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Technical note

Synergy of subgroup J avian leukosis virus and *Eimeria tenella* to increase pathogenesis in specific-pathogen-free chickens

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ABSTRACT

To investigate the effects of co-infections of subgroup J avian leukosis virus (ALV-J) and *Eimeria tenella* on the pathogenesis in specific-pathogen-free (SPF) white leghorn chickens, groups of chickens were infected with ALV-J strain NX0101 at one day of age or with *E. tenella* at 14 days of age or both. The control group was left uninfected and was mock-inoculated with phosphate buffer saline (PBS). Mortality rates, body weights, cecal lesions, and viremia of infected chickens in each group were evaluated. Immune status was evaluated by measuring several parameters: immune organ weight/body weight index, specific humoral responses to inactivated NDV vaccine and to inoculated *E. tenella*, proportions of blood CD3 + CD4+ and CD3 + CD8α+ lymphocytes and transcriptional levels of cytokines in blood and cecal tonsils. The results show that co-infections of ALV-J and *E. tenella* induced a higher mortality rate and a lower body weight in SPF chickens compared to single-pathogen infection. In co-infected chickens, ALV-J accelerated the disease symptoms induced by *E. tenella*, and the *E. tenella* extended the ALV-J viremia. Thymus atrophy, decrease in the humoral response levels to pathogens and the NDV vaccine, modifications in the blood lymphocyte sub-populations and transcriptional cytokine disorders were found in co-infected chickens compared to chickens infected with one pathogen alone and to controls. We underline a synergy between ALV-J and *E. tenella* that results in increasing pathogenesis in SPF chickens.

1. Introduction

Eimeria parasites and avian leukosis viruses such as ALV-J are major pathogens in chicken breeding worldwide. Coccidiosis caused by various *Eimeria* species leads to tremendous economic losses (Zhang et al., 2013; Haug et al., 2008; Sharma et al., 2015). These parasites are ubiquitous in environment. Up to 90% of chicken flocks can be exposed (Györke et al., 2013), and the prevalence of diseased chickens can be high. Uncontrolled outbreaks cause high morbidity and mortality, especially when *E. tenella* infects young chickens. *Eimeria* enters the host by penetration of intestinal epithelial cells causing serious damage to the physical integrity and mucosal immunity of the gut. *Eimeria*-infected chickens are often co-infected with many other intestinal parasites, bacteria or viruses (Fukata et al., 1984; Motha and Egerton, 1984; Ruff and Rosenberger, 1985). Avian leukosis viruses (ALVs) belong to the genus *Alpharetrovirus*, part of the *Retroviridae* family, and it includes six subgroups (A–E and J) in chickens (Payne and Fadly, 1997; Sandelin and Estola, 1974). Economic losses due to ALV include tumor-related mortality (Payne and Nair, 2012) and adverse effects to production performance under subclinical infection (Landman et al., 2002). Immunosuppressive effects of ALV infection are important aspects of the disease because of increased rate of secondary infections, resulting in immeasurable losses (Williams and Sellers, 2012). The ALV-J was first isolated in 1988 from meat-type chickens in Great Britain, and it has been reported in many areas of the world during the last 10 years, leading to a serious problem in poultry industry in many countries (Payne et al., 1991; Cui et al., 2003; Lai et al., 2011; Thapa et al., 2004). Co-infection with different pathogens (bacteria, viruses, yeasts, or parasites) is commonly observed, resulting most of the time in higher pathogenesis. In this

Abbreviations: ALV-J, avian leukosis virus subgroup J; *E. tenella*, *Eimeria tenella*; SPF, specific-pathogen-free; $TCID_{50}$, 50% tissue culture infective dose; ELISA, enzyme linked immunosorbent assay; DMEM, Dulbecco modified Eagle medium; PBS, phosphate buffer saline; g, gram; NDV, Newcastle disease virus; HI, hemagglutination inhibition; IFN-γ, interferon-gamma; IL-6, interleukin-6; IL-17A, interleukin-17A.

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study, we aimed to evaluate the potential additive effects of ALV-J (strain NX0101) and *E. tenella* on pathogenesis in the chicken after infection at a young age.

2. Materials and methods

2.1. Virus

The ALV-J field strain NX0101 (Cui et al., 2003) was newly propagated in chicken DF-1 cell line. Virus titer was defined as the 50% tissue culture infective dose ($TCID_{50}$) ml^{-1} using the Reed-Muench formula after enzyme linked immunosorbent assay (ELISA) determination.

2.2. Parasite

The wild type *E. tenella* strain SD-01 isolated from a layer chicken flock in Shandong province was purified from newly infected chickens and stored in 2.5% potassium dichromate at 4 °C.

2.3. Experimental design

All animal protocols were approved by the Shandong Agricultural University Animal Care and Use Committee (SACUC Permission number: AVM140301-20). Temperature and humidity of the isolators were monitored every day to provide a stable and constant living environment for all experimental chickens. Health status of all experimental chickens were monitored by checking feeding, feces and respiratory symptoms and so on throughout the experiment course.

