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CONSTRUCTION AND CHARACTERIZATION OF STEE DELETION MUTANT OF SALMONELLA PULLORUM

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Salmonella Pullorum (S. Pullorum) is one of the host-restricted serotypes causing systemic infection in poultry. After S. Pullorum infection, chicks and turkeys usually have acute systemic infection. The main clinical symptoms are white dysentery and dyspnea, and the mortality can be as high as 100%. In adult chickens, local and chronic infections are the most common without obvious clinical symptoms, and can be transmitted vertically to offspring through ovary. Although the use of antibiotics reduces the death of sick chickens, it can not completely eliminate the pathogenic microorganisms in hosts, and is prone to public health problems such as drug resistance and drug residues. No study has ever reported the role of steE in HD-11 cells infected by S. Pullorum. The growth and biochemical characteristics of S. Pullorum ΔsteE were similar to that of S. Pullorum. Furthermore, we also observed the effects of steE on cell proliferation and apoptosis in S. Pullorum-infected HD-11 cells.In order to define the pathogenicity of steE gene of S. Pullorum, the steE deletion strain of S. Pullorum and its complemented strain were successfully constructed, and then its characterization were analyzed. S. Pullorum was preserved by the microbiology laboratory of the college of animal science and veterinary medicine, Henan Institute of Science and Technology. The pKD4, pKD46 and pCP20 or pBR322 plasmids were used for the λ-Red recombination system or complementary strain. The biological characteristics of S. Pullorum ΔsteE were consistent with those of its parent strain S. Pullorum and complementary strain S. Pullorum ΔsteE (pBR322-steE). Construction and confirmation of the ΔsteE strain. To identify the roles of steE in S. Pullorum, the steE deletion mutant of S. Pullorum was correctly constructed.The virulence test showed S. Pullorum ΔsteE decreased the proliferation and apoptosis of HD-11 cells compared to that of S. Pullorum and S. Pullorum ΔsteE (pBR322-steE). Taken together, our data demonstrate that the deletion of steE in S. Pullorum had no effect the growth and biochemical characteristics, but its proliferation ability decreased significantly in HD-11 cells, which decreased cell apoptosis, indicating that steE was closely related to virulence of S. Pullorum. Altogether, our research suggest that the steE gene was required for S. Pullorum virulence, which laid a foundation for further related research in S. Pullorum vaccine strains.

Key words: *Salmonella Pullorum, steE, biological characteristics, HD-11 cells, apoptosis, virulence.*

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Introduction. Chicken pullorum disease is an important bacterial infectious disease caused by Salmonella enterica serovar Pullorum (S. Pullorum) (Ding et al., 2021). It is an important cause of disease commonly existing in modern intensive chicken farms. After S. Pullorum infection, chicks and turkeys usually have acute systemic infection. The main

clinical symptoms are white dysentery and dyspnea, and the mortality can be as high as 100%. In adult chickens, local and chronic infections are the most common without obvious clinical symptoms, and can be transmitted vertically to offspring through ovary (Fei et al., 2020; Matos et al). Therefore, the prevalence of S. Pullorum has brought serious economic losses to the poultry industry.

At present, there are few reports on the pathogenic mechanism of S. Pullorum. The current effective measures to prevent and control S. Pullorum are biosafety control and purification measures, mainly to eliminate the diseased chickens, continuously monitor the healthy chickens and cut off the route of transmission (Ter et al., 2022; Islam et al., 2020). However, the full implementation of these measures is difficult and costly in many developing countries (Vaid et al., 2021). Although the use of antibiotics reduces the death of sick chickens, it can not completely eliminate the pathogenic microorganisms in hosts, and is prone to public health problems such as drug resistance and drug residues. Therefore, it is very necessary to explore prevention and control measures including new vaccines. As a new virulence phenotype, steE is encoded by Gifsy-1 through the regulation of type III secretion system 1 and 2 (T3SS1 and T3SS2) (Brodsky et al., 2020). steE plays an important role in the evolution of Salmonella host specificity. In a mouse infection model, steE increased the virulence of Salmonella and the expression of anti-inflammatory cytokines (Johnson et al., 2018). However, the role of steE in Salmonella pathogenesis needs to be further studied.

