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Autoinducer-2 of quorum sensing is involved in cell damage caused by avian pathogenic *Escherichia coli*

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ABSTRACT

Avian pathogenic *Escherichia coli* (APEC) infections are responsible for great losses in the poultry industry. Quorum sensing (QS) acts as a global regulatory system that controls genes involved in bacterial pathogenesis, metabolism and protein biosynthesis. However, whether QS of APEC is related to cell damage has not been elucidated. In the present study, we explored the correlation between the damage of chicken type II pneumocytes induced by APEC and the autoinducer-2 (AI-2) activity of APEC. The results showed that when chicken type II pneumocytes were co-cultured with 10^8 CFU/ml of APEC-O78 for 6 h, the release of LDH reached the highest level (192.5 ± 13.4 U/L) ($P < 0.01$), and the percentages of dead cells followed the same trend in trypan blue exclusion assay. In addition, the AI-2 activity of cell-free culture fluid (CF) reached the maximum value after 6 h co-culture with 10^8 CFU/ml of APEC-O78. At the same time, the mRNA expressions of eight virulence genes (*papC*, *fimA*, *fimC*, *hlyE*, *ompA*, *luxS*, *pfs*, and *qseA*) of 10^8 CFU/ml APEC-O78 were significantly increased compared with those of 10^7 CFU/ml, and the mRNA expressions of four virulence genes (*hlyE*, *tsh*, *iss*, and *luxS*) of 10^8 CFU/ml APEC-O78 were higher than those of 10^9 CFU/ml ($p < 0.05$) after incubation for 6 h. These results suggested that AI-2-mediated QS is involved in the cell damage induced by APEC-O78, indicating AI-2 may be one new potential target for preventing chicken colibacillosis.

1. Introduction

Avian pathogenic *Escherichia coli* (APEC) causes a wide range of localized or systemic diseases in avian species with a variety of clinical signs, including septicemia, air sacculitis and salpingitis [1]. In Asia, O78 is the most frequently detected serotype [2]. APEC contains a number of virulence factors, including iron uptake chelate gene D (*iucD*), iron regulatory protein 2 (*irp-2*), type 1 pili fimA gene (*fimA*); type 1 pili fimC gene (*fimC*), pyelonephritis-associated pili papC (*papC*), temperature-sensitive hemagglutinin gene (*tsh*), hemolysin E (*hlyE*), serum survival gene (*iss*), outer membrane proteins A (*ompA*), and vacuolating autotransporter toxin gene (*vat*), among others. These virulence factors are associated with bacterial iron acquisition, metabolism, adhesion and invasion, and serum survival to attack the host [3,4]. APEC infection begins in the upper respiratory tract [5] and breaches the blood-air barrier to induce septicemia [6]. Type II pneumocytes play an important role of maintaining the function of blood–air barrier [7,8], and APEC can invade chicken type II pneumocytes [9].

Quorum sensing (QS) is a bacterial intercellular communication system, involving the production and detection of autoinducers (AIs). Microorganism use QS to control the multiple virulence expression of genes, regulate survival [10] and coordinate particular phenotypic features [11]. Autoinducer-2 (AI-2) is a furanosyl boronated diester molecule, and has been deemed as a language between Gram-negative and Gram-positive bacteria for intra-species and inter-species communication [12,13], which is synthesized dependent on the enzyme LuxS and pfs [12].

Quorum sensing was demonstrated to act as a key player in the expression of virulence genes at stationary phase in diffusible *Escherichia coli* [14–18]. In addition, it is reported that K88 ETEC (JG280)-induced cell death was cell density dependent, 10^8 CFU/ml of strain caused more death of IPEC-J2 cells than did 10^9 CFU/ml, and, there is a positive correlation between AI-2 activity of JG280 and death of IPEC-J2 cells during the infection [19]. However, the role of QS in APEC pathogenesis also has not been clearly

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elucidated. Therefore, in this study, we set out to investigate the association between AI-2 and cell damage caused by APEC-O78.

2. Materials and methods

2.1. Bacterial strains and culture conditions

APEC-O78 strain (CVCC1418) was purchased from the China Veterinary Culture Collection Center (CVCC, Beijing, China), which was isolated from the heart of chicken with septicemia signs. The bacteria were grown routinely in peptone culture media agar plates at 37 °C. *Vibrio harveyi* BB152 (*V. harveyi* BB152) (sensor^{1+}sensor^{2+}) strain was provided by Dr. Han, Xian-Gan of Shanghai Veterinary Research Institute (CAAS, Shanghai, China), *Vibrio harveyi* BB170 (*V. harveyi* BB170) (sensor^{1-}sensor^{2+}) strain was donated by Dr. Ke, Cai-Huan of the College of Ocean & Earth Sciences (Xiamen University, Xiamen, China) and cultivated in modified autoinducer bioassay [19] medium at 30 °C [20]. *E. coli* DH5α was purchased from Takara Bio Inc. Chemical reagents were of analytical quality where available.

