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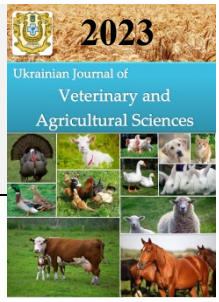

SteE enhances the colonization of *Salmonella* Pullorum in chickens

Zhike Liu[1,2], Anatoliy Fotin[1], Roman Petrov[1], Jinyou Ma[2], Tetiana Fotina[1]

[1]*Faculty of Veterinary Medicine, Sumy National Agrarian University, Herasima Kondratieva Str., 160, Sumy, 40021, Ukraine*
[2]*College of Animal Science and Veterinary Medicine, Henan Institute of Science and Technology, Hualan Str., 90, Xinxiang, 453003, China*

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Correspondence author
Tetiana Fotina
Tel.: +38-099-238-62-24
E-mail: tif_ua@meta.ua

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Abstract

Salmonella pullorum (*S. pullorum*) is the causative agent of pullorum disease and results in severe economic losses in poultry, and can long-term survival by colonizing host organs. *steE* is an effector protein secreted by *Salmonella* pathogenicity island 2. It is not clear in vivo for the colonization of *Salmonella*. To investigate the role of *steE* on the colonization of *S.* Pullorum in the principal organs of chicken, we used *S. pullorum* and *S. pullorum* Δ*steE* strains immunized chickens, respectively. The results of the virulence assay showed that the LD_{50} of *S. pullorum* Δ*steE* was 22.8 times higher than that of *S. pullorum* in chickens. The colonization experiment of bacteria showed that the overall change trend of the number of *S. pullorum* and *S. pullorum* Δ*steE* strains were similar in chicken liver, spleen, heart, bursa, and cecum, which increased first and then decreased. However, the deletion of *steE* caused significantly reduced colonization, pathological change, and virulence of *S. pullorum* in a chicken infection model. Our findings provide exciting insights into the pathogenic mechanism and live attenuated vaccine associated with *steE* in *S. pullorum*.

Keywords: *Salmonella pullorum*; *steE*; virulence; chicken; colonization.

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1. Introduction

Salmonella enterica serovar Pullorum (*S. pullorum*) is an intracellular pathogen of host specificity (Li et al., 2018; Chechet et al., 2022). It mainly infects chickens within three weeks of age, resulting in acute systemic diseases and high mortality in poultry. The growth and production performance of young and adult chickens infected with *S. pullorum* will be badly affected (Li et al., 2019; Foster et al., 2021). *S. pullorum* can be transmitted vertically, and the hatching rate of contaminated breeding eggs will significantly reduce. Sick chickens and infected chickens are the primary sources of *S. pullorum*. The cocks carrying *S. pullorum* can also transmit to the hens through vertical transmission. One of the essential measures to control the occurrence and prevalence of *S. pullorum* is the purification of *S. pullorum* in breeding chickens and the disinfection of breeding eggs. At present, *S. pullorum* is still occurring and prevalent in many countries, especially in developing countries, including Brazil and India, which has caused substantial economic losses to the poultry industry (Geng et al., 2019; Xian et al., 2020). Therefore, further study of the pathogenic mechanism of *S. pullorum* is necessary.

The survival and proliferation of *Salmonella* are related to a particular cell membrane region formed in the host cell, namely the *Salmonella*-containing vacuole (SCV). The formation of SCV is inseparable from type III secretion system 2 (T3SS2) encoded by *Salmonella* pathogenic island 2 (SPI-2). Therefore, T3SS2 plays an essential role in the pathogenesis of *S. pullorum* (Figueira et al., 2013; Knuff-Janzen et al., 2021; Cohen et al., 2021). T3SS2 is an injection device in that the Salmonella-secreted effector is translocated into the host cells through the device to provide a favorable environment for *Salmonella* invasion (Greene et al., 2021; Fang & Méresse, 2021). As a novel effector protein of *Salmonella* T3SS2, *steE* is encoded in *Salmonella* prophage gifsy-1,

which helps to survive and replicate in macrophages and plays a vital role in the evolution of *Salmonella* and the regulation of host innate immune response (Coombes et al., 2005).

