Part 2. Veterinary medicine

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CELLULAR AND HUMORAL MEDIATED IMMUNITY AND DISTRIBUTION OF VIRAL ANTIGENS IN CHICKENS AFTER INFECTION WITH A LOW PATHOGENIC AVIAN INFLUENZA VIRUS (LPAIV H4N6) ISOLATED FROM WILD DUCKS

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Summary. Four-week-old commercial chickens were intranasally inoculated with a H4N6 low pathogenic avian influenza virus (LPAIV) isolated from a garganey in Ukraine. The virus did not cause disease in chickens and no pathological changes were observed in inoculated birds. No virus was isolated from internal organs, but 3 chickens of 5 showed antibodies to influenza virus at intranasal infection. Cecum, spleen, lung, and trachea samples were collected from LPAIV-infected chickens from 1 to 14 days post inoculation (dpi) and examined by immunohistochemical techniques to determine the distribution of LPAIV and evaluate the host immune response using various immune markers. From 7 to 14 dpi, a sharp increase of the number of cells bearing CD4, IgM, IgG, and IgA was observed. In spleen, the number of CD4 T lymphocytes and macrophages were increased in immunohistochemical staining when compared to controls. In the lung, B lymphocytes expressing IgM (6.8±0.5%), IgG (9.4±1.3%), and IgA (8.6±0.1%) were detected in higher numbers than in controls. High levels of IFN-γ, IL-2, IL-15 were present on 7 dpi. We also found LPAIV nucleoprotein (NP) staining in the trachea observed on 10 dpi (2.7±0.4% of antibody-stained areas) as well as in the spleen on 5 dpi (3.3±0.2%). There was no NP antigen in other organs. In conclusion, although infection with a LPAIV did not cause obvious clinical disease, viral replication was seen in the trachea and spleen and both local and systemic cellular and humoral immune responses were elicited in these LPAIV-infected chickens.

Keywords: avian influenza, low pathogenic avian influenza virus, cellular immunity, humoral immunity, viral antigen, wild birds

Introduction. Avian influenza viruses (AIV) (family Orthomyxoviridae, genus *Influenza virus* A) are an important cause of economic losses in the poultry industry. Members of the order Anseriformes (i.e. ducks, swans, geese) and Charadriiformes (i.e. shorebirds, gulls, terns) are the principal reservoirs of influenza A viruses in nature and are often the focus of research and surveillance (Nemeth et al., 2010). LPAIVs produce subclinical infections in SPF chickens, but under commercial rearing conditions, these viruses can produce mild to moderate respiratory disease when there are secondary infections, environmental stress and/or immunosuppression (Costa-Hurtado et al., 2014).

When low pathogenic avian influenza viruses (LPAIVs) were first isolated from wild water birds in the 1970s, the absence of clinical signs in both naturally and experimentally infected chickens led to the conventional wisdom that LPAIV in wild water birds is avirulent, possibly owing to the adaptation of the virus to its host over many centuries (Kuiken, 2013). Neither clinical signs nor histopathological findings were observed in LPAIV-infected chickens. In addition, only a short-term viral shedding which was accompanied by seroconversion was detected in some LPAIV-infected chickens (Bertran et al., 2011).

This study was conducted to better understand the underlying host-pathogen interaction in infections with wild bird AIV in chickens. The dynamics of viral spread and local and systemic host immune responses in infected chickens were examined. The changes in cellular immune response triggered by LPAIV infection by measuring lymphocytes that express CD4, CD8, macrophages, IgM, IgG, and IgA have been

studied in chickens after infecting them with LPAIV subtype H4 isolated from wild ducks.

Reason to do this type of study is to understand infection of chickens with wild bird AIVsince it can help explain introduction or not of these viruses in poultry.

