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Screening host proteins required for bacterial adherence after H9N2 virus infection

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ABSTRACT

H9N2 subtype low pathogenic avian influenza virus (LPAIV) is distributed worldwide and causes great economic losses in the poultry industry, especially when complicated with other bacterial infections. Tissue damages caused by virus infection provide an opportunity for bacteria invasion, but this mechanism is not sufficient for low pathogenic strains. Moreover, although H9N2 virus infection was demonstrated to promote bacterial infection in several studies, its mechanism remained unclear. In this study, infection experiments *in vivo* and *in vitro* demonstrated that the adhesion of *Escherichia coli* (*E. coli*) to host cells significantly increased after H9N2 virus infection, and this increase was not caused by pathological damages. Subsequently, we constructed a late chicken embryo infection model and used proteomics techniques to analyze the expression of proteins associated with bacterial adhesion after H9N2 virus infection. A total of 279 significantly differential expressed proteins were detected through isobaric tags for relative and absolute quantitation (iTRAQ) coupled with nano-liquid chromatography-tandem mass spectrometry (nano-LC–MS/MS) analysis. The results of Kyoto encyclopedia of genes and genomes (KEGG) enrichment analysis showed that differentially expressed proteins were enriched in host innate immunity; cell proliferation, differentiation, and apoptosis; and pathogenicity-related signaling pathways. Finally, we screened out several proteins, such as TGF-β1, integrins, cortactin, E-cadherin, vinculin, and fibromodulin, which were probably associated with bacterial adhesion. The study analyzed the mechanism of secondary bacterial infection induced by H9N2 virus infection from a novel perspective, which provided theoretical and data support for investigating the synergistic infection mechanism between the H9N2 virus and bacteria.

1. Introduction

The H9N2 influenza virus is an RNA virus from the *Orthomyxoviridae* family. Prior to 1990, cases of H9N2 influenza virus infection were mainly reported in avian species in North America (Guo et al., 2000) and only in ducks in southeastern China (Shortridge, 1992). In the 1990s, the H9N2 virus had been isolated worldwide (Arafa et al., 2012). Currently, it circulates not only in avian hosts but also crosses the species boundary to infect mammals (Shanmuganatham et al., 2013). Moreover, increasing evidences showed that H9N2 avian influenza virus (AIV) can act as internal gene donor of some novel human influenza viruses (Deng et al., 2015; Lam et al., 2015), indicating its critical role in influenza ecosystems. Up to now, outbreaks of H9N2 low pathogenic avian influenza virus (LPAIV) in poultry have resulted in enormous economic losses due to decreased egg production and increased mortality. Moreover, most of influenza-related deaths and disabilities results not from primary viral disease but from mixed infection between the H9N2 virus and other bacterial pathogens (Pan et al., 2012). However, the interaction between the H9N2 AIV and bacterial infection is still not well understood.

Many clinical cases confirmed that H9N2 virus promotes the susceptibility of chickens to bacterial infections, such as *Escherichia coli* (*E. coli*) infections, which is a major cause of increased morbidity and mortality in poultry. Using data from virus isolation experiments and sero monitoring, Bano et al. (2003) revealed the significant role of *E. coli* in aggravating the clinical conditions of the commercial broiler

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chickens earlier infected with H9N2 virus. Nili and Asasi (2002) showed that high mortality rates (20%–65%) caused by H9N2 viruses with a low intravenous pathogenicity index of 0.00/3.00 were primarily due to environmental stress and secondary bacterial infections. In addition, H9N2 virus greatly promotes the adhesion of *Streptococcus pneumoniae* to pulmonary epithelial cells in humans and mice (Chockalingam et al., 2012). All these findings implied an inevitable connection between H9N2 virus and secondary bacterial infection.

Air-exchange regions (especially the bronchus and lung) are important invasion sites of opportunistic pathogens during primary infection (Dho-Moulin and Fairbrother, 1999). Secondary bacterial pneumonia caused by pandemic influenzas also plays an important role in increasing morbidity and mortality in humans (Weinberger et al., 2012). Initially, bacteria such as *Pneumococcus*, *Staphylococcus*, and *Streptococcus*, were assumed to invade a body by thriving on bronchial and pulmonary mucosas damaged by virus infections (Morens et al., 2008). However, some studies showed that low-dose Sendai virus and influenza virus, which hardly caused pathological damages, also contributed to secondary bacterial infection (Alymova et al., 2005). Thus, the secondary infection mechanism mentioned above can be supported by the highly pathogenic strains but not by the low pathogenic strains. The most likely explanation for the secondary bacterial infection caused by the low pathogenic strains is that the virus infection shows not only single cytotoxicity but also facilitation to bacterial colonization (Alymova et al., 2005). Active materials on the surfaces of respiratory epithelia are destroyed in the early phase of virus infection, thereby resulting in small-airway obstruction and increasing the secretion of fibrin-rich mucus, which provides a beneficial space and medium for the propagation of bacteria. However, how these bacteria further invade the lower respiratory tract remains unknown, but it is certain that epithelial cells in the lower respiratory tract will change such that bacterial receptors are exposed and activated, thereby facilitating the adhesion of bacteria to host cells.