In the present study, little work is reported about the *steE* of *S. pullorum*. Continuing to explore the relationship between *steE* and the virulence of *S. pullorum* contributes to revealing the pathogenic mechanism of *S. pullorum*. Therefore, chickens were infected with *S. pullorum* and *S. pullorum* Δ*steE* strains as an animal models to analyze their potential role in the virulence and colonization of *S. pullorum*.

2. Materials and methods

2.1 Strains and Animals

S. pullorum and *S. pullorum* Δ*steE* strains were preserved and constructed in our laboratory. Our laboratory hatched healthy 2-day-old Jinghong laying hens.

2.2 Recovery and Counting of Bacteria

Take out the frozen *S. pullorum* or *S. pullorum* Δ*steE* strain glycerol bacteria at -80 ℃, rejuvenate it in xylose lysine deoxycholate (XLD, Hopebio Bio-Technology, Qingdao, China) agar plate at 37 °C for 18–24 h. The next day, a single colony of *S. pullorum* was added to 1 ml of Luria-Bertani (LB) broth at 37 °C and 180 rpm for 16–18 h. Overnight cultures of the *S. pullorum* and *S. pullorum* Δ*steE* strains with 10-fold serial dilutions (1×10^{-6}, 1×10^{-7}, and 1×10^{-8}) were enumerated by plating on XLD agar. All dilution was repeated three times.

2.3 Analysis of Clinical Symptoms and Autopsy

The clinical symptoms and morbidity of the chicks were observed every day after *S. pullorum* or *S. pullorum* Δ*steE* strain infection and recorded and photographed.

2.4 *S.* Pullorum Virulence Assay

S. pullorum and *S. pullorum* Δ*steE* strains were inoculated respectively in LB broth for 12 h. The bacteria cultures were washed three times with PBS and suspended to adjust the bacterial concentration. One hundred commercial 2-day-old chickens were randomly divided into ten groups. Each group was infected orally with 10-fold serial dilutions of *S. pullorum* or *S. pullorum* Δ*steE* strain (1×10^6, 1×10^7, 1×10^8, 1×10^9, 1×10^{10} CFU or 1×10^7, 1×10^8, 1×10^9, 1×10^{10}, 1×10^{11} CFU). Ten chickens received 100 μL of PBS as a control group. Deaths were recorded until 14 days, and the half-lethal dose (LD_{50}) of each strain was calculated to evaluate the virulence of the strain to chickens using Karber's method.

2.5 Bacterial Colonization in Organs

S. pullorum and *S. pullorum* Δ*steE* strains were cultured in LB broth for 12 h. The bacteria cultures were washed three times with PBS and suspended to adjust the bacterial concentration. Sixty chickens were randomly divided into three groups (n = 20). Chicken from each group was infected orally with 1×10^8 CFU of *S. pullorum* or *S. pullorum* Δ*steE* strain in 100 μl of PBS, according to the LD_{50} assay as mentioned previously. Twenty chickens received 100 μL of PBS as a control group. Chickens were deprived of food and water for 12 hours before and after chicken immunization. At 12 h, 24 h, 36 h, 2 d, 3 d, 4 d, and 7 d post-challenge, the cecum, liver, spleen, bursa, and heart organs were harvested from each chicken. After weighing, organs from each group were homogenized mechanically, and diluted serially for the subsequent cultivation on XLD agar plates at 37 °C for 12−16 h. The bacterial number was counted and displayed as log10 CFU/g. The dynamic distribution of *S. pullorum* and *S. pullorum* Δ*steE* strains in various organs was analyzed by plating on XLD agar. The bacterial number was counted and expressed as log10 CFU/g at 12 h, 24 h, 36 h, 2 d, 3 d, 4 d, and 7 d.

3. Results and discussion

3.1 Results

3.1.1 *S. pullorum* Δ*steE* reduces Virulence in Chicken

The virulence of *S. pullorum* and *S. pullorum* Δ*steE* strains were analyzed in chicken. As shown in Table 1, the LD_{50} of *S. pullorum* was 9.14×10^7 CFU, but the LD_{50} of *S. pullorum* Δ*steE* was 2.08×10^9 CFU. The LD_{50} of *S. pullorum* Δ*steE* was 22.8 times higher than that of *S. pullorum*. The result showed that *steE* could decrease the virulence of *S. pullorum*.