Material and methods. ***Virus and virological study.*** A low pathogenic avian influenza virus, A/Garganey/Chervonooskilske/4-11/2009 (H4N6), isolated from the cloacal swab of clinically healthy garganey in 2009 in Ukraine was used in this study. Virus was isolated and identified by the Department of Avian Diseases of National Scientific Center 'Institute of Experimental and Clinical Veterinary Medicine' (NSC 'IECVM'). Virus stocks were made from the first and second embryo passages of archived virus stocks which have been deposited in the collection of viral pathogens in the Department of Avian Diseases of NSC 'IECVM'. AI virus was propagated and titrated in 9-to-11-days-old embryonated specific-pathogen-free (SPF)-chicken eggs (Valo BioMedia GmbH, Germany) according to the recommendations of the OIE. Titer calculation was carried out by Reed and Mench (Dufour-Zavala, 2008). Pathogenicity of the virus was determined by intravenous pathogenicity index (IVPI) test in accordance with the methods recommended by the OIE (Dufour-Zavala, 2008; OIE, 2012).

Virus isolation from internal organ samples, cloacal and nazopharyngial swabs were performed using SPF CE as recommended by the OIE (Dufour-Zavala, 2008; OIE, 2012).

Experimental design. Forty-two 6-weeks-old chickens were obtained from Poultry Farm 'Frunze' of Southern Branch of the National University of Life and Environmental Sciences of Ukraine 'Crimean Agrarian-and-Technological University', and were used to study immunity after infection with the LPAIV. Chickens which were used for the main experiment were not SPF, but they did not have antibodies to the influenza virus both, and in AI, and ELISA. Two groups of experimental birds included: 1) Chickens experimentally inoculated with low pathogenic avian influenza virus A/Garganey/Chervonooskilske/4-11/2009 (H4N6) intranasally with a dose of $10^{6,0}$ EID_{50} per chicken, and 2) Control non-inoculated chickens. Water and feed were provided *ad libitum* and experiments were approved by the institution's animal care committee.

Inoculated chickens were monitored for 14 days after infection. On 1, 2, 4, 7, 10, and 14 days post infection birds were necropsied (3 birds per day in each group), cloacal and nasopharyngeal swabs, samples of trachea, lungs, spleen, small and large intestine tissues were collected. On these days, we collected blood samples from live birds for serological analysis.

Serological studies. Detection of antibodies to avian influenza virus subtype H4 was performed with chicken serum samples by HI test and ELISA. HI test was performed using A/Garganey/Chervonooskilske/4-11/2009 (H4N6) inactivated antigens (produced by NSC 'IECVM'). The hemagglutination (HA) assay and HI test were performed in V-bottom microtiter plates using standard protocol recommended by the OIE (Dufour-Zavala, 2008; Rath et al., 1995). Antibodies in blood samples were detected by a commercial AI ELISA kit (IDEXX).

Immunohistochemistry. Spleen, caeca, trachea and lung tissue samples collected on 1, 3, 5, 7, 10 and 14 days post AI infection were quickly frozen in the liquid nitrogen. Samples were placed in cryotubes and immersed in the Dewar vessel with liquid nitrogen. Frozen tissues were stored at — 196 °C.

Histological sections were made by cryostat microtome HM-525 MICROM (Germany) at the appropriate temperature. Sections were mounted on Star Frost glass microscope slides (Knittel Glaser), treated with L-polylysine to reduce the risk of tissue damage in subsequent stages of processing. Sections were dried at room temperature overnight, treated with a fixative liquid 10 minutes at –4 °C. They were hydrated in two portions of 0.1 M phosphate buffer saline pH 7.2 with Twin 20 for 5 min, sections were subsequently washed twice with buffer after each step of the immunohistochemical reaction. Cryostat sections of 7-μm thickness were cut and immunohistology was carried out using unlabelled primary mouse monoclonal antibodies detecting CD4 (thymocytes and T-helpers), CD8 (cytotoxic T-cells, natural killer cells, cortical thymocytes), KUL01 (monocytes and macrophages), IgM, IgG, IgA (chicken Ig or isolated lymphocytes), and FluA-NP 2C9 (recombinant influenza virus type A nucleoprotein) (Southern Biotechnology Associates, Eching, Germany) followed by a commercially available staining kit (LSAB, ChemMate Detection kit, peroxidase antiperoxidase, rabbit/mouse; DakoCytomation, Hamburg, Germany) according to the manufacturer's recommendations. Monoclonal antibodies to IFN-γ, IL-2, Il-15 (T-lymphocytes) were produced by Dr. Hyun S. Lillehoj (Animal Biosciences and Biotechnology Laboratory, Beltsville Agricultural Research Center, Beltsville, Maryland, USA). As a negative control, slides were incubated with PBS instead of the monoclonal antibodies. Sections were counterstained with hematoxylin and mounted with Canadian balsam (Riedel de Haen AG, Seelze-Hannover, Germany).