Based on the above analysis, we used co-infected H9N2 virus and *E. coli* from clinical isolation to investigate the effect of H9N2 virus infection on *E. coli* adhesion to host by *in vitro* and *in vivo* experiments. Our results confirmed the relationship between H9N2 virus infection and bacterial adherence to the respiratory tract. In addition, we further used proteomics techniques to analyze the changes in protein expression levels between H9N2-infected and normal chicken embryo lungs, an artificial infection model at the later stage of chicken embryo development, thus to screen the host proteins required for bacterial adherence.

2. Materials and methods

2.1. Virus, bacterial strains, and cells

The co-infected H9N2 viruses (A/Chicken/Shandong/01/2016) and *E. coli* SD1 strain were isolated in our laboratory. H9N2 virus was identified by gene sequencing. The viruses were propagated in the allantoic cavities of 9-day-old specific pathogen-free (SPF) chicken embryos and stored at −80 °C. The median tissue culture infectious dose ($TCID_{50}$) of virus was determined using Madin–Darby canine kidney (MDCK) cells. *E. coli* was grown on Luria–Bertani (LB) agar and eosin-methylene blue medium for bacterial isolation and identification, respectively. Moreover, *E. coli* was identified by gene sequencing of 16S rDNA. Chicken embryo fibroblast (CEF) cells, DF-1 cells, and MDCK cells were cultured in Dulbecco's modified Eagle's medium (Gibco) containing 10% fetal calf serum (Gibco), penicillin (500 IU/ml), and streptomycin (100 ug/ml).

2.2. Analysis of E. coli adhesion to host cells

CEF and DF-1 cells were cultured on three 6-well cluster plates, respectively. The cells were infected with 100 multiplicity of infection (MOI) of H9N2 virus and grown to about 90% confluence. The cells were then incubated with 100 MOI of *E. coli* at 24, 48, and 72 h post infection (hpi). Three cell wells were set in a group, and cells with no virus infection served as the control. The cells were collected after incubation at 4 °C for 1.5 h. The serial dilutions of the cell suspension were plated onto LB agar for the quantitation of colony-forming unit (CFU). A P-value of < 0.05 was considered significant for bacteria counting. At 72 hpi, immunofluorescence assay was performed to examine the virus infected cells by using anti-influenza A virus nucleoprotein monoclonal antibody, and Wright's staining was performed to examine the adhesive bacteria to cells.

Twenty-four 5-weeks-old SPF chickens were kept in the sterile isolator. Twelve chickens were intranasally inoculated with 10^6 $TCID_{50}$ of H9N2 virus. At 24, 48, 72, and 96 hpi, three chickens were randomly selected and placed in a new isolator and then intranasally challenged with 10^6 CFU of *E. coli* per chicken. The other twelve chickens with no virus infection served as the control. *E. coli* was counted as CFU after 24 h of challenge. A P-value of < 0.05 was considered significant for bacterial counting. Meanwhile, the remaining lung tissues were fixed with 10% formalin. Hematoxylin-eosin (HE) staining and immunofluorescence histochemical staining were then performed with the use of anti-influenza A virus nucleoprotein monoclonal antibody to examine the pathological damages and virus infection, respectively.

2.3. Protein preparation and isobaric tags for relative and absolute quantitation (iTRAQ)-proteomics analysis

Twelve 15-day-old chicken embryos were randomly divided into two groups (named group S and group C). Each chicken embryo in group S was inoculated with 10^6 $TCID_{50}$ of H9N2 virus into the allantoic cavity. The embryos in group C were inoculated with the same volume of normal saline into the allantoic cavity. When the embryos were 19 days old, three embryonic lungs in each group were randomly collected and fixed with formalin. HE staining and immunofluorescence histochemical staining were then performed with the same methods mentioned above.

The other three embryonic lungs in each group were used in iTRAQ proteomics analysis (Shanghai Majorbio Bio-Pharm Technology Co., Ltd). In briefly, 60 mg of tissue sample from each lung was collected and lysed in 7 M urea and 4% SDS on ice for 30 min. Proteins were centrifuged at 15000 × g for 60 min at 4 °C. The protein in the supernatant was quantified using the enhanced bicinchoninic acid protein assay kit (Beyotime). The obtained protein samples were labeled with the iTRAQ tags. The labeled samples were vacuum dried and subsequently redissolved using a high-performance liquid chromatography loading buffer. Finally, the six iTRAQ-labeled peptide samples in the two groups were separated and analyzed by the first dimensional reversed-phase liquid chromatography and nano-liquid chromatography-tandem mass spectrometry (nano-LC–MS/MS). The isolated peptides were directly placed on a quadrupole Exactive mass spectrometer (Q-Exactive MS) for online detection.

2.4. Statistical and bioinformatics analysis

The data were processed using SPSS 16.0 statistics software. Student's t-test was performed to statistically analyze the data of the two groups. The raw files of the datasets produced on Q-Exactive MS were searched against the UniProt chicken complete proteome sequence database with Proteome Discoverer software (version 1.4.0.288). According to the ratio of the areas under the peaks at H9N2 virus-infected group and control group, all identified proteins must have ≥95% confidence. To designate significant changes in protein expression, we considered the proteins with P value of < 0.05 were considered the believed proteins. The proteins with fold changes of > 1.2 or < 0.83 relative to the normal control were considered up-regulated and down-regulated, respectively, based on the published