So far, most studies on steE have mainly relied on S. Typhimurium infection models, whereas little work has been performed in chicken infection models (Panagi et al., 2020). No study has ever reported the role of steE in HD-11 cells infected by S. Pullorum. In this study, we successfully constructed the steE deletion strain of S. Pullorum by λ-Red recombination system. The growth and biochemical characteristics of S. Pullorum ΔsteE were similar to that of S. Pullorum. Furthermore, we also observed the effects of steE on cell proliferation and apoptosis in S. Pullorum-infected HD-11 cells.

Materials and methods. *Strains and pasmids.* S. Pullorum was preserved by the microbiology laboratory of the college of animal science and veterinary medicine, Henan Institute of Science and Technology. The pKD4, pKD46 and pCP20 or pBR322 plasmids were used for the λ-Red recombination system or complementary strain. S. Pullorum was cultured in Luria-Bertani(LB)broth. The LB broth was supplemented with ampicillin (Amp; 100 μg/mL) or kanamycin (Kan; 50 μg/mL) as required. The pBBR1MCS2-Tac-mCherry plasmid carrying the mCherry gene was transformed into the wild type (WT) or S. Pullorum ΔsteE (ΔsteE) strain to provide the red fluorescence in S. Pullorum, respectively.

Cells culture and primers. HD-11 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Solarbio, Beijing, China) supplemented with 10% fetal bovine serum, 100 μg/mL penicillin, and 100 μg/mL streptomycin. The sequence of the kanamycin resistance cassette (KanR) was amplified from pKD4 plasmid, including 46-bp homology extensions at the 5′ and 3′ ends of the steE gene. According to the published sequence of S. Pullorum (GenBank: LK931482.1), the primers were designed to amplify the gene from the S. Pullorum using PCR method (Table 1). The above primers were synthesized in the study by Sangon Biotech Co., Ltd (Shanghai, China).

Small letter: KanR cassette amplification; *A 1089/683/2169 bp fragment was obtained by PCR method from WT, ΔsteE and ΔsteE::kan strains, respectively; 1Underlined nucleotides denote the XhoI restriction site; 2Underlined nucleotides denote the BamH I restriction site.

Generation of the steE-deficient S. Pullorum and its complementation. The steE deletion mutant of S. Pullorum was constructed by λ-Red recombination system as previously described (Ho et al., 2021). Briefly, the kanamycin resistance cassette (KanR) was amplified from pKD4 plasmid using the primers P1/P2. The PCR products were purified and transferred into S. Pullorum containing pKD46 plasmid by electroporation. The steE gene was replaced to construct the S. Pullorum ΔsteE::kan strain, and then the ΔsteE strain was obtained through FLP recombinase expressed by pCP20 plasmid. The ΔsteE strain was comfirmed by PCR method using the primers CX1/CX2 or N1/N2. The steE gene fragment was amplified by PCR method using primers steE-F/steE-R, and then cloned into pBR322 plasmid to construct the pBR322-steE plasmid. The pBR322-steE recombinant plasmid was then transformed into the ΔsteE strain to construct the S. Pullorum ΔsteE (pBR322-steE). The ΔsteE (pBR322-steE) strain was confirmed by PCR method using primers CX1/CX2 or N1/N2.

Identification of growth curve and biochemical characteristics of the ΔsteE strain. The WT, ΔsteE and ΔsteE (pBR322-steE) strains were inoculated into LB broth at 37 °C with shaking at 180 r/min for 15 h and subcultured 1:100 into LB broth as previously described (Yin et al., 2016). At a starting time point (0 h), the optical density was measured to achieve an approximate concentration (OD600 = 0.01). The

Table 1

The primers used in this study

Primer	Sequences (5′-3′) F /R	Size (bp)
P1	CGGGTGGCGATTTTAACGCCAGTGCGACGTTAGTCGTGGATTACCAgtgtaggctggagctgcttc	1567
P2	AACATTACGCCTCCGATCAAATGCCCGGCAGTTTGAAAAATACGGTcatatgaatatcctccttag	
CX1/ CX2	ATTCAGGGAACCACCACCAT/ACGCCAATCGCAAAACCACT	1089/683/2169*
N1/N2	ACGGTGAAATGCTGGAGGTC/CGTGCCGTTCTGTTGAAGTT	224
steE	C<u>CTCGAG</u>[1]ATGATGGAGAGATTCATAGTG/CG<u>GGATCC</u>[2]AGACCATTGGTAATCCACCTGTAACG	507

OD600 nm value of the bacterial cultures was measured at 2 h interval for 14 h by the Biodrop spectrophotometer (BioDrop, Cambridge, England). Biochemical characteristics of the WT, ΔsteE and ΔsteE (pBR322-steE) strains were performed, following the manufacturer's protocol, including glucose, lysine decarboxylase, sucrose, mannose, mannitol, sorbitol, lactose, maltose, malonate, ornithine decarboxylase, urease, arabinose and hydrogen sulfide.