Immunohistological tissue preparations were examined by light-microscopic image analysis ('VideoTest Morphology — 5.0'). At least 3 regions of interest of each tissue section and antibody were

scanned, the percentage of antibody-stained areas was determined, and the mean values were calculated (Berndt, Pieper and Methner, 2006).

Statistical analysis. Statistical analysis was performed using SPSS 17.0 software for Windows. All data for each group expressed as means ± SEM. The difference between the means considered at $p<0.05$.

Results. To study host immune response in chickens experimentally infected with a LPAIV, we used A/Garganey/Chervonooskilske/4-11/2009 virus (H4N6) isolated from cloacal swab of clinically healthy garganey in the Donetsk region of Ukraine in 2009. This virus was low pathogenic based on criteria of pathogenicity definition. Upon intravenous and intranasal infection with this virus, no clinical signs were observed in chickens and no pathological lesions were found at necropsy. Infection of poultry with this virus provoked an antibody response at 10 days after intranasal inoculation which ranged from 1:8 to 1:32 serum antibody titers. Only 2 of 5 chickens were positive by the HI test and 3 of 5 were positive by ELISA at intranasal inoculation. All 10 chickens were positive both and by HI test, and by ELISA. When chickens were given an intravenous infection with this virus, specific antibodies to influenza virus H4 were detected in titer ranges of 1:128 to 1:1024.

In immunohistological studies, the respiratory tract organs (lungs and trachea) showed higher level of humoral immunity (IgM, IgG, IgA-expressing cells) in the lung compared to the trachea. Also, indicators of cell-mediated immunity as measured by the CD4 and macrophage markers were higher in LPAIV-infected chickens in the lungs at 14 days post infection compared to uninfected control chickens AIV-infected chickens showed 1.5 times higher ($p<0.05$) CD4 cells than healthy chickens. The number of positive cells continued to increase until 14 dpi when it reached the maximum level as shown in Fig. 1.

Lymphocytes expressing CD8 were increased starting from 7 dpi. The chickens in the infected group showed 2 times higher levels of CD8 cells ($p<0.05$) compared to the non-infected control chickens. The time of CD8 cell increase coincided with the reduced CD4 cells which may suggest that CD8 cells play an important role in host defense against AI. Furthermore, CD8 cells declined as the number of CD4 cells increased perhaps indicating an important role of antibodies that follow cytotoxic activity of CD8 cells.

IgM cells increased in the lungs of AIV-infected chickens at 7 dpi. Level of IgM expressing cells in the lungs of AIV-infected chickens was 1.5 times ($p<0.05$) higher than in the non-infected control chickens. The level of IgM in AI-infected chickens on 3 dpi was almost 2 times higher than in the chickens of uninfected control group (4,3±0,5% vs 2,9±0,2%). From 10 dpi, less IgM cells were shown.

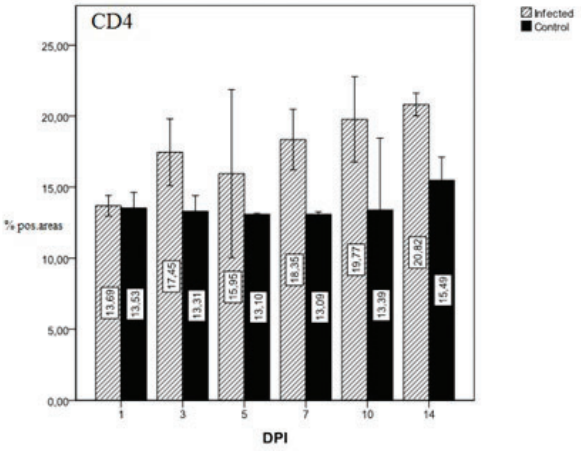

Figure 1. Dynamic response of CD4 T-cells subsets in the lung after infection with LPAIV. Each bar represents the percentage of antibody-stained areas from 1 to 14 dpi. Significant impact of LPAIV on immune response is shown from 7 dpi. The Student *t* test for comparison of two independent samples was used for statistical evaluation of differences between the groups (each infected group against the control group)

