DOI: 10.29326/2304-196X-2022-11-1-70-76

Testing of chickens experimentally infected with A/H9N2 avian influenza virus isolates for their immune responses

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SUMMARY
Data on tests of chickens for their immune responses to infection with low pathogenic A/H9N2 avian influenza virus isolates belonging to Y-280 and G1 genetic lines are presented in the paper. $CD4^+/CD8^+$ ratios were determined with flow cytometry for initial immune status examination and for detection of apparent immune system disorders. Quantitative analysis of peripheral blood lymphocyte subpopulations in chickens revealed changes characteristic of the immune suppression. Analysis of dynamics of T- and B-lymphocyte levels in blood of the infected chickens revealed decrease in relative T-lymphocyte counts and increase in relative B-lymphocyte counts. T-lymphocyte subpopulation composition expressed as $CD4^+/CD8^+$ ratio (%) changed after the infection: $CD4^+$ cell proportion was found to decrease whereas $CD8^+$ cell proportion increased. According to literature data, immune response activated by vaccination induces the reverse dynamics towards to increase in $CD4^+/CD8^+$ ratio. Both cell-mediated immunity and humoral immunity play role in development of the immune response in chickens infected with avian influenza viruses. Apparent humoral immune response was detected by serological tests of sera taken from chickens on day 14 after infection. Mean specific anti-A/H9N2 AIV antibody titre in all groups of test chickens infected with low pathogenic avian influenza virus isolates was higher than 6 $\log_2$. High level of specific antibodies to avian influenza virus was indicative of postvaccinal humoral immune response development.

Keywords: avian influenza virus (AIV), H9N2, T-cells

Acknowledgements: The study was funded by the FGBI "ARRIAH" within the framework of "Veterinary Welfare" research work.

For citation: Osipova O. S., Volkova M. A., Frolov S. V., Andreychuk D. B., Chvala I. A. Testing of chickens experimentally infected with A/H9N2 avian influenza virus isolates for their immune responses. *Veterinary Science Today*. 2022; 11 (1): 70–76. DOI: 10.29326/2304-196X-2022-11-1-70-76.

Conflict of interest: The authors declare no conflict of interest.

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УДК 619:616.98:578.832.1:636.52/.58.053:616-085.37:612.017.11/.12

Изучение иммунного ответа цыплят после экспериментального заражения изолятами вируса гриппа птиц A/H9N2

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РЕЗЮМЕ

Представлены данные по изучению параметров иммунного ответа цыплят после инфицирования изолятами низкопатогенного вируса гриппа птиц подтипа A/H9N2, относящимися к генетическим линиям Y-280 и G1. Для первичного исследования иммунного статуса и выявления выраженных нарушений иммунной системы были определены соотношения $CD4^+/CD8^+$ клеток методом проточной цитофлуориметрии. В результате количественного анализа субпопуляций лимфоцитов периферической крови цыплят обнаружено наличие изменений, характерных для иммунной супрессии. При изучении динамики уровня Т- и В-лимфоцитов в крови инфицированных цыплят установлено снижение относительного количества Т-лимфоцитов и увеличение относительного количества В-лимфоцитов в крови. После инфицирования изменение субпопуляционного состава Т-лимфоцитов в процентном соотношении $CD4^+/CD8^+$ клеток отмечено в сторону уменьшения процента $CD4^+$ клеток и увеличения процента $CD8^+$ клеток. Согласно литературным данным, при иммунизации вакцинными препаратами активация иммунного ответа приводит к обратной динамике в сторону увеличения отношения $CD4^+/CD8^+$ клеток. В формировании иммунного ответа у цыплят после инфицирования вирусами гриппа птиц играет роль не только клеточно-опосредованный, но и гуморальный иммунитет. В результате серологических исследований сывороток крови цыплят после инфицирования на 14-е сут установлен выраженный гуморальный иммунный ответ. Средний титр специфических антител к вирусу гриппа птиц подтипа A/H9N2 во всех группах цыплят, зараженных изолятами низкопатогенного вируса гриппа птиц, был выше 6 $\log_2$. Высокий уровень специфических антител к вирусу гриппа птиц показал развитие постинфекционного гуморального иммунного ответа.