The experimental plan was illustrated in the Supplementary figure. Eighty hatched SPF White Leghorn chicken WL-M/O (C/O) were randomly divided into 4 groups (20 birds per group). Chickens in the group 1 were infected with 0.4 ml of $10^{3.5}$ $TCID_{50}$ ml^{-1} of ALV-J per bird via intra-abdominal inoculation at 1 day of age, those in the group 2 with 6000 sporulated oocysts of *E. tenella* per bird orally at 14 days of age, those in the group 3 with both 0.4 ml of $10^{3.5}$ $TCID_{50}$ ml^{-1} of ALV-J at one day of age and 6000 sporulated oocysts of *E. tenella* at 14 days of age per bird, and those in the group 4 were inoculated with phosphate buffer saline (PBS) as controls. Feed and water free of coccidiostat were used for chicken rearing. At 21 days of age, mortality rates, body weight gains, indexes of immune organs (thymus, spleen and bursal atrophy) were evaluated. The index of immune organ was expressed as the weight of immune organ relative to body weight. The animal experiment was repeated with the same number of chickens to test the consistency and the reliability between two independent experiments, concerning mortality rates, viremia rates and oocyst shedding.

2.4. Viremia

The anticoagulant blood was collected randomly from eight chickens from each virus infected group for viremia detection on days 7, 17, 27, 37 post-inoculation. Centrifugal separated plasma were then inoculated into 80% confluent DF-1 cells and the cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2 for two hours. Cells were washed with sterile PBS, and maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 2% fetal bovine serum (FBS) in humidified atmosphere for 7–9 d. Cell cultures were tested with an ALV Ag Test kit (IDEXX, USA) following the manufacturer's instruction. Viremia rate was defined as rate of ALV positive chickens in ALV infected groups.

2.5. Cecal lesion and oocysts shedding

Feces of six chickens in each group were collected during five to seven days post-challenge. Feces of each group were mixed and the oocyst numbers per gram (g) of the feces per chicken per day were counted microscopically using a McMaster chamber (Hodgson, 1970). Six chickens per group were sacrificed at 21 days of age for cecal lesion scoring using a numerical scale from 0 (normal) to 4 (severe) following the method described by Johnson and Reid (Johnson and Reid, 1970).

2.6. Antibody determination

To compare effects of the two pathogens on the antibody response to vaccination, all chicks from each treatment were inoculated subcutaneously with inactivated Newcastle disease virus (NDV) emulsion vaccine of 0.2 ml at the age of 10 days. On days 21 and 28 post-vaccination, sera from eight chickens of each group were collected to measure the hemagglutination inhibition (HI) antibody titers to NDV.

Sera collected above (17 and 24 days post-challenge of *E. tenella*) were also used to determine the antibody response to *E. tenella* by ELISA. Briefly, 96-well microtiter plates were coated with sporulated oocysts antigen (200 ng/well). Fifty-fold diluted sera were used as a primary antibody and HRP-conjugated rabbit anti-chicken IgY at a dilution of 1:5000 as a second antibody in the reaction system. Optical density values at 450 nm (OD 450) were measured using an automated microplate reader (Biotek, USA). All samples were analyzed in triplicates.

2.7. Fluorescent cell sorting analysis of peripheral blood lymphocyte subpopulations

For the analysis of the subsets of CD3 + CD4+ and CD3 + CD8+ lymphocytes in chicken peripheral blood mononuclear cells (PBMCs) at 21 days of age, lymphocytes from four chickens for each group were isolated by gradient density centrifugation using a lymphocyte separation medium (density: 1.09g/ml) (Solarbio, China) according to the manufacturer's protocol. The specific monoclonal antibodies including chicken CD3, CD4 (Chen et al., 1986) and α chain of CD8 (Tregaskes et al., 1995) were purchased from SouthernBiotech (USA). 10^6 lymphocytes were incubated with 0.2 μg PE-anti-CD3 antibody/0.1 μg FITC-anti-CD4 antibody or 0.2 μg PE-anti-CD3 antibody/0.1 μg FITC-anti-CD8α antibody respectively. Flow cytometric analysis was performed with a fluorescence activated cell sorting (FACS) Calibur cell sorter (Merck, San Jose, CA, USA) and analyzed with CellQuest software. The value of CD4/CD8α lymphocyte was determined by the ratio of CD3 + CD4+ T cells to CD3 + CD8α+ T cells.

2.8. Analysis of cytokine gene transcription

PBMCs and cecal tonsils were collected from three chickens of each group at 14, 18, and 21 days of age, quickly frozen in liquid nitrogen and stored at −80 °C for a RNA extraction. Expression of Th1-associated gene (interferon-gamma, IFN-γ, primer pair: F-AGCCGCACATCAAACACATA/R-CGCTGGATTCTCAAGTCGTT), Th2-associated gene (interleukin-6, IL-6, primer pair: F-CTCCTCGCCAATCTGAAGTC/R-GGCACTGAAACTCCTGGTCT) and Th17-associated gene (interleukin-17A, IL-17A, primer pair: F-TATCAGCAAACGCTCACTGG/R-AGTTCACGCACCTGGAATG) were detected by RT-qPCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was served as a reference gene (primer pair: F-GAACATCATCCCAGCGTCCA/R-CGGCAGGTCAGGTCAACAAC).

qPCR was performed on an ABI 7500 RT-qPCR system using SYBR® *Premix Ex* Taq™ (Tli RNaseH Plus) (Takara, China). Samples were