached 0.3 to 0.4, the temperature was shifted from 28 °C to 42 °C for 3–4 h to produce DE17Δ*luxS*Δ*aroA*-BG. Then, DE17Δ*luxS*Δ*aroA*-BG was centrifuged (1800 × *g*, 10 min) and washed thrice with sterile PBS (pH 7.2). Finally, the cell pellets were re-suspended in sterile PBS and stored at −80 °C until use. After three rounds of freeze–thawing, a final concentration of 40 μg/mL gentamicin was added. Then, DE17Δ*luxS*Δ*aroA*-BG was inoculated into LB solid medium and incubated at 37 °C for 24 h for a sterility test. BGs that passed the sterility test were adjusted to an appropriate concentration according to the optical density of the bacteria, adjuvant was added (Montanide™ ISA 70 V G, SEPPIC, Paris, France) for emulsification and the BGs were stored at -4 °C until use. Furthermore, the DE17 wild-type strain was cultured and adjuvant was added to prepare traditional heat or formalin-inactivated forms of the strain.

2.8. Immunization and challenge

Sixty 14-day-old Sanhuang chickens were divided randomly into three groups for the experiments (n = 20). The PBS control group (non-immune) was used as a control and animals were inoculated subcutaneously with sterile PBS. The chickens of the BG and formalin-inactivated vaccine groups were vaccinated with DE17Δ*luxS*Δ*aroA*-BG (10^9 CFU/0.3 mL) vaccine and DE17-formalin-inactivated (10^9 CFU/0.3 mL) vaccine, respectively, via the subcutaneous route. Free access to food and water was provided during the study period. At 2-weeks post vaccination, all groups were challenged with 5×10^7 CFU/0.3 mL of a virulent APEC strain (DE17) via the intramuscular (IM) route. Then, the chickens were monitored and mortality of the chickens was recorded daily for 7 days, at the end of which, the surviving chickens in the BG and formalin-inactivated vaccine groups were humanely euthanized using CO_2 in an inhalation chamber according to the approved protocol. Then the challenge isolate (DE17) was recovered from the liver and kidneys of chickens based on a previously published protocol (Han et al., 2015). Briefly, animal tissue samples from the liver and kidney were homogenized and then serially diluted using PBS. The diluted samples were plated onto LB agar and cultured at 37 °C overnight and the number of bacteria was counted in each sample using the plate counting method.

For evaluation of the immune protection offered by BG vaccine to heterologous APEC strain, APEC isolate CE35 (serotype O2) was selected for heterologous challenge. Based on the methods described above, sixty 14-day-old Sanhuang chickens were divided into three groups (n = 20), and inoculated subcutaneously with sterile PBS, DE17Δ*luxS*Δ*aroA*-BG or DE17-formalin-inactivated vaccine. At 2-weeks post vaccination, all groups were challenged with 4×10^7 CFU/0.3 mL of a virulent APEC strain (CE35) via the intramuscular (IM) route. Then chickens were monitored and mortality of chickens was recorded daily for 7 days.

2.9. Histopathology

For histopathological analysis, three chickens were randomly chosen from each group of the DE17 strain challenge model inoculated with DE17Δ*luxS*Δ*aroA*-BG, DE17-formalin-inactivated vaccine or PBS. Then the three chickens were humanely euthanized with CO_2 in an inhalation chamber according to the approved protocol. Heart, liver and spleen tissues were collected a week post-challenge, and then fixed in 15% neutral buffered formaldehyde for 24–48 h, sliced to thicknesses of 3–4 mm with a fully automated Leica RM2255 rotary microtome (Leica Microsystems GmbH, Wetzlar, Germany), and stained with hematoxylin and eosin, as described in our previous report (Liu et al., 2013).

2.10. Antibody assay

Blood samples from the wing veins of each chicken were collected 2-weeks post-immunization. The blood samples were centrifuged and the sera samples were collected. The serum IgG titers were determined by indirect enzyme-linked immunosorbent assay (ELISA). Briefly, the 96-well ELISA plates (Corning Inc., NY, USA) were coated with 10^7 CFU/well of APEC strains (DE17) in PBS overnight at 4 °C. The plates were then washed with PBS containing 0.05% Tween 20 and blocked with PBS containing 5% skim milk for 2 h at 37 °C. Sera were added at an initial dilution of 1:100 in duplicate, with 1:5 serial dilutions performed in PBS containing 5% skim milk. All plates were incubated for 1 h at 37 °C and then washed five times with PBS containing 0.05% Tween 20. A 1:5000 dilution of horseradish peroxidase-labeled goat anti-chicken IgG (Bethyl Laboratories, Montgomery, TX, USA) was added to the plates for 1 h at 37 °C. All plates were washed again, then developed with TMB (Tiangen, Beijing, China) for 15 min, and the reaction was stopped with 2 M H_2SO_4. The optical density of each well was determined at 450 nm on a microplate reader (BioTek, Winooski, VT, USA).

2.11. Cytokine assay

Blood samples from the wing veins of each of the chickens were collected at 2-weeks post-immunization. The blood samples were centrifuged and the sera samples were collected and the concentrations of cytokines (interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) and interleukin 6 (IL-6)) were determined according to the manufacturer's instructions using a chicken cytokine detection system (ANRC, Tianjin, China). The optical density of each well was read and data were analyzed using BioTek software (BioTek).

2.12. Statistical analysis

Statistically significant differences between mean values were identified using the Student's t-test. A probability (p) value of < 0.05 was considered statistically significant.

3. Results

3.1. Construction of plasmid pUC19-ΔcI857-E-rrnB-pL-SN

Using the constructed pUC19-ΔcI857-E-rrnB-pL-SN plasmid as a template, PCR amplification was performed to confirm the identity of the target fragment with different primers, and the results are shown in Fig. 1. Restriction endonuclease analysis was also performed to confirm the identity of the target fragment in the pUC19-ΔcI857-E-rrnB-pL-SN plasmid. The lysis plasmid was digested with restriction endonucleases, and the expected fragments were obtained (as shown in Fig. 1).