Analysis of HD-11 cells apoptosis. HD-11 cells were seeded on 6-well plates at a density of 1 × 105 cells/well and cultured for 18-20 h and reached 80%-90% confluence as previously described (Wang et al., 2016). Briefly, the overnight cultured of mCherry-WT and mCherry-ΔsteE strains in LB broth with kanamycin (50 mg/mL) were washed with PBS for 3 times to adjust the concentration of bacteria. HD-11 cells were infected with the indicated S. Pullorum at a multiplicity of infection (MOI) of 10:1. The cells were incubated for 3 h at 37 °C and then were fixed in 4% paraformaldehyde, and were stained with 4',6-diamidino-2-phenylindole (DAPI) staining. Staining was assessed by laser scanning confocal microscopy.

HD-11 cells infection assay. HD-11 cells were seeded on 6-well plates at a density of 1 × 105 cells/well and cultured for 18-20 h and reached 80%-90% confluence as previously described (Xian et al., 2020). Briefly, the cells were infected with WT, ΔsteE and ΔsteE (pBR322-steE) strains at a MOI of 10:1, the plates were then incubated at 37 °C for 1 h. To kill extracellular Salmonella, the cells were washed three times with PBS and incubated for 1 h with 100 μg/mL gentamicin of fresh medium, and then incubated with the 10 μg/mL gentamicin of fresh medium for the indicated durations. The bacteria number was counted at 6, 9, 12 and 24 h post infection. Intracellular growth was expressed as the fold-change in the bacterial number at different time points relative to the bacteria number at 2 h post infection.

Statistical analysis. All data were expressed as mean ± standard error of the mean (SEM) unless otherwise specified. All statistical analysis was performed using GraphPad Prism 8 software. The significance of the difference was determined between two samples using one-way analysis of variance as *p < 0.05 and **p < 0.01.

Results. Construction and confirmation of the ΔsteE strain. To identify the roles of steE in S. Pullorum, the steE deletion mutant of S. Pullorum was correctly constructed. A schematic diagram depicting the deletion strategy for ΔsteE strain generation using λ-Red recombination technology is shown in Fig. 1.The upstream and downstream homologous arms of the steE gene and Kan gene with fragment size of 1567 bp were obtained from pKD46 plasmid by PCR method using primers P1/P2 (Fig. 2A). The ΔsteE::kan strain has a length of 2169 bp by PCR method using primers CX1/CX2 (Fig. 2B). The ΔsteE strain was verified by PCR using primers CX1/CX2 or N1/N2 to generate a 1089 bp or 224 bp fragment (Fig. 2C). These results indicated that the ΔsteE strain was successfully constructed.

Confirmation of the ΔsteE (pBR322-steE) strain

The ΔsteE (pBR322-steE) strain was verified by PCR method using primers CX1/CX2 or N1/N2. As shown in Fig. 3, the WT and ΔsteE strains has a length of 1089 bp