2.2. Culture of chicken type II pneumocytes

This assay was performed as described previously [9] with modifications. Briefly, lung tissue samples of 13-day-old chicken embryos were cut into small tissue blocks of about 1 cubic centimeter, and then the 0.25% trypsin and 0.1% IV collagenase (Invitrogen-Gibco, Grand Island, NY, USA) were added for digestion at 37 °C for 10 min and 15 min, respectively. Cell suspensions were filtrated by 200 mesh sieve, re-suspended with 10% fetal bovine serum (FBS) in a 100-mm culture plate, incubated for 1 h, and then the supernatants with the unattached cells were collected for three times. The unattached cells were centrifuged at 1200 r/min for 5 min, re-suspended in fresh Dulbecco's modified Eagle's medium (DMEM) for three times, filtrated by 400 mesh sieve, and cells were incubated for 18 h at 37 °C. The attaching cells on culture dish were chicken type II pneumocytes.

2.3. Lactate dehydrogenase (LDH) activity detection

Chicken type II pneumocytes were seeded in 24-well plates and incubated in DMEM with 20% FBS for 18 h to 90% confluence. The cells of each well were infected with 400 μl APEC-O78 (10^7–10^9 CFU/ml) for 3, 6 and 9 h. Cells handled with DMEM with 20% FBS served as controls. The supernatants were collected after centrifugation at 300 × g for 5 min and 12,000 × g for 10 min. The LDH activity was determined according to the manufacturer's protocol (Jiancheng Technology Co., Nanjing, China). LDH activity was calculated as follows: LDH activity (U/L)= (Aexp − Acon)/(Asta − Abla) × 0.2 × 1000, where Aexp is the absorbance of test samples, Acon is the absorbance of control samples, Asta is the absorbance of standard hole, and Abla is the absorbance of blank wells.

2.4. Cell viability assay

Cell death of chicken type II pneumocytes was initially assessed by Trypan blue staining for quantitative analysis of cell viability [19]. Cells were treated as described in LDH detection. After being harvested, cells were washed twice with phosphate buffer saline (PBS), then concentrated to 100 μl, and stained with 0.4% trypan blue for 5 min at room temperature. To assess cell viability, approximately 100 cells were counted with a hemocytometer for each experiment. The total numbers of cells and blue cells (dead cells) were counted. The percentage of cytotoxicity was calculated as follows: Cytotoxicity (%) = (blue cells/total cells counted) × 100.

2.5. Preparation of cell-free culture fluid (CF)

Cell-free culture fluid (CF) was performed as described previously [21]. *V. harveyi* BB152 were grown to an optical density 600 nm (OD_{600}) of 2.0 in 50 ml of AB medium contained in 250-ml flasks. CF were collected by being centrifuged at 12,000 × g for 10 min at 4 °C, and the resulting supernatant was further filtered through a 0.22 μm filter (Pall Corporation, Ann Arbor, MI, USA). CF preparations of DH5α and APEC-O78 were collected in a similar way.

2.6. AI-2 bioluminescence assay

The AI-2 bioluminescence assay was performed as described previously [22], with modifications. Cell-free culture fluids from *E. coli* and V. harveyi strains BB152 were tested for the presence of signaling substances that could induce luminescence in the V. harveyi reporter strain BB170. CF of V. harveyi BB152 was used as a positive control, and CF of DH5α served as a negative control, CF of APEC-O78 (40 μl) boiled at 121 °C for 15 min was used as a blank control. In order to establish the optimum AI-2 bioluminescence assay, we chose 0–40 μl of CF from APEC-O78strains, added to the black flat-bottomed 96-well plates (Corning Costar, Fisher Scientific, Canada), the reporter strain *V. harveyi* BB170 was grown in AB medium to OD_{600} of 2.0, diluted at 1: 5000 with fresh AB medium, and then 160–200 μl inoculum of the V. harveyi BB170 was supplemented, total reaction volume is 200 μL per well and incubated at 30 °C for 2–5 h. Bioluminescence was measured via BHP9504 microplate luminescence analyzer (Beijing Hamamatsu Photonics Technology Co., Beijing, China). The AI-2-mediated bioluminescence was expressed as induction (n-fold) over the negative control. The experiments were repeated three times.

2.7. The growth curve and AI-2 activity of APEC-O78 in different growth phases

APEC-O78 were grown at 37 °C overnight with aeration in Luria–Bertani (LB) broth. The next morning fresh LB medium used for the overnight growth was inoculated at a 1:100 dilution with the overnight-grown cultures. Then extracting the 5.0 mL mixture to 14 tubes. The 14 tubes inoculated in a shaker, at 37 °C, 200 rpm. Respectively at 0–26 h with the number corresponding to the time of the test tube removed, taking 1.0 mL–1.5 mL extraction centrifuge tube, for measuring the optical density, fresh LB medium as control, using the BioPhotometer analyzer in 600 nm wavelength. The remaining 4 mL broth were subjected to centrifugation for 10 min at 12,000 × g, the supernatant were filtered through a 0.22 μm filter membrane, −80 °C stored for use. CF samples were prepared from different phases, corresponding at 0–26 h. The AI-2 activities in CF samples were tested as described above. The experiments were done in triplicate.

2.8. AI-2 activity of APEC-O78 co-cultured with chicken type II pneumocytes

The chicken type II pneumocytes were incubated with 10^7, 10^8, and 10^9 CFU/ml of APEC-O78 for 3, 6 and 9 h, cells handled with equivalence medium were used as controls. The supernatant was collected by centrifuging at 12,000 × g for 10 min and further filtered through a 0.22 mm filter. The reporter strain V. harveyi BB170 was grown in AB medium to OD600 of 2.0, diluted at 1:5000 with fresh AB medium. The 20 μl above supernatant was diluted 10-fold with 180 μl V. harveyi BB170 dilution, then added to black flat-