3.2. Assay to determine the cleavage efficiency of DH5α and DE17ΔluxSΔaroA

Strains DH5α (pUC19-ΔcI857-E-rrnB-pL-SN) and DE17Δ*luxS*Δ*aroA* (pUC19-ΔcI857-E-rrnB-pL-SN) were grown at 37 °C until the OD_{600} reached 0.4, then the temperature was rapidly increased to 42 °C to start induction. In the first 90 min, the OD_{600} of the DE17Δ*luxS*Δ*aroA* (pUC19-ΔcI857-E-rrnB-pL-SN) strain increased to ~1.4, then decreased to 0.8 and remained relatively constant. After 4 h of induction, the cleavage efficiency was 99.99%. In the first 120 min, the OD_{600} value of the DH5α (pUC19-ΔcI857-E-rrnB-pL-SN) strain increased to ~1.1, and then decreased to 0.8 and remained relatively constant. After 4 h of induction, the lysis efficiency was 99.99%. However, the OD_{600} values of the DH5α (pUC19) and DE17Δ*luxS*Δ*aroA* (pUC19) strains continued to increase, and no significant decrease was observed (Fig. 2 and Table 3).

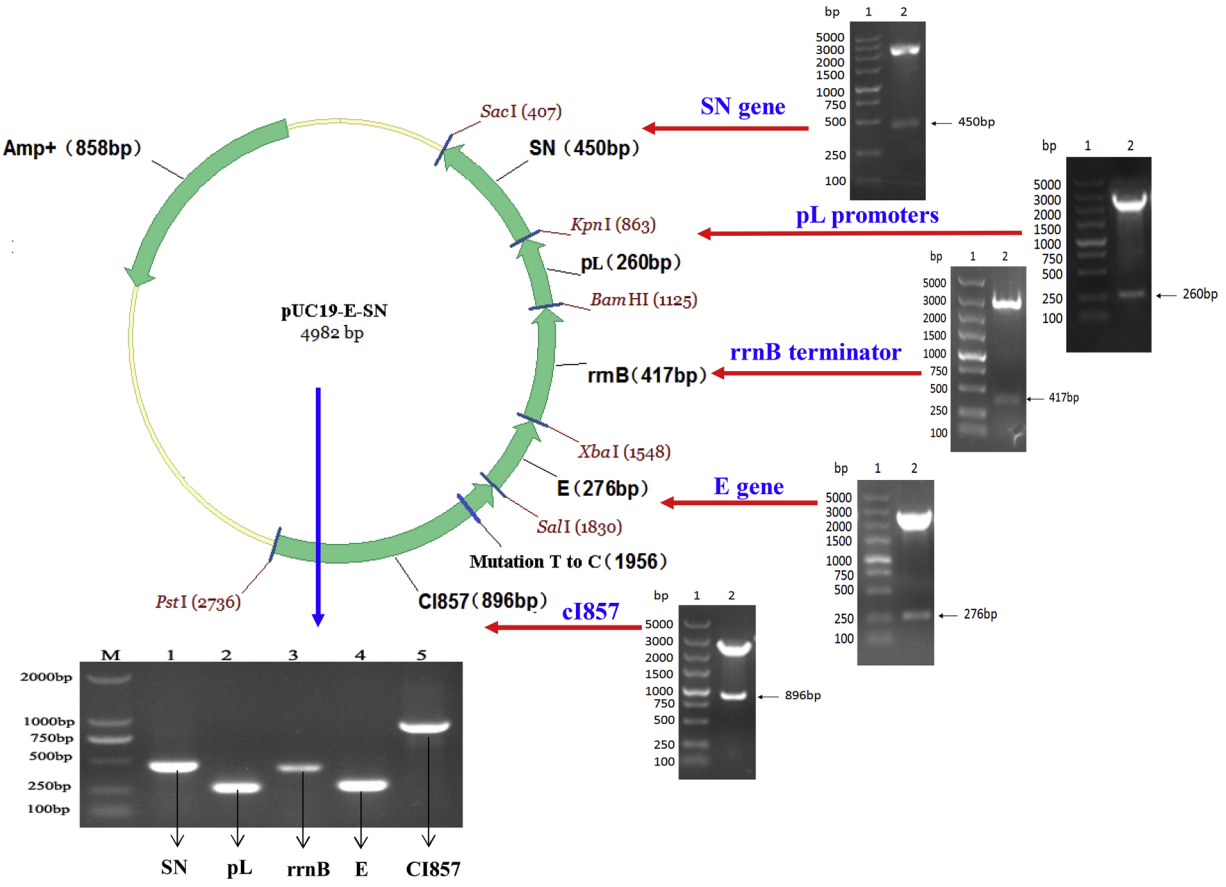

Fig. 1. Schematic representation of the construction of the lysis plasmids, and verification with PCR and restriction endonuclease analysis.
The lysis plasmid was mainly based on the temperature-controlled expression vector of the rightward phage λpL/pR promoter and the corresponding temperature-sensitive repressor cI857. The λpL/pR promoter was disassembled, and the λpR promoter was used to initiate the expression of the *E* gene. The pL promoter initiated the expression of the *SN* gene. The 9th base of the λpR promoter 2nd operator (OR2) was mutated from T to C.
PCR analysis of the pUC19-ΔcI857-E-rrnB-pL-SN plasmid:
Lanes M: DL 2000 DNA marker, Lane 1: The *SN* gene fragment shows a 450-bp PCR product using the primer pair SN-F/SN-R, Lane 2: The pL promoter fragment shows a 260-bp PCR product using the primer pair pL-F/pL-R, Lane 3: The rrnB fragment shows a 417-bp PCR product using the primer pair rrnB-F/rrnB-R, Lane 4: The *E* gene fragment shows a 276-bp PCR product using the primer pair E-F/E-R, Lane 5: The cI857 fragment shows an 896-bp PCR product using the primer pair cI857-F/cI857-R.
The lysis plasmid was digested with the corresponding restriction endonucleases. An 896-bp (cI857) product was digested from the plasmid by *PstI/SalI*. A 276-bp (*E*) product was digested from the plasmid with *SalI/XbaI*. A 417-bp product (rrm B) was digested from the plasmid using *XbaI/Bam*HI. A 260-bp product (pL) was digested from the plasmid with *Bam*HI/*Kpn*I. A 450-bp product (*SN*) was digested from the plasmid using *KpnI/Sac*I.

3.3. Nuclease activity assay

The nucleases of strains DH5α (pUC19-ΔcI857-E-rrnB-pL-SN) and DE17Δ*luxS*Δ*aroA* (pUC19-ΔcI857-E-rrnB-pL-SN) were detected in the culture supernatants after 60 min of induction by warming. The levels of nucleases gradually increased, then became stable. After 6 h of induction, the diameters of the pink circles around strains DH5α (pUC19-ΔcI857-E-rrnB-pL-SN) and DE17Δ*luxS*Δ*aroA* (pUC19-ΔcI857-E-rrnB-pL-SN) were 17 and 18 mm, respectively (Fig. 3A). Intracellular nucleases were detectable after induction for 30 min, and then gradually increased. After 2 h, the pink circles reached 22 and 20 mm, respectively (Fig. 3B). However, the diameters of the pink circles around strains DH5α (pUC19) and DE17Δ*luxS*Δ*aroA* (pUC19) were 0 in all previous tests, indicating that nuclease activity was not detectable.