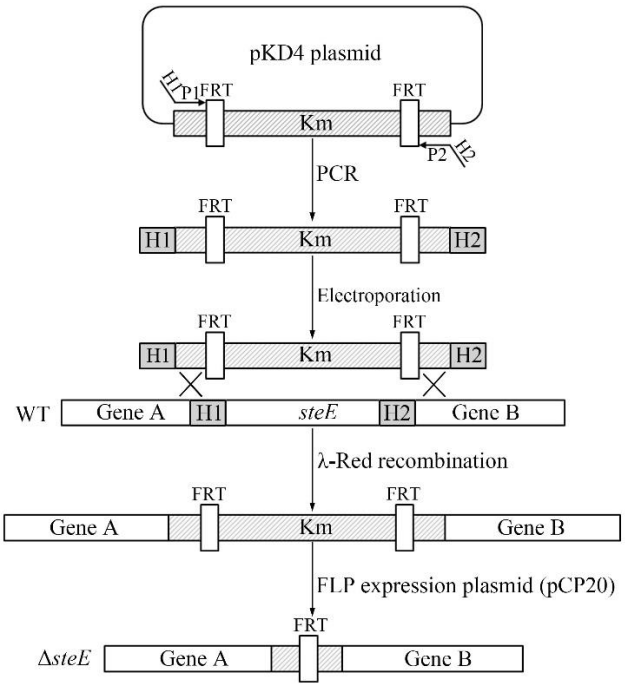

Fig. 1. Schematic diagrams for the construction of the ΔsteE strain

or 683 bp by PCR method using primers CX1/CX2, respectively. In addition, the WT and ΔsteE (pBR322-steE) strains has a length of 224 bp by PCR method using N1/N2 primers. These results indicated that the ΔsteE (pBR322-steE) strain were successfully constructed.

The growth curve and biochemical characteristics of the ΔsteE strain. The growth curve analysis revealed no significant differences among the WT, ΔsteE and ΔsteE+steE strains cultured in LB broth at different time points at 37 °C (Fig. 4). Similar to the WT and ΔsteE strains, the ΔsteE strain was able to ferment mannitol, glucose, arabinose, ornithine, mannose, decarboxylase and lysine decarboxylase activity, but could not utilize sucrose, hydrogen sulfide, maltose, sorbitol, lactose, malonate and urease. It shows that the deletion of steE gene in S. Pullorum does not affect the biochemical characteristics of the WT strain.

SteE promoted apoptosis of HD-11 cells. To evaluate the effect of steE on the apoptosis of HD-11 cells induced by mCherry-S. Pullorum infection, DAPI staining was observed by laser scanning confocal microscopy. The result showed a large number of early apoptotic HD-11 cells were observed in WT strain infected group compared to that of the cells infected with the ΔsteE strain (Fig. 5). In addition, the cell apoptosis was not significant difference in HD-11 cells infected the ΔsteE strain than that of blank control group.

Deletion of steE decreased proliferation of S. Pullorum in HD-11 cells. To evaluate the influence of steE on the S. Pullorum infection in the HD-11 cells, we compared the proliferation of the WT, ΔsteE and ΔsteE+steE strains in HD-11 cells. As shown in Fig. 6, the proliferation fold of WT strain was higher than that of ΔsteE strain in HD-11 cells. Significant difference was detected at 6 h and 24 h