Ключевые слова: вирус гриппа птиц, H9N2, Т-клетки

Благодарности: Работа выполнена за счет средств ФГБУ «ВНИИЗЖ» в рамках тематики научно-исследовательских работ «Ветеринарное благополучие».

Для цитирования: Осипова О. С., Волкова М. А., Фролов С. В., Андрейчук Д. Б., Чвала И. А. Изучение иммунного ответа цыплят после экспериментального заражения изолятами вируса гриппа птиц A/H9N2. *Ветеринария сегодня*. 2022; 11 (1): 70–76. DOI: 10.29326/2304-196X-2022-11-1-70-76.

Конфликт интересов: Авторы заявляют об отсутствии конфликта интересов.

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INTRODUCTION

Low pathogenic A/H9N2 avian influenza virus is a RNA virus belonging to *Orthomyxoviridae* family, *Alphainfluenzavirus* genus, *Influenza* A virus species [1]. The virus was reported for the first time in birds in Wisconsin State, USA, in 1966 [2, 3]. Since then, the low-pathogenic A/H9N2 avian influenza virus has become widespread worldwide, especially in Asia and the Middle East [4–6]. In mainland China, A/H9N2 influenza virus was first isolated in 1994 and has become the most common subtype of avian influenza virus in poultry [4, 7].

This virus is responsible for development of clinically pronounced disease in case of concurrent infection with opportunistic viral and bacterial pathogens and poses a constant threat to poultry industry [8–11]. Economic losses are resulted from the following: increased mortality in young birds, decrease in egg and meat production in poultry establishments.

Preventive immunization against A/H9N2 avian influenza aimed at reducing economic losses is used by many countries (China, Pakistan, Iran, Israel, South Korea, etc.) for this disease control [2, 12–15]. In the Russian Federation, programmes for health status improvement and infection eradication can include preventive immunization with inactivated vaccines due to A/H9N2 AI virus circulation [16].

Vaccination induces both humoral and cell immunity. Cell mechanisms play the major role in immune response to viruses. T-lymphocytes are the main cells of acquired anti-virus immunity. Among them, $CD8^+$ T-lymphocytes recognize foreign viral antigens associated with class I histocompatibility molecules and kill cells infected with viruses. $CD4^+$ T-lymphocytes (T-helper cells) recognize viral antigens located on antigen-presenting cells associated with class II histocompatibility molecules and act as assistants in the synthesis of specific antiviral antibodies by B-lymphocytes [17]. Antigen-recognizing $CD8^+$ T-lymphocytes play a critical role in specific cell-mediated response [18, 19]. Increase in relative $CD8^+$ T-cell count was observed in chickens infected with A/H7N9 and A/H9N2 AI viruses and these cells were demonstrated to confer antivirus protection [20, 21]. However, according to literature data, immunization of chickens against A/H9N2 AI virus was found to result in increase in relative $CD4^+$ T-cell count and decrease in $CD8^+$ T-cell count [21, 22]. $CD4^+/CD8^+$ ratio of T-cells are determined for initial immune status examination and for apparent immune system disorder detection. According to the published data, $CD4^+/CD8^+$ ratio markedly increased after immunization and apparently decreased after infection that supposed immunity enhancement after immunization and immunity suppression after infection [23–26]. $CD8^+$ T-cell deficiency could be a reason for insufficient anti-virus immune response after poultry vaccination and infection of poultry in vaccinated flocks in China.

It is also important to study humoral response since low pathogenic AI virus is able to induce immune suppression in case of co-infection with other pathogens. Various infectious diseases become more severe when the immunity is suppressed.

Thus, a comprehensive study of the immune response features in chickens experimentally infected with low-pathogenic A/H9N2 avian influenza virus is of considerable interest.