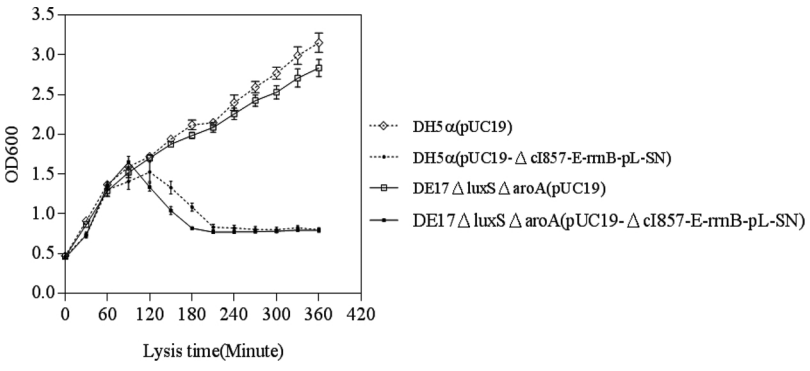

Fig. 2. Growth and lysis curves of bacteria.
The temperature was shifted from 37 °C to 42 °C over 360 min. The OD600 decreased dramatically due to gene *E*-mediated lysis of DH5α(pUC19-ΔcI857-E-rrnB-pL-SN) and DE17Δ*luxS*Δ*aroA*(pUC19-ΔcI857-E-rrnB-pL-SN). The OD600 values of the DH5α (pUC19) and DE17Δ*luxS*Δ*aroA* (pUC19) strains continued to increase.

Table 3
The cleavage efficiency of BGs.

Bacteria (plasmid)	0 h (CFU)	4 h (CFU)	Cleavage efficiency
DH5α (pUC19)	2.8×10^6	1.2×10^8	–
DH5α (pUC19-ΔCI857-E-rrnB-pL-SN)	2.6×10^6	2×10^2	99.99%
DE17△luxS△aroA (pUC19)	3.1×10^6	1.1×10^8	–
DE17△luxS△aroA (pUC19-ΔCI857-E-rrnB-pL-SN)	3.0×10^6	2.3×10^2	99.99%

Notes: "-" indicates no statistics.

3.4. Genomic detection

Fig. 4 shows the results of electrophoresis of the bacterial genomes of DH5α (pUC19-ΔcI857-E-rrnB-pL-SN) and DE17Δ*luxS*Δ*aroA* (pUC19-ΔcI857-E-rrnB-pL- SN) over 0, 1, 2, 3 and 4 h induction. With increased induction time, the genome was gradually degraded after up to 4 h of lysis. The DH5α and DE17 bacterial genomes were almost completely degraded over this timescale.

3.5. Electron microscopy

To investigate the morphology changes of BGs, an electron microscopic analysis of DE17Δ*luxS*Δ*aroA*-BG was performed and compared with that of wild-type strain DE17. By SEM, no changes in the morphology of wild-type DE17 cells were observed (Fig. 5A), with the cell and cytoplasmic membranes remaining intact in unlysed bacterial cells (Fig. 5C). By contrast, lysis holes were detected within the DE17-Δ*luxS*Δ*aroA*-BG envelope (Fig. 5B), either in the middle of the bacterial cell or at the polar sites. TEM analysis of DE17Δ*luxS*Δ*aroA*-BG sections showed that the cytoplasmic membrane and cell wall were partially disrupted, accompanied by the loss of cytoplasmic and nucleoplasmic contents (Fig. 5D). However, there is no obvious difference in the fimbriae and flagella between DE17 wild-type strain (E) and DE17-Δ*luxS*Δ*aroA*-BG (F) by TEM analysis.

3.6. Immune protection experiment

Two weeks after immunization, the chickens in the PBS (n = 20), BG immune (n = 20) and formalin-inactivated vaccine immune (n = 20) groups were challenged with 5×10^7 CFU (about 45 times the median lethal dose, LD_{50}) of DE17. Survival is shown in Fig. 6A. The PBS control group all died within 2 days. No deaths occurred in the BG immune group, i.e., the survival rate was 100%. Deaths in the formalin-inactivated vaccine immune group occurred within the first 4 days, with a final survival rate of 85%. The immunization protection rate was the highest in the BG immune group, followed by the formalin-inactivated vaccine immune group, then the PBS group. The BG vaccine therefore offered 100% protection against challenge with the APEC strain DE17, compared with 85% protection with formalin-inactivated vaccine.

For evaluation of the immune protection offered by BG vaccine to heterologous APEC strains, the APEC isolate CE35 (serotype O2) was selected for heterologous challenge. The survival rates are shown in Fig. 6B. The results showed that the BG vaccine offered 90% protection against challenge with APEC strain CE35, compared with 80% protection with formalin-inactivated vaccine.

The challenge isolate (DE17) was recovered from the liver and kidney of chickens that survived in the BG and formalin-inactivated vaccine immune groups. The results showed that the amount of DE17 in the BG immune group was decreased by 16-fold and 11-fold in the liver and kidney, respectively, compared with the formalin-inactivated vaccine immune group (Fig. 6C).

3.7. Histopathological analysis

One week after challenge, the chickens that died in the challenge group were dissected. The surviving chickens in the challenge group were also dissected following euthanization. The pathological changes observed are shown in Fig. 7. In the PBS control group, typical perihepatitis in the liver, fibrinous pericarditis in the heart, and pneumonia and enlargement of the lungs were observed, along with thickening and turbidity in the abdominal air sac. However, no obvious pathological changes were found in the BG immune group. In the formalin-inactivated vaccine immune group, no obvious pathological changes were found in the heart and liver, but pneumonia was detected and the lungs became black and stiff, in addition, the abdominal air sac contained caseous exudates (Fig. 7).

Histopathological lesions in the heart in the BG immune group and the formalin-inactivated vaccine immune group were compared with those in the PBS control group (Fig. 8). In the PBS control group, local myocardial cell degeneration, connective tissue hyperplasia and large regional myocardial tissue necrosis were observed, whereas the heart cells of the BG immune group and the formalin-inactivated vaccine immune group showed no obvious pathological changes.