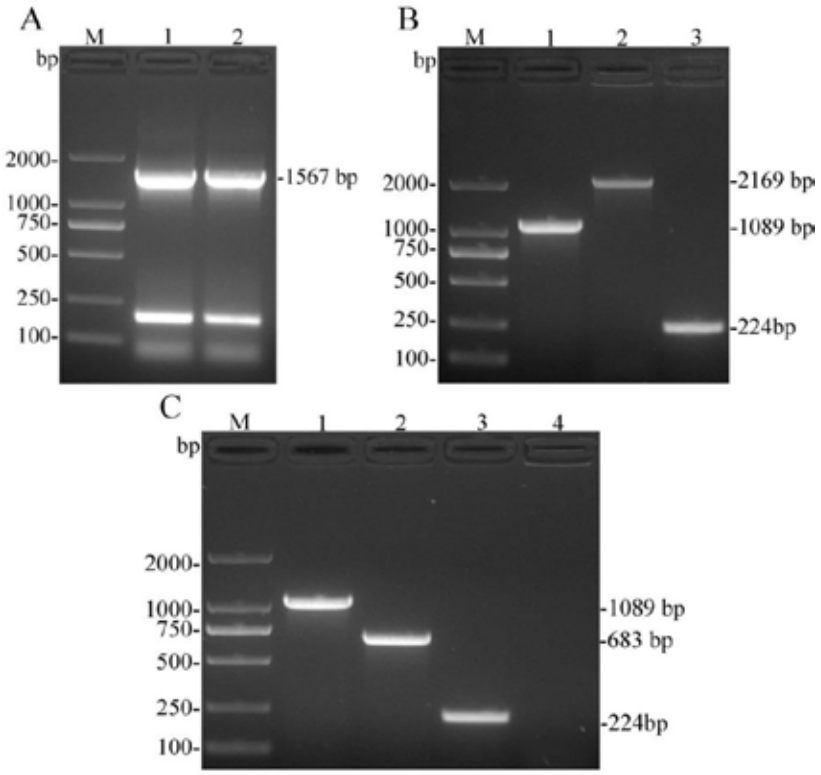

Fig. 2. Identification of the ΔsteE strain by PCR method. M: DL 2000 DNA marker. A. PCR identification of pKD4 plasmid with primers P1/P2; 1, 2: PCR products from pKD4 plasmid. B. PCR identification of the ΔsteE::kan strain with primers CX1/CX2 or N1/N2; 1: WT; 2, 3: ΔsteE::kan. C. PCR identification of the ΔsteE strain with primers CX1/CX2 or N1/N2; 1: WT; 2, 3: ΔsteE; 4: Negative control

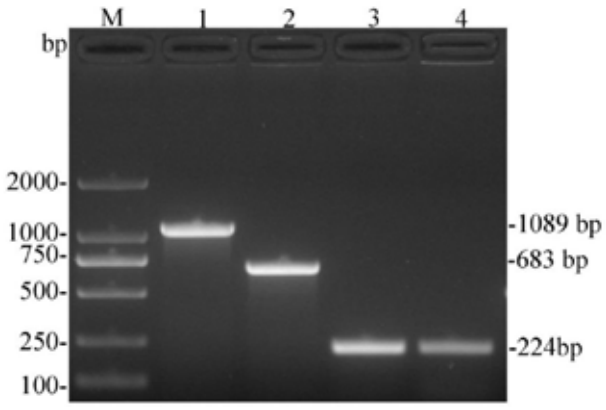

Fig. 3. PCR identification of the ΔsteE (pBR322-steE) strain. M: DL 2000 DNA marker; 1,3: WT; 2, 4: ΔsteE (pBR322-steE)

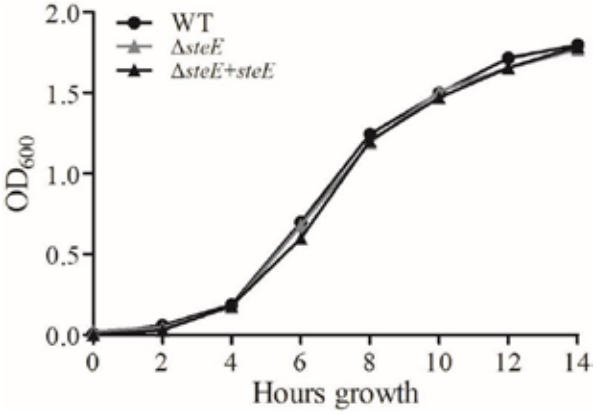

Fig. 4. Growth curves of the WT, ΔsteE and ΔsteE +steE strains in LB broth. The OD600 values of Salmonella cultures were determined in 2 h intervals by spectrophotometry

in HD-11 cells. These results indicated that the steE deletion of S. Pullorum has reduced bacterial colonization in HD-11 cells.

Discussion. Salmonella is an important intracellular pathogen. After S. Pullorum infection, Salmonella can form Salmonella-containing vacuole (SCV) in the host cell, and proliferate and diffuse in the SCV (Li et al., 2018; Yu et al., 2016). This characteristic is very important for the pathogenesis of S. Pullorum. Salmonella pathogenesis are two T3SSs encoded in Salmonella pathogenicity islands 1 and 2 (SPI-1 and SPI-2) that are responsible for the secretion and translocation of a set of bacterial proteins termed effectors into host cells with the intention of altering host cell physiology for bacterial entry and survival.

The maintenance of SCV function is inseparable from the participation of a series of Salmonella virulence factors,