IgA cells were also seen in the lungs of infected chickens within 10 days post infection its level fluctuating until 10 dpi but reached at its maximum level at 14 dpi with AIV-infected chickens showed 2–3 times higher ($p<0.05$) IgA cells than uninfected control chickens (Fig. 2).

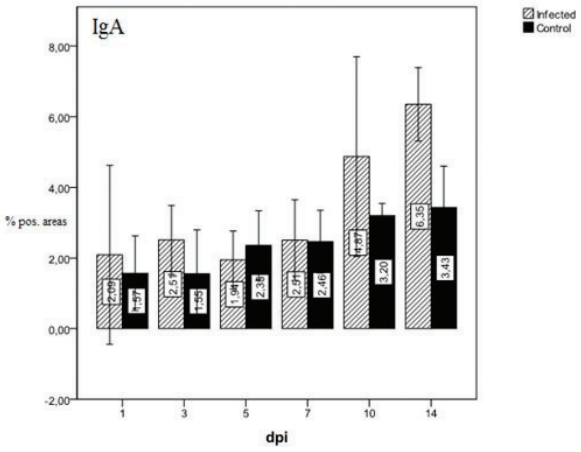

Figure 2. Dynamics of IgA changes in the lungs of AI-infected chickens. Each bar represents the percentage of antibody-stained areas from 1 to 14 dpi. Significant impact of LPAIV on immune response is shown from 10 dpi. The Student *t* test for comparison of two independent samples was used for statistical evaluation of differences between the groups (each infected group against the control group)

IFN-γ transcripts were observed in the AI-infected chickens starting at 7 dpi that coincides with the increasing level of CD4 cells. In Fig. 3 the changing levels of IFN-γ are shown.

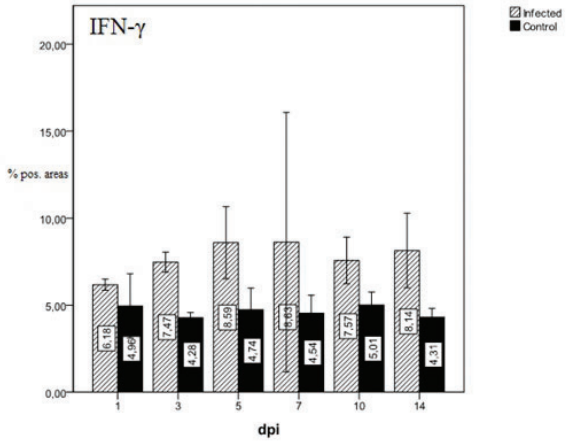

Figure 3. Dynamics of IFN-γ changes in lungs after infection with LPAIV. Each bar represents the percentage of antibody-stained areas from 1 to 14 dpi. Significant impact of LPAIV on immune response is shown from 5 dpi. The Student *t* test for comparison of two independent samples was used for statistical evaluation of differences between the groups (each infected group against the control group)

The number of lymphocytes which secrete IL-2 and IL-15 in AI-infected chickens was in general 1.5 to 2 times higher ($p<0.05$) compared to the uninfected control chickens (Fig. 4). Increased levels of these cytokines coincided with the enhanced number of CD8 cells in AIV-infected chickens.

The percentage of IgM-expressing cells was markedly increased during the first seven days post infection in the trachea. AIV-infected chickens showed 2 times higher IgM-expressing cells ($p<0.05$) compared to the uninfected control. Level of IgG-expressing cells in AIV-infected chickens increased starting from 7 dpi reaching a peak at 14 dpi (Fig. 5).

The level of cells expressing IFN-γ, IL-2, and IL-15 increased in AIV-infected chickens at 7-days after infection. The peak time coincided with a period of increasing CD8 cells. However, there was no significant difference in these cytokine levels between the AIV-infected and uninfected groups.