In the PBS control group, local lymphocyte infiltration, local hepatocyte degeneration, nuclear condensation, and cytoplasmic acidophilic degeneration were observed in the liver, whereas the liver tissues from the BG immune group and the formalin-inactivated vaccine immune group showed no pathological changes (Fig. 8).

In the PBS control group, the white pulp and red pulp partition of the spleen was blurred, and the red pulp was damaged by the membrane structure. In contrast there were no obvious pathological changes in the spleen cells collected from the BG immune group or the formalin-inactivated vaccine immune group (Fig. 8).

In the PBS group, the epithelial cells of the bronchioles in the lung were degenerated and necrotic, and infiltration of inflammatory cells was evident. In the formalin-inactivated vaccine immune group, the epithelial cells of the bronchioles in the lung were slightly degenerated

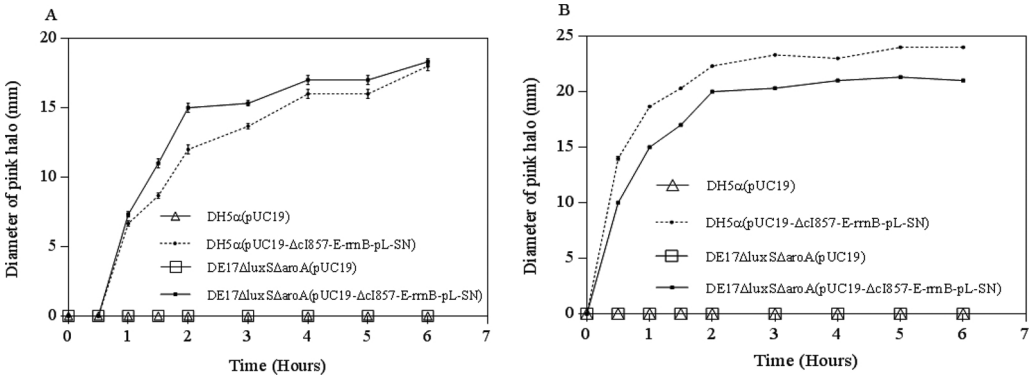

Fig. 3. Nuclease activity of the culture supernatants (A) and intracellular nuclease activity (B) of *E. coli*.
For the bacteria DH5α (pUC19-ΔcI857-E-rrnB-pL-SN) and DE17Δ*luxS*Δ*aroA* (pUC19-ΔcI857-E-rrnB-pL-SN) (containing the lysis plasmid), nuclease activity was detected in the culture supernatants, and the intracellular concentration was higher than that of the supernatant. Whereas, for the bacteria DH5α (pUC19) and DE17Δ*luxS*Δ*aroA* (pUC19) (containing the empty plasmid), no nuclease activity was detected during the whole process.

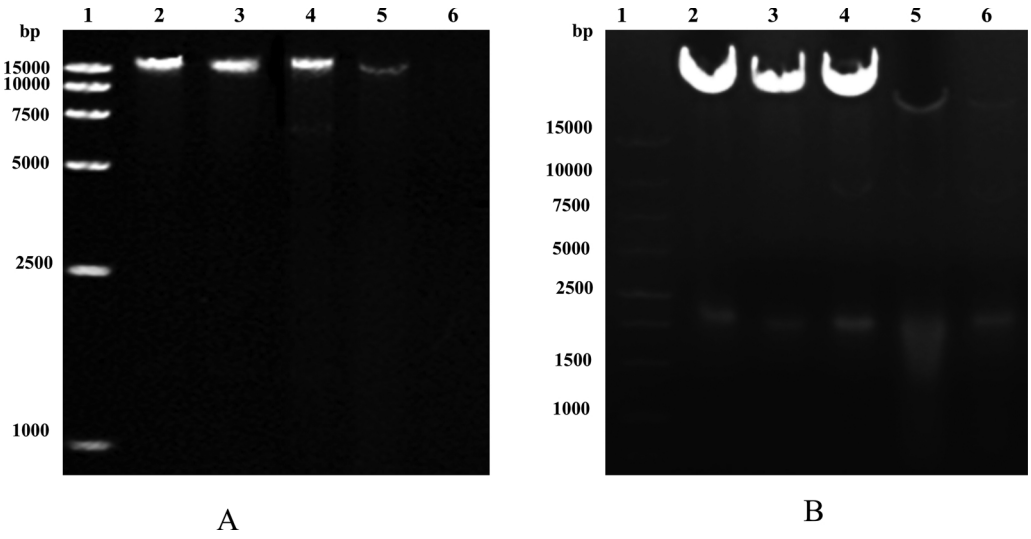

Fig. 4. Electrophoretic analysis of bacterial genomic DNA.
(A) Genomic DNA of DH5α (containing the lysis plasmid). Lane 1: DNA marker DL-15000, Lanes 2–6: DH5α genome at 0, 1, 2, 3, and 4 h, respectively. The DH5α genome was almost completely degraded in 4 h. (B) Genomic DNA of DE17Δ*luxS*Δ*aroA* (containing the lysis plasmid). Lane 1: DNA marker DL-15000, Lanes 2–6: DE17Δ*luxS*Δ*aroA* genome at 0, 1, 2, 3, and 4 h, respectively. The DE17Δ*luxS*Δ*aroA* genome was almost completely degraded in 4 h.