Table 1
LD_{50} of *S. pullorum* and *S. pullorum* Δ*steE* strains

Strain	Challenge doses	Dead counts/Chicken counts	LD_{50}/CFU
S. Pullorum	1×10^{10}	10/10	9.14×10^7
	1×10^9	10/10	
	1×10^8	6/10	
	1×10^7	2/10	
	1×10^6	0/10	
S. Pullorum Δ*steE*	1×10^{11}	10/10	2.08×10^9
	1×10^{10}	9/10	
	1×10^9	3/10	
	1×10^8	0/10	
	1×10^7	0/10	

3.1.2 Clinical Symptoms and Changes of Autopsy

One day after the chickens were infected with *S. pullorum* or *S. pullorum* Δ*steE* strain, the chicks were depressed and had a poor appetite, accompanied by the phenomenon of gathering together. Some chicks could not stand steadily, discharged white sticky feces, and the feathers around the anus were covered with feces. As shown in Fig. 1, the pathological change of chicken organs infected with *S. pullorum* was more severe than that of the *S. pullorum* Δ*steE* and control groups as follows: there were dark red needle tips and large bleeding spots at the edge of the liver; splenomegaly, dark red; bursal enlargement; the cecum has puffed and bleeding; heart congestion, swelling with blood filaments.

3.1.3 *steE* Enhances the Colonization of *S.* Pullorum in Chicken Cecum

The results of bacterial colonization showed that the bacterial number of *S. pullorum* and *S. pullorum* Δ*steE* strains in the cecum were increased from 12 h to 3 d but decreased from 3 d to 7 d (Fig. 2). At three days post-challenge, the colonization of *S.* Pullorum and *S. pullorum* Δ*steE* stains reached the peak in the chicken cecum, and the settlement amount of *S. pullorum* and *S. pullorum* Δ*steE* strains reached the lowest at 7 d. The changing trend of *S.* Pullorum and *S. pullorum* Δ*steE* strains was increased at first and then decreased in the whole process of the infection. Still, the settled quantity of *S. pullorum* Δ*steE* was consistently lower than that of *S. pullorum*.