MATERIALS AND METHODS

Virus. Low pathogenic A/H9N2 avian influenza virus isolates belonging to Y-280 genetic line (A/chicken/Tadjikistan/2379/2018, A/chicken/Primorsk/419/2018) and G1 genetic line (A/chicken/Chelyabinsk/30/2019) recovered and identified in the FGBI "ARRIAH" Reference Laboratory for Avian Viral Diseases were used for infection during the experiment.

Virus isolation was carried out in 10 day-old specific pathogen free (SPF) chicken embryonated eggs. 10–20% suspension was prepared from the biological material with phosphate buffered solution (pH 7.2–7.4) and injected into allantoic cavity of chicken embryonated eggs, 0.2 cm^3 per egg. Extraembryonic fluid was collected from the chicken embryonated eggs with embryos dead 24 or more hours of incubation for further examinations. Extraembryonic fluid with infectivity of 10^6 EID_{50}/cm^3 and hemagglutination titre of 9 log_2 was used for infection of chickens.

Experiment in animals. Egg cross 30 day-old chickens without antibodies to avian influenza virus obtained from infectious disease-free holdings were used for the experiment. Chickens were divided into three groups, 5 chickens per group, and kept in isolators. Virus-containing extraembryonic fluid with infectivity of 10^6 EID_{50}/cm^3 was injected to chickens intramuscularly in a volume of 0.5 cm^3. Blood samples were collected from chickens before infection and for 14 days after infection for serological tests for