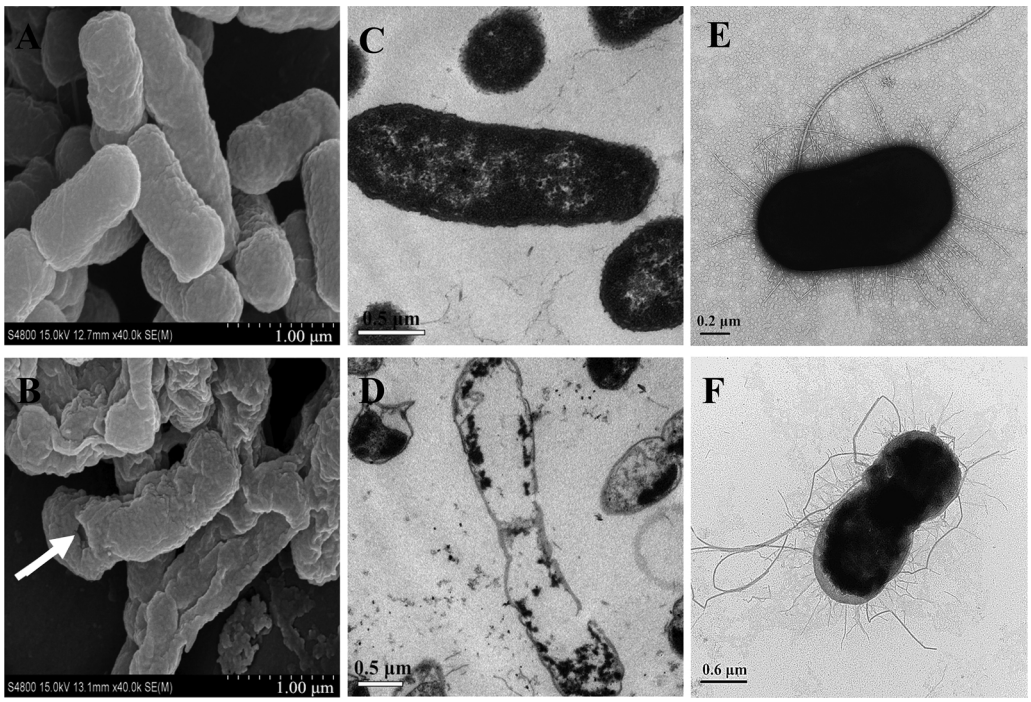

Fig. 5. TEM and SEM of DE17 and DE17Δ*luxS*Δ*aroA* bacterial ghosts.
No morphological changes were observed with SEM (40,000×) for the DE17 wild-type strain (A), whereas lysis hole structures were observed within the DE17Δ*luxS*Δ*aroA*-BG envelope (B, white arrowhead). TEM analysis showed that the cell and cytoplasmic membrane were intact in unlysed bacterial cells (C). The cytoplasmic membrane and cell wall of DE17Δ*luxS*Δ*aroA*-BG was partially disrupted, accompanied by the loss of cytoplasmic and nucleoplasmic contents (D). However, there is no obvious difference in the fimbriae and flagella between DE17 wild-type strain (E) and DE17ΔluxSΔaroA-BG (F) by TEM analysis.

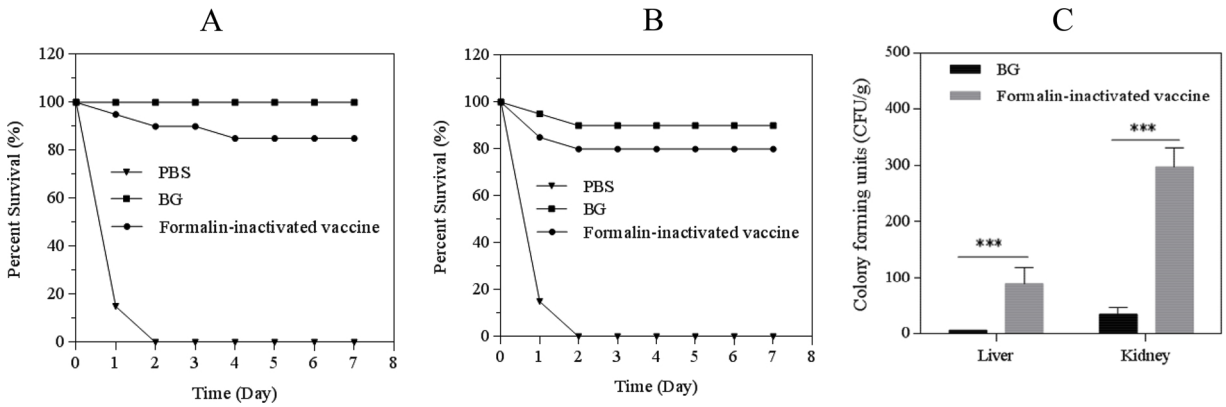

Fig. 6. Survival rate curves and isolated the challenge isolate DE17.
Two weeks after immunization, the chickens were challenged with 5×10^7 CFU of strain DE17 (A) or 4×10^7 CFU of strain CE35 (B). The survival status of the chickens was observed for 7 days. The challenge isolate (DE17) was recovered from the liver and kidney of chickens that survived in the BG and formalin-inactivated vaccine immune groups, respectively (C).