Interestingly, nucleoprotein-expression in the LPAIV-infected chickens was strong at 10 dpi in AI-infected chicken trachea. All chickens showed negative response in virological examination. At virological investigations, we have not identified the virus in all investigated organs. This indicates the absence of virus isolation in the environment, as evidenced by previous studies (Morales et al., 2009; Spackman et al., 2010).

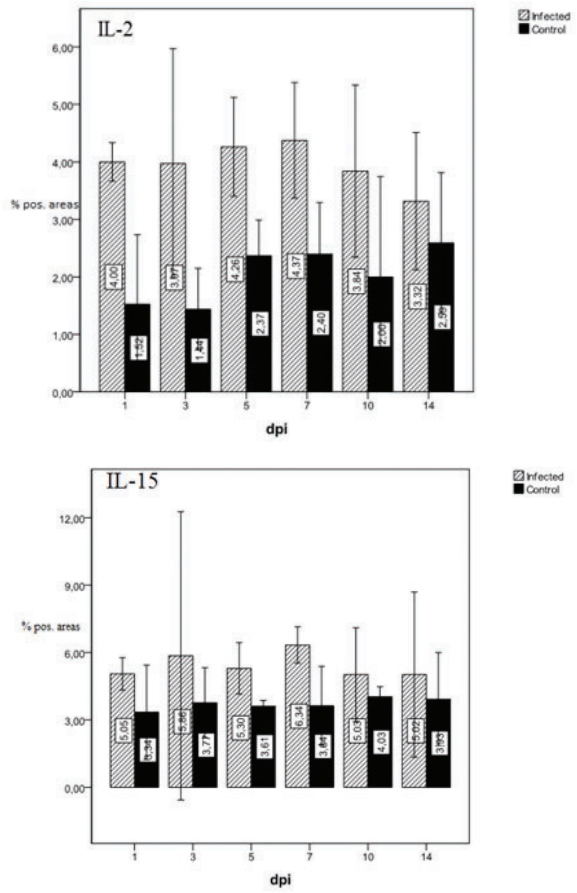

Figure 4. Dynamics of lymphocyte changes secreting IL-2 and IL-15 in the lung after infection with LPAIV. Each bar represents the percentage of antibody-stained areas from 1 to 14 dpi. Significant impact of LPAIV on immune response is shown from 5 dpi. The Student *t* test for comparison of two independent samples was used for statistical evaluation of differences between the groups (each infected group against the control group)

In the cecum, lower levels of CD4 cells were seen on 5 dpi but levels slowly increased from 7 dpi to 14 dpi following AIV infection.

In the ceca, a significant (1.5–2 times, $p<0.05$) increase in the number of cells expressing IgM and IgG was found (Fig 6).

LPAIV infection induced an increasing in macrophages and lymphocytes expressing CD4 and CD8 in the spleen throughout the period examined in this study indicating their role in host response to viral infection. Macrophage and heterophils play an important role in innate immune response in poultry and are able to ingest and kill a variety of microbial pathogens (Kogut and Klasing, 2009). The levels of macrophages in chickens of AIV-infected group were 2 times ($p<0.05$) higher than the non-infected control after the 1 dpi. This may suggest a role of macrophages

in initiating innate immune response to AIV infection. In the cecum, AIV infection enhanced lymphocytes especially the ones expressing IgG and IgA. At 10 dpi, there was a significant increase in cells expressing IgG and IgA ($p<0.05$) in infected chickens compared to uninfected control chickens.