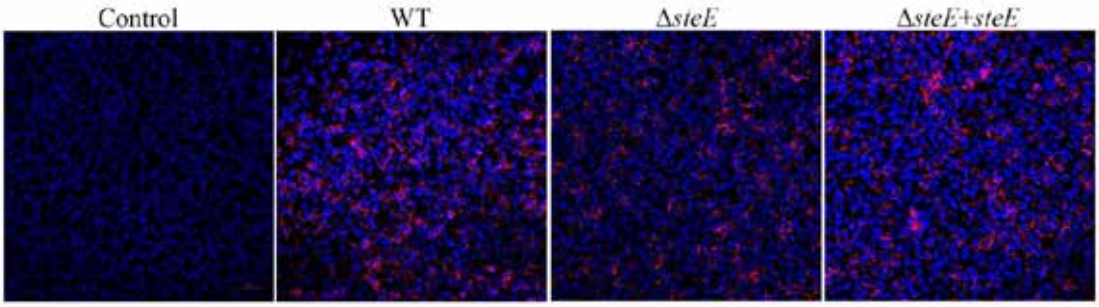

Fig. 5. Effector protein steE promotes the apoptosis of HD-11 cells infected by S. Pullorum. The morphological changes of HD-11 cells during apoptosis were observed by confocal laser scanning microscopy (20 ×). HD-11 cells were infected with mCheery-WT or mCheery-ΔsteE strain at a MOI of 10:1 showed red fluorescence at 3 hpi

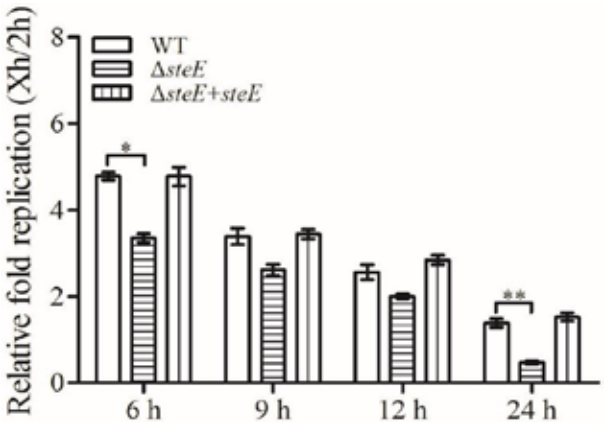

Fig. 6. steE inhibits proliferation of S. Pullorum in HD-11 cells. Intracellular bacterial growth at indicated times is shown as the fold change compared to the primary intracellular bacteria (2 h)

in which the T3SS2 encoded by SPI-2 and its secreted effector protein (Kodama et al., 2015). As a potential virulence protein of T3SS2, steE was found to regulate macrophage activation and host immune response. Some studies have found that Salmonella can be colonized in the intestine and spleen, and directly take macrophages as target cells (Geng et al., 2019; Lin et al., 2017). After S. Pullorum infection, the bacterium can not only avoid the killing of intracellular active substances, but also proliferate and spread in macrophages (Gulati et al., 2019). steE is necessary for the replication and virulence of Salmonella in macrophages (Stapels et al., 2018). Therefore, we speculated that the pathogenic mechanism of S. Pullorum infection in chickens may be the same as that in HD-11 cells.

In this study, steE was selected as the research gene based on λ-Red recombination system to construct the S. Pullorum ΔsteE strain. The results showed that the growth and biochemical characteristics of S. Pullorum and S. Pullorum steE strains are similar, which is consistent with the research results of Pham et al (2020), indicating that steE is not necessary for the growth and metabolism of S. Pullorum. The results showed that steE would reduce the colonization ability and virulence of S. Pullorum in HD-11 cells. S. Pullorum induced apoptosis of HD-11 cells is a special virulence mechanism, which could promote the spread of bacteria between cells. Recent studies have shown that the deletion of S. Pullorum SPI-2 significantly reduced the pathogenicity of chicks, which is consistent with steE belonging to SPI-2 effector protein (Niemann et al., 2011; Lawley et al., 2006; Gibbs et al., 2019). In addition, Pham et al reported that steE can drive macrophages to polarize to M2 type and increased the ability of Salmonella infection-permissive state (Pham et al., 2020). Therefore, we suspected that steE may be related to the virulence of Salmonella. The results of S. Pullorum infecting HD-11 cells also showed that steE enhanced the intracellular viability of S. Pullorum and promoted the apoptosis of HD-11 cells.

Conclusion. In conclusion, we demonstrated that the deletion of steE in S. Pullorum had no effect the growth and biochemical characteristics, but its proliferation ability decreased significantly in HD-11 cells, which decreased cell apoptosis, indicating that steE was closely related to virulence of S. Pullorum. Altogether, our research suggest that the steE gene was required for S. Pullorum virulence, which laid a foundation for further related research in S. Pullorum vaccine strains.

Conflict of interest

The authors declare that there is no conflict of interest.