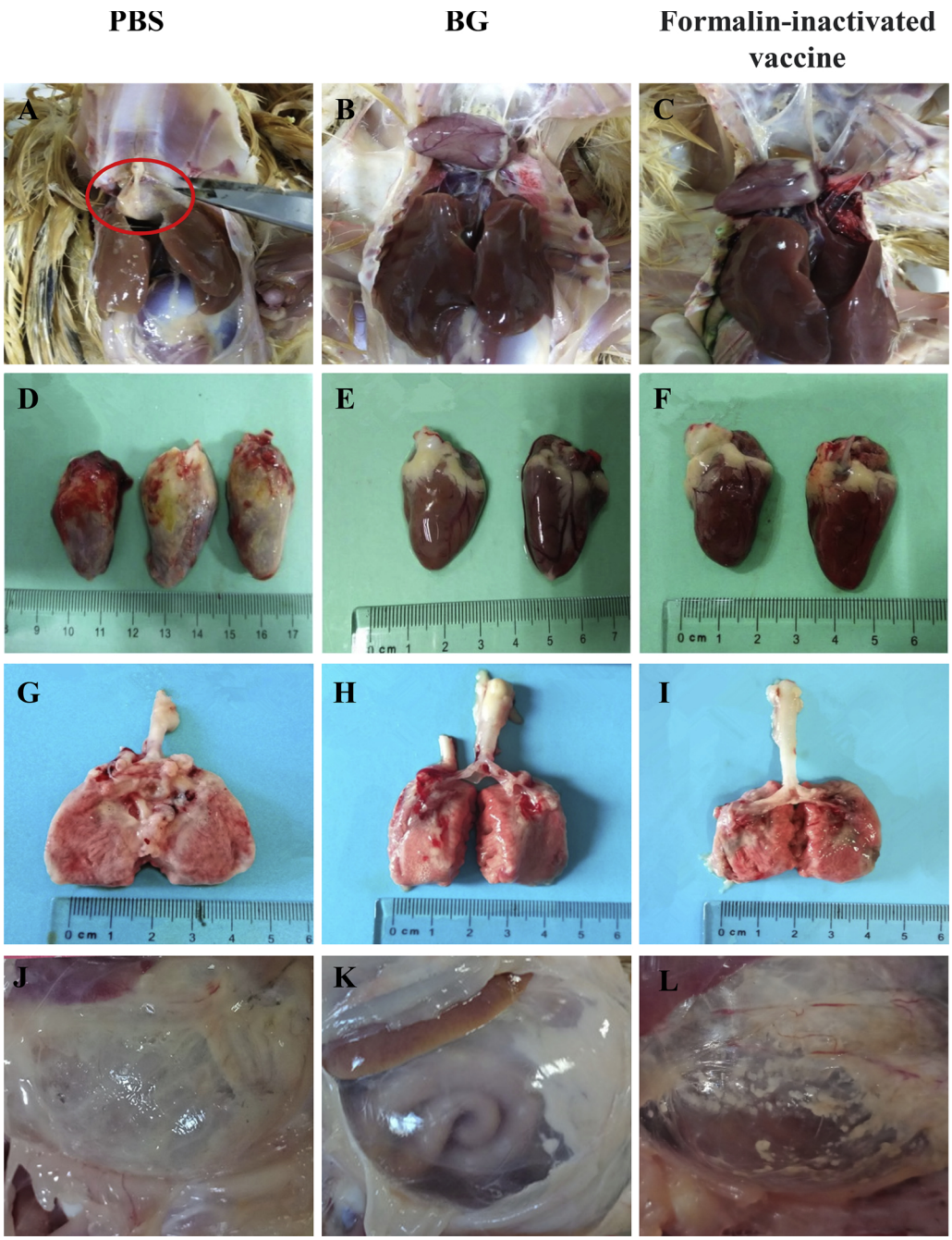

Fig. 7. Analysis of the pathological anatomy of chicks after challenge.
A, D, G and J show pathological changes of the PBS control group, typical perihepatitis in the liver, fibrinous pericarditis in the heart, and pneumonia and enlargement of the lungs were observed, along with thickening and turbidity in the abdominal air sac. However, no obvious pathological changes were found in the BG immune group (B, E, H and K). In the formalin-inactivated vaccine immune group, no obvious pathological changes were found in the heart and liver, but pneumonia was detected and the lungs became black and stiff, in addition, the abdominal air sac contained caseous exudates (C, F, I and L).

and necrotic compared with the PBS group. By contrast, there were no obvious pathological changes in the lung cells from the BG immune group (Fig. 8).

3.8. Antibody assay

The results of antibody detection are shown in Fig. 9. Antibody levels in the BG immune group and the formalin-inactivated vaccine immune group were higher than those in the PBS control group, and this difference was significant ($p < 0.01$). Between the immunized groups, the antibody level of the BG immune group was higher than that of the formalin-inactivated vaccine immune group, but this difference was not significant ($p > 0.05$).

3.9. Cytokine assay

The cytokine detection results are shown in Fig. 10. The IFN-γ levels in the BG immune group were higher than in the PBS group ($p < 0.01$) and the formalin-inactivated vaccine immune group ($p < 0.05$), and the levels in the formalin-inactivated vaccine immune group were higher than in the PBS group ($p < 0.05$). There were no significant differences in IL-6 levels between the three groups ($p > 0.05$). By contrast, TNF-α levels were higher in the BG group than in the formalin-inactivated vaccine immune group and the PBS group ($p < 0.05$), whereas there was no significant difference between the formalin-inactivated vaccine immune group and the PBS group ($p > 0.05$).