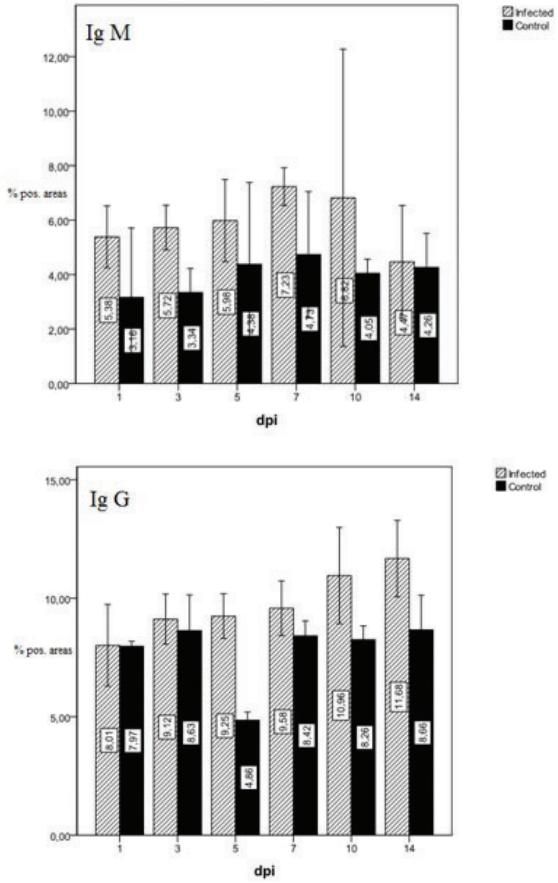

Figure 5. Dynamics of changes in lymphocytes expressing IgM and IgG in the trachea after infection with LPAIV. Each bar represents the percentage of antibody-stained areas from 1 to 14 dpi. Significant impact of LPAIV on immune response is shown from 7 to 10 dpi. The Student *t* test for comparison of two independent samples was used for statistical evaluation of differences between the groups (each infected group against the control group)

Discussion. There are no data on role of different immune cells in immune response at avian LPAI. It served as a reason to carry out current research. In this study, we assessed the clinical response, viral shedding, antibody production and immune response of chickens after infection with LPAIV A/Garganey/Chervonooskilske/4-11/2009 (H4N6) isolated from a wild bird. Our study shows that this LPAIV infection does not cause clinical signs of disease in infected chickens.

Starting at 7 dpi to 14 dpi, there was a sharp increase in the percentages of lymphocytes expressing CD4, IgM, IgG and IgA. Moreover, in the lung of AI-infected chickens, the levels of these cells were significantly higher compared to those of the noninfected control chickens. On the 3rd day after infection, the number of CD4 cells was significantly higher in the AI-infected chickens compared to the uninfected control ($p<0.05$).

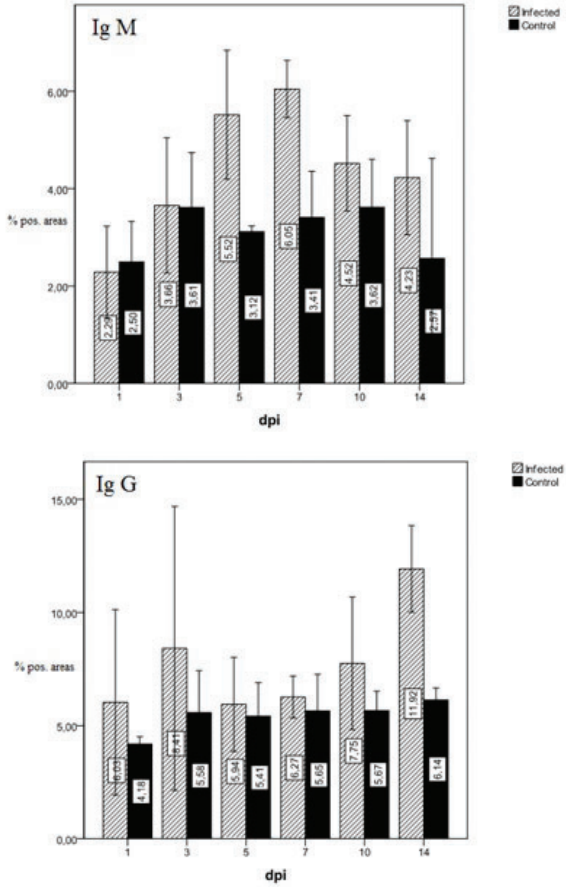

Figure 6. Dynamics of changes of cells expressing IgM and IgG in the caeca after infection with LPAIV. Each bar represents the accumulation of cells from 1 to 14 dpi. Each bar represents the percentage of antibody-stained areas from 1 to 14 dpi. Significant impact of LPAIV on immune response is shown from 7 to 10 dpi. The Student *t* test for comparison of two independent samples was used for statistical evaluation of differences between the groups (each infected group against the control group)

After AI infection, CD8 cells decreased until 6 dpi but showed a peak increase at 7 dpi (6.976±1.765 % at 3.056±0.704 %) ($p<0.05$) and then slowly declined but remained high until the end of experiment.