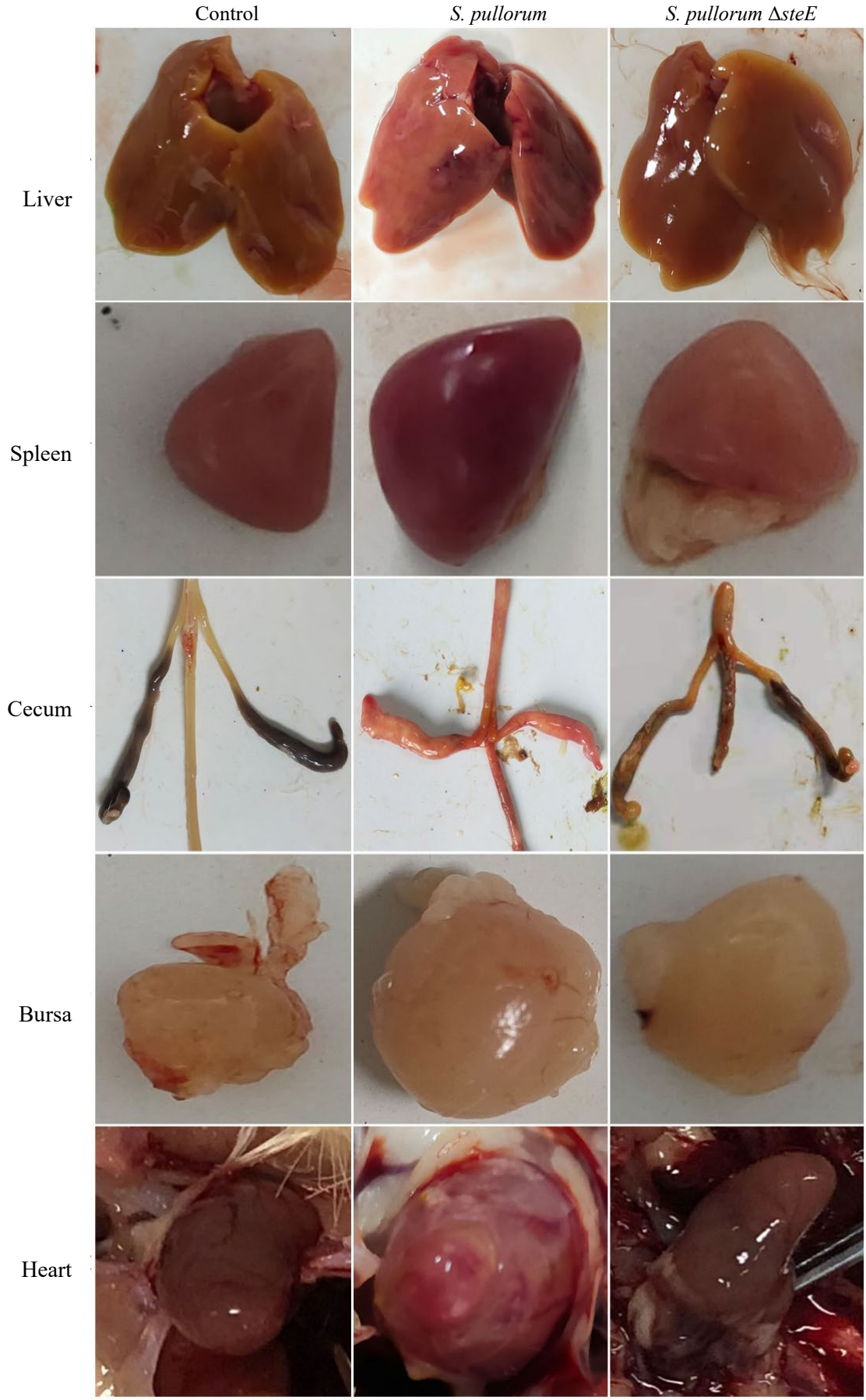

Fig. 1. The macroscopic image of different organs of chickens

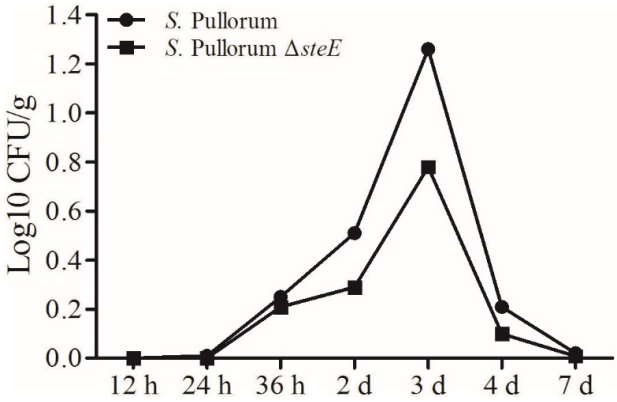

Fig. 2. The change of bacterial colonization in the chicken cecum

3.1.4 *steE* Enhances the colonization of *S. pullorum* in chicken liver

The results of bacterial colonization showed that the bacterial number of *S.* Pullorum was increased from 12 h to 4 d in the whole process of the infection and decreased significantly from 4 d to 7 d (Fig. 3). The total amount of *S. pullorum* Δ*steE* was increased from 12 h to 3 d after inoculation, and decreased from 4 d to 7 d. The colonization of *S.* Pullorum and *S. pullorum* Δ*steE* strains reached the peak r about four days after chicken inoculation. The changing trend of *S. pullorum* and *S. pullorum* Δ*steE* strains increased first and then decreased throughout the experiment. However, the settled quantity of *S. pullorum* Δ*steE* was consistently lower than that of *S. pullorum*.