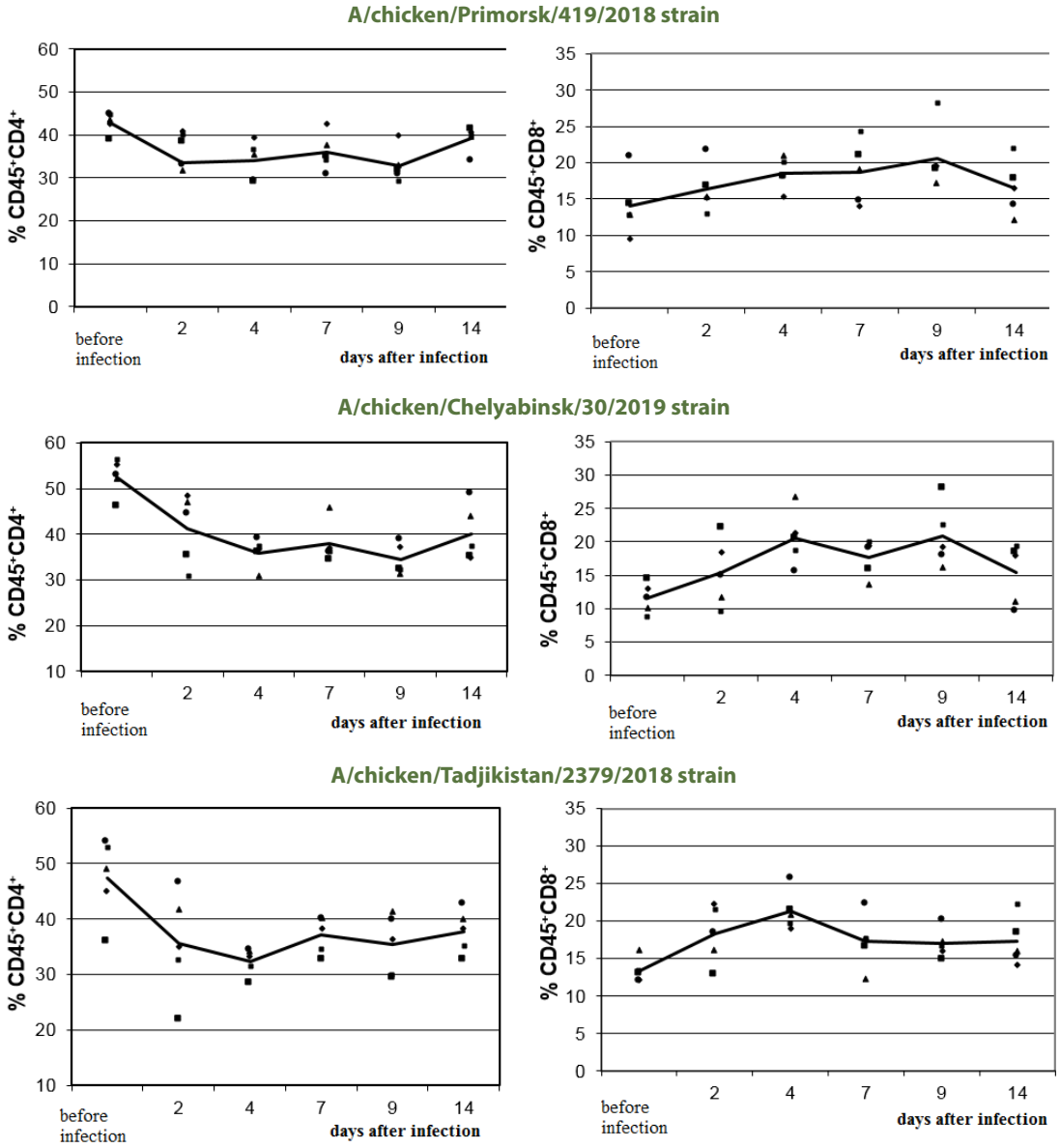

Fig. 1. Dynamics of T-cell subpopulations in chickens after their infection with H9N2 avian influenza virus isolates.
Solid line – arithmetic mean for group of 5 chickens; individual symbols – the percentage of cells for each chicken in the group

immune response with hemagglutination inhibition test and with flow cytometry.

All experiments in animals were carried out in strict accordance with the interstate standards on laboratory animal keeping adopted by the Interstate Council for Standardization, Metrology and Certification as well as according to Directive 2010/63/EU of European Parliament and the Council of 22 September 2010 about protection of animals used for scientific purposes.

Serological tests. Sera collected before and 14 days after infection were tested for antibodies to A/H9N2 avian influenza virus with hemagglutination inhibition (HI) test using commercial test-kit produced by the FGBI "ARRIAH" (Vladimir) in accordance with the instruction for its use. Before testing, sera were inactivated by heating at 56 °C for 30 minutes. Test results were recorded visually after complete red blood cell sedimentation in control wells (in the form of "button"). Test results were considered positive when the test sera contained specific antibodies to A/H9N2 avian influenza virus at a titre of 1:16 (4.0 $\log_2$) or higher.

Quantitative analysis of lymphocyte subpopulations. Dynamics of changes in the proportions of T-lymphocyte ($CD45^+CD3^+$, $CD45^+CD4^+$ and $CD45^+CD8^+$) and B-lymphocyte ($CD45^+$, $CDBu1a^+$) populations in the peripheral blood of chickens was examined by flow cytometry. For this purpose, blood samples were collected from chickens before and on day 2, 4, 7, 9 and 14 after infection in tubes containing K3-EDTA anticoagulant.

Lymphocytes were isolated from chicken peripheral blood according to standard method [27] using Ficoll-Paque™ PLUS medium for lymphocyte separation (BioWest, France). Labeled monoclonal antibodies, CD45-FITC, CD4-PE, CD8α-PE, CD3-PE and Bu1a-PE (Southern Biotech, USA), were used for sample preparation for lymphocyte surface marker detection. Lymphocyte samples (50 µl) were added to microtubes in several replicates (depending on number of used antibody panels). Fluorochrome-conjugated monoclonal antibodies (2 µl) were added and the tubes were incubated for 30 minutes at temperature of 4–8 °C. Unbound monoclonal antibodies were removed by centrifugation with phosphate buffered solution at 260 g for 10 minutes. BD FACS Calibur flow cytometer (Becton Dickinson, USA) was used for quantitative analysis of cells. Cell Quest Pro 1.0 software was used for obtained result assessment and processing.