In general, the levels of cells expressing IgM, IgG and IgA were higher in the lung and caeca in the AI-infected chickens compared to those in the trachea. The percentages of IgM+ and IgA+ B cells increased with infection reaching almost two times higher than that of uninfected control chickens ($p<0.05$). After 7 dpi, IgG+

cells began to decrease. But then the percentage of IgG increased from 10 to 14 dpi when the levels of these cells in all experimental groups reached a maximum rate. On the 14 dpi, the percentage of IgA in AI-infected chickens was twice higher than the control group ($p<0.05$). However, on 10 dpi, there was a sharp decrease of IgM+ cells which continued until 14 dpi which may suggest that the isotype switching from IgM to IgG class antibodies may have occurred during the later phase of B cell immune response. As for cell-mediated immune response in the cecum, CD4+ cells were decreased on 5 dpi in AI-infected chickens ($p<0.05$) compared to uninfected control chickens.

Increases in cells expressing CD4 and CD8 as well as macrophages were evident in the spleen of AI-infected chickens, which indicates an enhanced AI-induced cell-mediated immunity. Macrophages were significantly enhanced on 3 dpi and persisted until 7 dpi suggesting their active role in processing viral antigens in the initiation of local immunity. CD4+ cells reached a stably high level compared to uninfected control chickens at 14 dpi ($p<0.05$).

Increased CD4+ cells and IL-2 level clearly indicate AI-induced cell-mediated immune response that can further activate CD8+ cells. IL-2 has a relatively narrow range of cells expressing IL-2 immune cells. The main ones are activated T- and B-lymphocytes and natural killer cells (NK-cells) which are induced to proliferate in the presence of IL-2. IL-2 also acts on monocytes bearing the receptor for IL-2 on its surface enhancing the generation of reactive oxygen and peroxides. Its effect can be direct or indirect. IL-2 operates at all stages of host immune response against pathogen invasion (Amrani, Mauzy-Melitz and Mosesson, 1986; Gendron et al., 1991; Klasing, 1994; Rath et al., 1995).

Following AIV infection, infected chickens showed high levels of IFN-γ, IL-2, and IL-15 expressing cells on 7 dpi when there was an increased CD8+ cells. Interferons are important cytokines in host defense against viral infections during the early phase of infection (Baron et al., 1987; Farrar and Schreiber, 1993; Grossber, 1987). Cells expressing IL-2 and IL-15 were found twice more In the lung of infected chickens, compared to uninfected control chickens especially on 7 dpi ($p<0.05$).

Infection with a low pathogenic avian influenza virus A/Garganey/Chervonooskilske/4-11/2009 (H4N6) did not cause any clinical disease at the infecting dose of $10^{6,0}$ EID_{50} when intranasally route was used. However, virus replicated in trachea and spleen inducing local and systemic cellular and humoral immune response in chickens. These results are in agreement with the previous observation (Spackman et al., 2010). No morbidity or mortality was seen when infecting doses of 10^6 or 10^8 EID_{50}/bird was administered by the upper-respiratory route suggesting that the virus is poorly adapted to chickens (Nemeth et al., 2010). Virus was shed at higher titers and spread to the kidneys in chickens when intravenous route was used (Spackman et al., 2015). We found nucleoprotein expression in the trachea at 10 dpi in the AI-infected chickens. In the spleen, NP was seen at 5 dpi. There was no NP antigen in other organs.

Conclusion. Our results indicate the potential possibility for infection of poultry with viruses isolated from wild birds. But currently, it is not completely known why some viruses from wild birds can cause infection in poultry while others can not. Further study the immune response will enable us to determine the features of the pathogenesis of low pathogenic avian influenza.

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