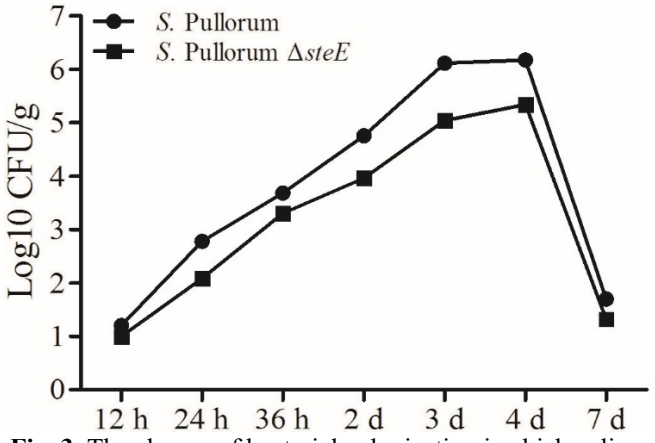

Fig. 3. The change of bacterial colonization in chicken liver

3.1.5 *steE* Enhances the Colonization of *S. pullorum* in Chicken Spleen

The colonization of *S. pullorum* and *S. pullorum* Δ*steE* strains increased from 12 h to 3d after chicken inoculation, and decreased significantly from 3 d to 7 d in the chicken spleen (Fig. 4). The colonization of *S. pullorum* and *S. pullorum* Δ*steE* strains reached the peak about 3 d in the chicken spleen after chicken inoculation. The changing trend of *S. pullorum* and *S. Pullorum* Δ*steE* strains increased initially and then decreased throughout the infection. Still, the settled quantity of *S. pullorum* Δ*steE* was consistently lower than that of *S. pullorum*.

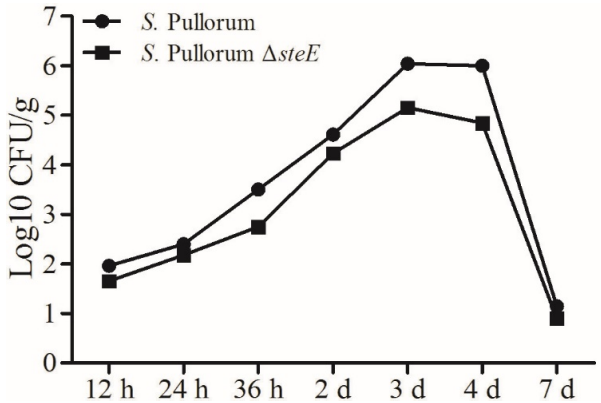

Fig. 4. The change of bacterial colonization in the chicken spleen

3.1.6 *steE* Enhances the Colonization of *S. pullorum* in Chicken Brusa

The colonization of *S.* Pullorum was increased from 12 h to 3 d in chicken bursa, increased slowly at 36 h, and decreased from 3 d to 7 d. The results showed that *S. pullorum* reached the peak of about 3 d in the bursa after chicken inoculation (Fig. 5). The changing trend of *S. pullorum* Δ*steE* in chicken bursa is consistent with that of *S. pullorum*. At the same time, the settled number of *S. pullorum* Δ*steE* was consistently lower than that of *S. pullorum*.

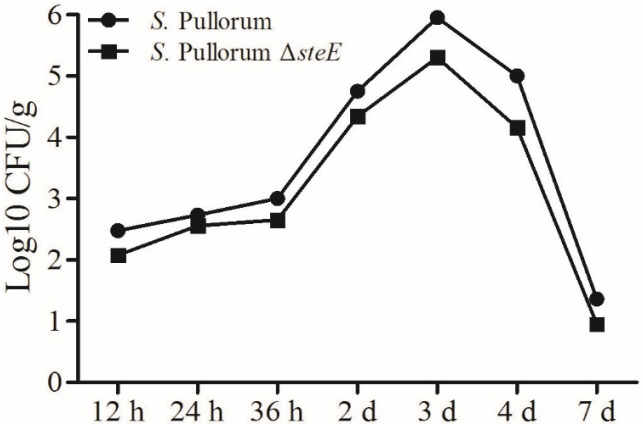

Fig. 5. The change of bacterial colonization in the chicken bursa

3.1.7 *steE* Enhances the Colonization of *S. pullorum* in Chicken Heart

The colonization of *S.* Pullorum and *S. pullorum* Δ*steE* strains was increased from 12 h to 4 d in the heart after chicken inoculation and decreased from 4 d to 7 d. As shown in Fig. 6, the bacterial number of *S. pullorum* and *S. pullorum* Δ*steE* stains peaked about four days in the heart after chicken inoculation. The settlement amount of *S. pullorum* and *S. pullorum* Δ*steE* strains reached the lowest at 7 d. The changing trend of *S. pullorum* and *S. pullorum* Δ*steE* strains was increased at first and then decreased in the whole process of the infection, but the settled quantity of *S. pullorum* Δ*steE* was consistently lower than that of *S. pullorum* in chicken heart.