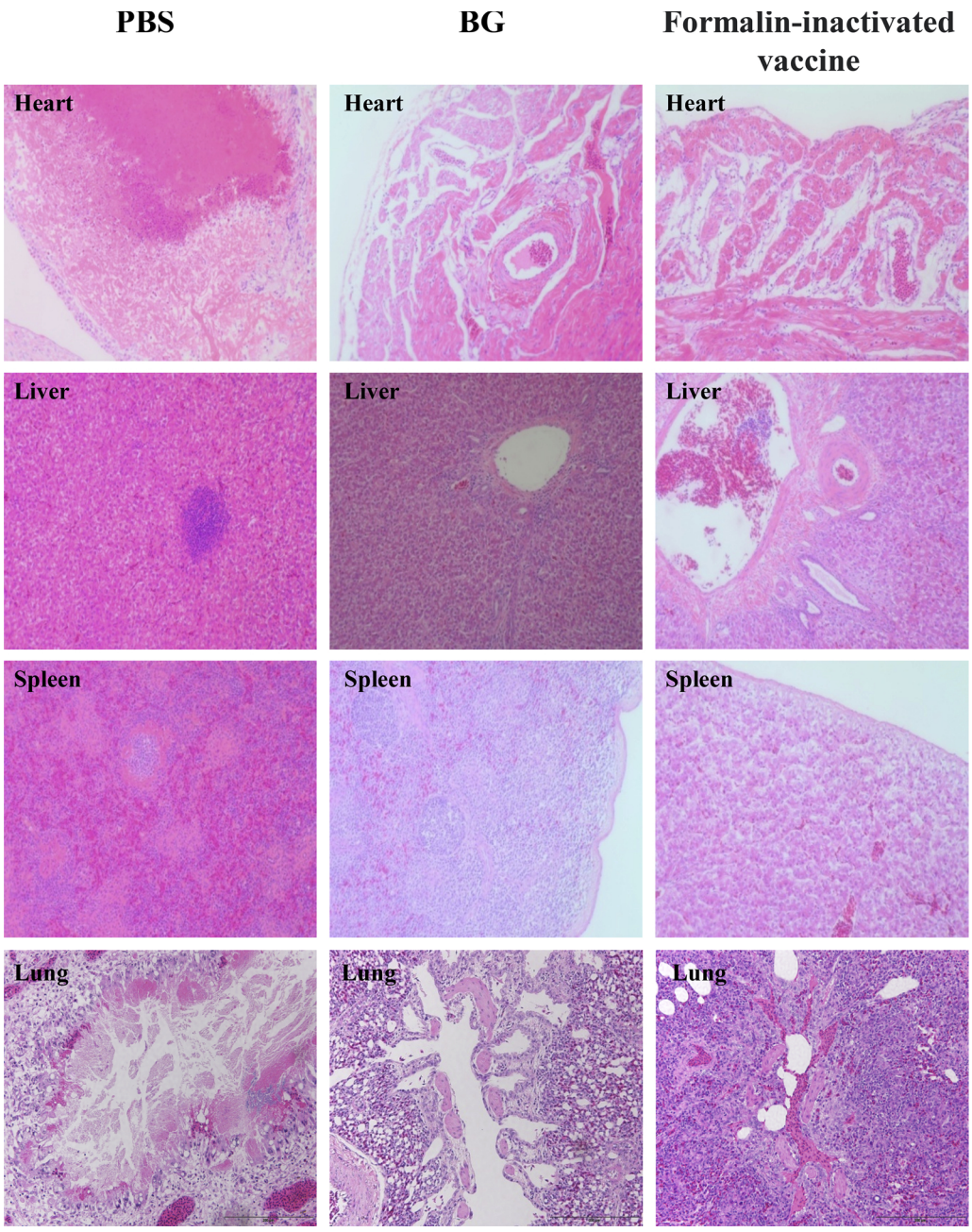

Fig. 8. Histopathological lesions in chickens challenged with strain DE17. In the PBS control group, local myocardial cell degeneration, connective tissue hyperplasia, large regional myocardial tissue necrosis and local lymphocyte infiltration in heart. Local hepatocyte degeneration, nuclear condensation and cytoplasmic acidophilic degeneration were observed in liver. The white pulp and red pulp partition of the spleen was also blurred, and the red pulp was damaged by the membrane structure in spleen. The epithelial cells of the bronchioles in the lung were degenerated and necrotic, and infiltration of inflammatory cells was evident.
There were no obvious pathological changes in the heart, liver and spleen cells collected from the BG immune group and the formalin-inactivated vaccine immune group. In the formalin-inactivated vaccine immune group, the epithelial cells of the bronchioles in the lung were slightly degenerated and necrotic compared with the PBS group, while no obvious pathological changes were observed in cells from the BG immune group.

4. Discussion

BGs are the empty bacterial envelopes of Gram-negative bacteria produced by controlled expression of the cloned gene *E*, forming a lysis tunnel structure within the envelope of the living bacteria. BGs can be used as a new type of bacterial vaccine and they offer advantages compared with traditional vaccines (Hajam et al., 2015; Jawale and Lee, 2016; Liu et al., 2015). In addition, BGs are cost-effective to prepare and store and offer an innovative alternative for vaccine, drug or active substance delivery and other biotechnological applications (Kudela et al., 2010; Lubitz, 2001).

Two unique features of BGs make them attractive for vaccine development. (1) BGs are inactivated vaccines for which proliferation cannot occur. Thus, BGs offer a safer alternative to live vaccines. (2) The fact that inactivation is not required in the preparation of BGs means that they avoid the effects of physical and chemical inactivation, ensuring that some relevant immunogenic determinants on the cell wall remain intact and representative of certain properties of live bacteria. For example, the inner and outer membrane structures, LPS and some pathogen-associated molecular patterns (PAMPs) of BGs remain intact (Mayr et al., 2005). Hence, BG-vaccinated animals exhibited a good immune response compared with inactivated vaccine. Some previous studies showed that the protective effect of the BG vaccine was higher than that of the inactivated vaccine (Mayr et al., 2005; Eko et al., 2011).

The recombinant plasmid pUC19-ΔCI857-E-rrnB-pL-SN was constructed in this study. The 9th base of the λpR promoter OR2 was mutated from T to C by site-directed mutagenesis, and the mutated λpR system continued to stably suppress lysis gene *E* expression at 37 °C. Thus, host bacteria containing gene *E* can achieve stable growth at 37 °C. Induction at 42 °C causes bacteriolysis of the bacteria and enables the preparation of BGs. This ensures that the host bacteria can be cultured at the optimum temperature for growth, and avoids severe thermal shocks from having an impact on the immunogenicity of BG vaccines, which may also be a reason for the superior immune effects of BG vaccines compared with formaldehyde vaccines. Furthermore, a strong promoter pL was inserted after the *E* gene and this promoter was

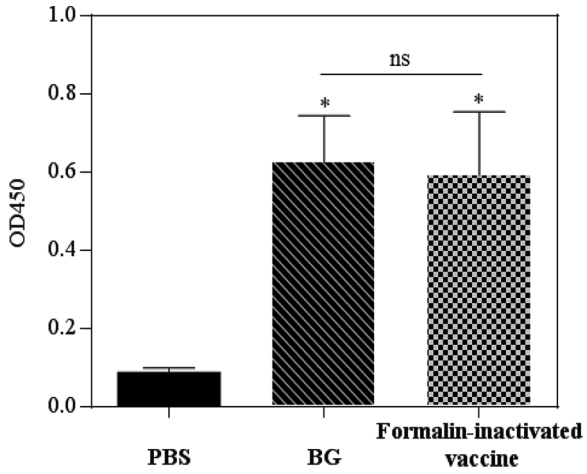

Fig. 9. Detection of the antibody levels in sera with ELISA.
Two weeks after immunization, sera samples were collected from the chickens. Antibody levels in the BG immune group were higher than in the other groups. Antibody levels were expressed as the mean ± S.D. Statistical significance was defined at p values ≤0.05 or 0.01. * $p < 0.01$, vs. unvaccinated control. ns indicates no significant differences between the two immunized groups.

not shared with the *E* gene, with *SN* gene expression being initiated by pL alone. The results showed that the *SN* gene mediated expression of a high concentration of nuclease in the cell, leading to successful degradation of the bacterial genome. The nuclease is likely to be excreted into the extracellular space through the transmembrane tunnel, thereby further degrading the extracellular genome and eliminating potential biosafety issues. This result was consistent with that of a previous study analyzing only SNA gene expression in host bacteria (Fu et al., 2016). In the present study, pUC19 was used as the vector for the lysis plasmid pUC19-ΔCI857-E-rrnB-pL-SN due to its high copy number, its ability to replicate in large numbers and its multiple cloning site that can be used to insert five genes. The use of this vector compensated for the deficiencies previously encountered in the preparation of BGs. The results indicated that the cleavage efficiency of BGs was 99.99% using this lysis plasmid, which was higher than that obtained when expressing the *E* gene alone (Hu et al., 2017), thereby improving the effectiveness of BGs.

With the development of biotechnology, the use of live attenuated vaccines has been shown to be an effective means of preventing APEC. Increasing numbers of APEC-attenuated strains are caused by the deletion of virulence genes, such as *cya*, *luxS*, *ibeA*, and particularly *aroA* (Han et al., 2015; Peighambari et al., 2002). Some bacteria are used as vaccine strains following attenuation of the *aroA* gene. The *aroA* gene encodes the 5-enopyruvylshikimate-3-phosphate synthase (EPSPS) (Xing et al., 2014), and after deletion of the *aroA* gene, the bacteria cannot synthesize aromatic amino acids and some growth and metabolic substances, causing their invasiveness to decline (Salehi et al., 2012). Deletion of the *aroA* gene is therefore used as a strategy for the development of live attenuated vaccine in various bacteria. In the present study, the DE17 *luxS-aroA* double-gene deletion strain (DE17-Δ*luxS*Δ*aroA*) was used as a vaccine strain for the following reasons. 1) Firstly, our previous study investigating the possibility of using mutant DE17Δ*luxS*Δ*aroA* as a live vaccine candidate, some phenotypic features of DE17Δ*luxS*Δ*aroA* were studied (such as its virulence, virulence factor transcription levels, and adhesive and invasive capacity). The results showed that the virulence of DE17Δ*luxS*Δ*aroA* was even weaker than that of DE17Δ*aroA*, making DE17Δ*luxS*Δ*aroA* a safer candidate vaccine strain (Han et al., 2015). 2) Secondly, the immunogenicity or protective efficacy of DE17Δ*luxS*Δ*aroA* was shown to be superior to the single *aroA* mutation for future vaccine development to increase vaccine safety (Han et al., 2015). 3) Finally, there was only one copy of *aroA*, which encodes EPSPS that is located in the cytoplasm according to bioinformatic analysis of the DE17 genome (https://www.uniprot.org/uniprot/P0A6D3#sequences). Hence, deletion of *aroA* could not change the surface structural properties of bacteria. In view of these reasons, the DE17Δ*luxS*Δ*aroA* mutant was selected for the preparation of a BG vaccine for APEC in this study.