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Конструкція та характеристика stee-делеційного мутанта Salmonella Pullorum

Сальмонельоз курей – це бактеріальне інфекційне захворювання, що спричинене сероваром Salmonella enterica Pullorum (S. Pullorum). Це захворювання, широко реєструється в сучасних птахівничих господарствах. Основними клінічними симптомами хвороби є пронос білого кольору та задишка, смертність може досягати 100 %. Поточні заходи для запобігання та боротьби з захворюванням – це контроль біобезпеки та заходів профілактики, які направлені на своєчасне виявлення хворої птиці, постійного спостереження за здоровими курчатами та виявлення шляхів передачі інфекції. Незважаючи на те, що використання антибіотиків зменшує загибель хворої птиці, але це не може 100% знищити патогенні мікроорганізми в організмі птиці, крім того це викликає виникнення антибіотикорезістентних штамів мікроорганізмів, що є загрозою для людини. Тому важливим є необхідність вивчення та розробки заходів профілактики та контролю, включаючи нові вакцини. Як новий фенотип вірулентності, steE кодується Gifsy-1 через регуляцію системи секреції III типу 1 і 2 (T3SS1 і T3SS2). SteE відіграє важливу роль в еволюції специфічності господаря мікроорганізмів роду Salmonella. На лабораторній моделі при зараженні мишей steE підвищується вірулентність Salmonella та експресія протизапальних цитокінів. Однак роль steE в патогенезі Salmonella потребує подальшого вивчення. На сучасному рівні більшість досліджень steE в основному проводиться на моделі інфекції S. Typhimurium, тоді як на моделях S. Pullorum було проведено мало досліджень. Немає повідомлень про роль steE в клітинах HD-11, інфікованих S. Pullorum. В своїх дослідженнях ми успішно сконструювали делеційний штам steE S. Pullorum за допомогою системи рекомбінації λ-Red. Ріст і біохімічні характеристики S. Pullorum steE були подібні до S. Pullorum. Крім того, ми також спостерігали вплив steE на проліферацію та апоптоз клітин HD-11, інфікованих S. Pullorum. Патогенез Salmonella – це два T3SS, закодовані в острівцях патогенності Salmonella 1 і 2 (SPI-1 і SPI-2), які відповідають за секрецію та транслокацію набору бактеріальних білків, які називаються ефекторами, у клітини-господарі з наміром змінити фізіологію клітини-господаря для проникнення та виживання бактерій. Підтримка функції SCV невіддільна від участі ряду факторів вірулентності сальмонели, в яких T3SS2, кодований SPI-2, і його секретований ефекторний білок. Що steE регулює активацію макрофагів та імунну відповідь господаря. Дослідження показали, що сальмонела може колонізуватися в кишечнику і селезінці та безпосередньо приймати макрофаги як клітини-мішені. Після зараження S. Pullorum бактерія може не тільки уникнути знищення внутрішньоклітинних активних речовин, але й проліферувати та поширюватися в макрофагах. SteE необхідний для реплікації та вірулентності Salmonella в макрофагах. Таким чином, ми встановили, що патогенний механізм інфекції S. Pullorum у птиці може бути таким же, як і в клітинах HD-11. SteE було обрано як дослідницький ген на основі системи рекомбінації λ-Red для конструювання штаму S. Pullorum steE. Результати показали, що ростові та біохімічні характеристики штамів S. Pullorum і S. Pullorum steE схожі. Доведено, що steE зменшить здатність до колонізації та вірулентність S. Pullorum у клітинах HD-11. Індукований S. Pullorum апоптоз клітин HD-11 є особливим механізмом вірулентності, який може сприяти поширенню бактерій між клітинами. Дослідження показали, що делеція S. Pullorum SPI-2 значно знижує патогенність курчат, що узгоджується з приналежністю steE до ефекторного білка SPI-2. Результати інфікування S. Pullorum клітин HD-11 також показали, що steE посилює внутрішньоклітинну життєздатність S. Pullorum і сприяє апоптозу клітин HD-11. У сукупності наші дані демонструють, що ген steE бере участь у вірулентності S. Pullorum і сприяє розробці ослабленої вакцини проти S. Pullorum.

***Ключові слова:** Salmonella Pullorum, steE, біологічні характеристики, клітини HD-11, апоптоз, вірулентність.*