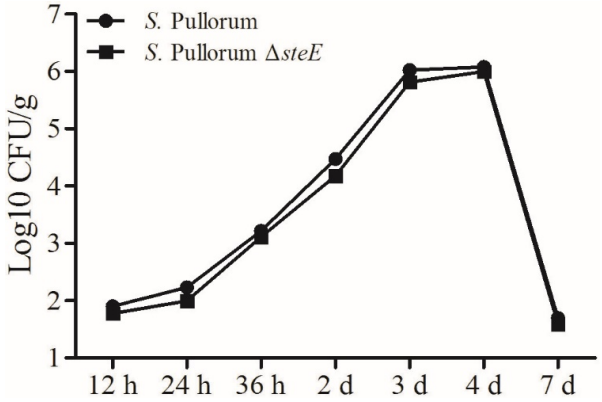

Fig. 6. The change of bacterial colonization in the chicken heart

3.2 Discussion

S. pullorum has brought substantial economic losses to the poultry industry. The pathogenic mechanism of *S.* Pullorum needs to be further studied. After the host cell is infected with *Salmonella*, *Salmonella* can form a *Salmonella*-containing vacuole (SCV) in the host cell and survive in it for immune escape (Walch et al., 2021). The maintenance of SCV function is inseparable from the participation of a series of *Salmonella* virulence factors, which play a significant role in the T3SS2 encoded by SPI-2 and its secreted effector protein (Röder et al., 2021; Morrison et al., 2022). SteE is an effector protein deeply involved in regulating the secretion of *Salmonella* pathogenic island two, and its role is significant for *Salmonella* virulence (Gibbs et al., 2020).

This study evaluated the virulence and dynamic distribution of *S. pullorum* and *S. pullorum* Δ*steE* strains in chicken organs after chicken immunization. The results of the virulence assay showed that the deletion of *steE* caused a decrease in the pathogenicity of *S. pullorum* in chickens, which proved the critical role of *steE* in the virulence of *S.* Pullorum (Fig. 1). At the same time, the colonization of bacteria in chicken organs showed that the overall change trend of *S.* Pullorum was similar to that of *S. pullorum* Δ*steE*. The colonization of *S.* Pullorum and *S. pullorum* Δ*steE* strains in the chicken cecum, spleen, and bursa peaked at 3d, while the number of bacteria in the liver peaked at 4 d. From the whole infection process, the number of *S. pullorum* Δ*steE* in various organs at the initial infection stage after chicken infection first increased, peaked at the middle infection stage, and decreased at the later infection stage. At the same time, the number of *S. pullorum* Δ*steE* in chicken organs was always significantly lower than that of *S. pullorum*. In addition, the colonization number of *S. pullorum* Δ*steE* in chicken organs was consistently lower than that of *S. pullorum*. These results show that the *steE* can promote the colonization of *S. pullorum* in chicken organs and contribute to the virulence of *S. pullorum*.

Recent studies demonstrated that the deletion of SPI-2 reduced the virulence of *Salmonella*, which is related to the fact that SPI-2 is a DNA fragment obtained by the superficial level in the process of *Salmonella* evolution. Some reported that the deletion of *steE* significantly attenuated colonization of mouse spleens and caused decreased virulence of *S. typhimurium* (Niemann et al., 2011). *SteE* had been considered an essential factor in host organs for persistent Salmonella infection (Pham et al., 2020). *steE* is secreted by *Salmonella* (Ruan et al., 2017; Stapels et al., 2018; Panagi et al., 2020), which reported activating the signal transducer and activator of transcription three signaling pathways and then produces anti-inflammatory cytokine IL-10, thereby promoting *Salmonella* replication in cells and increasing bacterial colonization in vivo (Jaslow et al., 2018). In this study, many factors affect the accuracy of the final results.

On the one hand, the interval between organ collections is relatively long, and the accurate time of bacteria invading various organs needs to be better determined. On the other hand, we can only determine when bacteria settle in organs from chickens to reach the peak by reducing the interval between collecting organs. In addition, other factors affect the number of bacteria, such as the cleanliness of equipment, reagents, Petri dishes, and so on in the test, and the errors caused by the operation in the test process.

4. Conclusions

In this study, the deletion of *steE* caused significantly decreased colonization and pathological change of *S. pullorum* in a chicken infection model, and its virulence was also considerably reduced. Altogether, our work shows that *steE* is closely related to the pathogenicity of *S. pullorum*, which provides exciting insights into the roles of *steE* in the pathogenic mechanism and the development of the live attenuated vaccine of *S. pullorum*.

Conflict of interest

The authors declare that there is no conflict of interest.

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