Immune protection experiments of the DE17Δ*luxS*Δ*aroA* BG vaccine were performed and this vaccine was found to offer higher immune protection than the DE17 formalin-inactivated vaccine, which could reduce the mortality of animals infected with APEC DE17 strain. Furthermore, the present study also showed that the IFN-γ and TNF-α levels in the BG immune group were higher than those in the formalin-inactivated vaccine immune group ($p < 0.05$). A possible reason is that BG vaccines can cause higher humoral and cell-mediated immune responses compared with formalin-inactivated vaccine. BG vaccines retain more immunogenic determinants (such as surface PAMPs, fimbriae, LPS and peptidoglycans) on the cell wall, which can mimic certain properties of live bacteria and stimulate the production of higher levels of antibody and cytokines (such as IFN-γ and TNF-α). As marker cytokines of Th1 immune responses, high levels of IFN-γ and TNF-α can activate monocytes and macrophages, enhancing their killing activity and thus improving the immune protection offered by BG vaccine (Balenovic et al., 2011; Brenner et al., 2015; Jawale et al., 2012). These findings were similar to previous reports that intravenously administered *V. cholerae* BGs to induce Th1-directed immune responses in C57BL/6 mice (Eko et al., 2011; Ekong et al., 2009). Recombinant *V. cholerae* ghosts carrying *Chlamydia trachomatis* antigens were shown to induce a *Chlamydia*-specific Th1 response by measuring the IFN-γ production by splenic T cells (Eko et al., 2003). Although BG vaccines can induce stronger immune responses compared with formalin-inactivated vaccines, they often show poor cross-protection. The

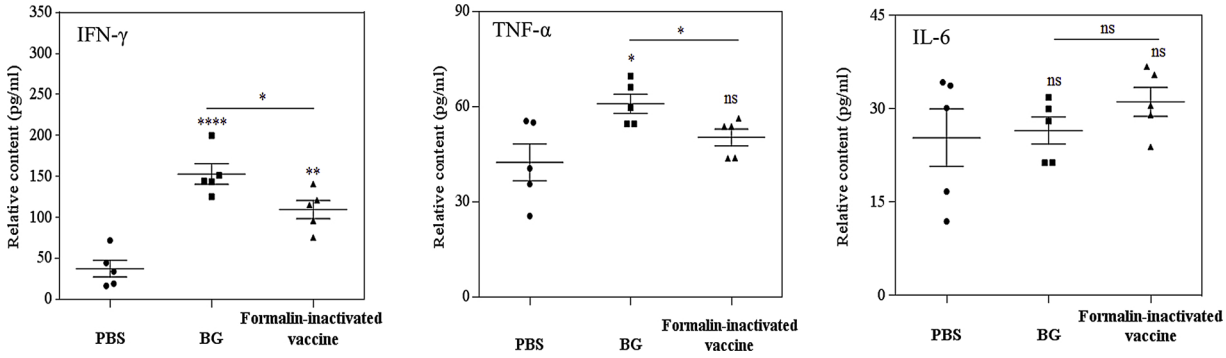

Fig. 10. Detection of cytokines in the sera with ELISA.
Two weeks after immunization, sera samples were collected from chickens to detect: IFN-γ, TNF-α and IL-6 concentrations with ELISA. Data were represented as the mean ± standard errors of the mean (SEM). Statistical significance was defined at p values≤0.05 or 0.01. * $p < 0.05$, ** $p < 0.01$ vs. unvaccinated control. ns indicates no significant differences between the two groups.

most commonly occurring serogroups of APEC are O1, O2 and O78, however, the cross-protection between these serogroups was found to be low (Ghunaim et al., 2014). In this study, although serotype O2 APEC BG vaccine was able to achieve over 90% immune protection against virulent challenge using the same serotype O2 strain (DE17 or CE35), it showed poor cross-protection against serotypes O_1 and O_{78} (data not shown). Strategies to improve the cross-protection of BG vaccine will be addressed in future studies.

In the present study, chickens were immunized with the vaccine via subcutaneous injection, which was consistent with some previous studies, for example, *Salmonella typhimurium* ghost vaccine in rats or chickens and *Salmonella gallinarum* ghost vaccine in chickens (Jawale and Lee, 2016; Vinod et al., 2017). For convenience of application, inhalation or oral administration may be preferred for large-scale poultry enterprises. However, it is noteworthy that a previous study with *Salmonella pullorum* ghost vaccine in rats showed that IM injection is more efficient than oral vaccination (Guo et al., 2016). Hence, the optimal immunization route for BG vaccine will be the subject of further study. In the current study, we attempted to challenge vaccinated chickens with DE17 via the air sac. However, the mortality rate of the PBS group of chickens (unimmunized group) was only 30% when using DE17 at a dose of 10^{10} CFU, which could not therefore be used to evaluate the immune protection of BG vaccine (data not shown). However, when the IM route was chosen for the challenge, the mortality rate of the PBS group was 100% at a dose of 5×10^7 CFU. Hence, the challenge model with strain DE17 via the IM route in chickens was selected in our laboratory for evaluation of the immune protection of APEC vaccine. Some previous studies have also used the IM route to evaluate the immune protection of APEC vaccine. In future studies, we will evaluate the immune protection of APEC BG vaccine by air sac challenge.

In conclusion, this study provides a modified BG lysis system for the construction of an inactivated APEC vaccine. It is a more effective vaccine that may replace traditional inactivated vaccine and provide a safer alternative to live vaccines. Furthermore, BGs can be used to design multivalent vaccines against avian colibacillosis.

Acknowledgments

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