Statistical analysis of the results. Statistica 10.0 programme was used for data statistical processing.

RESULTS AND DISCUSSION

Cell and humoral immunities were assessed in chickens after their infection with different three H9N2 avian influenza virus isolates.

The following clinical signs were observed in chickens on day 4 and day 7 after infection: depression, ruffled feathers, refusal from feed. No deaths were recorded in the infected chickens.

Blood samples collected from chickens before and on day 2, 4, 7, 9 and 14 after infection were examined with flow cytometry for quantitative analysis of lymphocyte subpopulations.

Significant decrease in $CD45^+CD4^+$ T-cell (T-helper) proportion and increase in $CD45^+CD8\alpha^+$ cytotoxic cell proportion were reported in chicken blood 2–4 days after their infection (Fig. 1). Relative counts of both populations in infected chickens differed from the initial levels (before infection) by 1.3–1.5 times for T-helpers and by 1.3–1.9 times for cytotoxic cells.

Increase in T-helper level in the blood was observed 9 days after infection. Both T-lymphocyte populations in infected chickens returned to normal levels by day 14 after infection but not in all tested chickens.

Progress of the infection had a suppressive effect on the immune system of the infected chickens. Obtained data on decrease of relative T-helper concentration in peripheral blood after infection confirm the data obtained by X. Hao et al. [21] and M. Dai et al. [22]. Significant increase

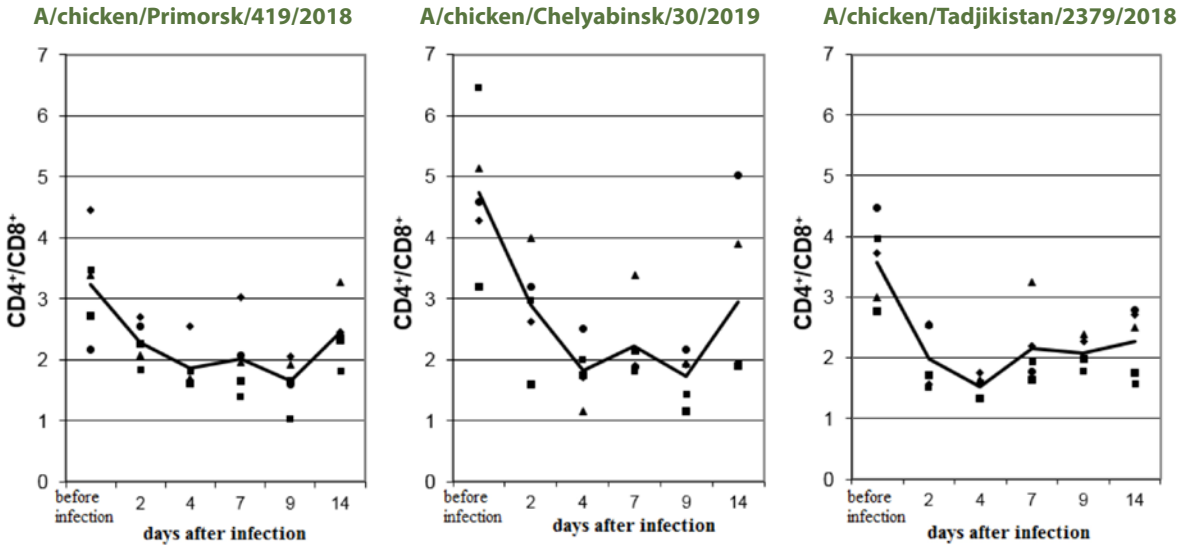

Fig. 2. Dynamics of $CD4^+/CD8^+$ ratio in chicken blood lymphocytes after infection with three H9N2 avian influenza virus isolates.
Solid line – arithmetic mean for group of 5 chickens;
individual symbols – the percentage of cells for each chicken in the group

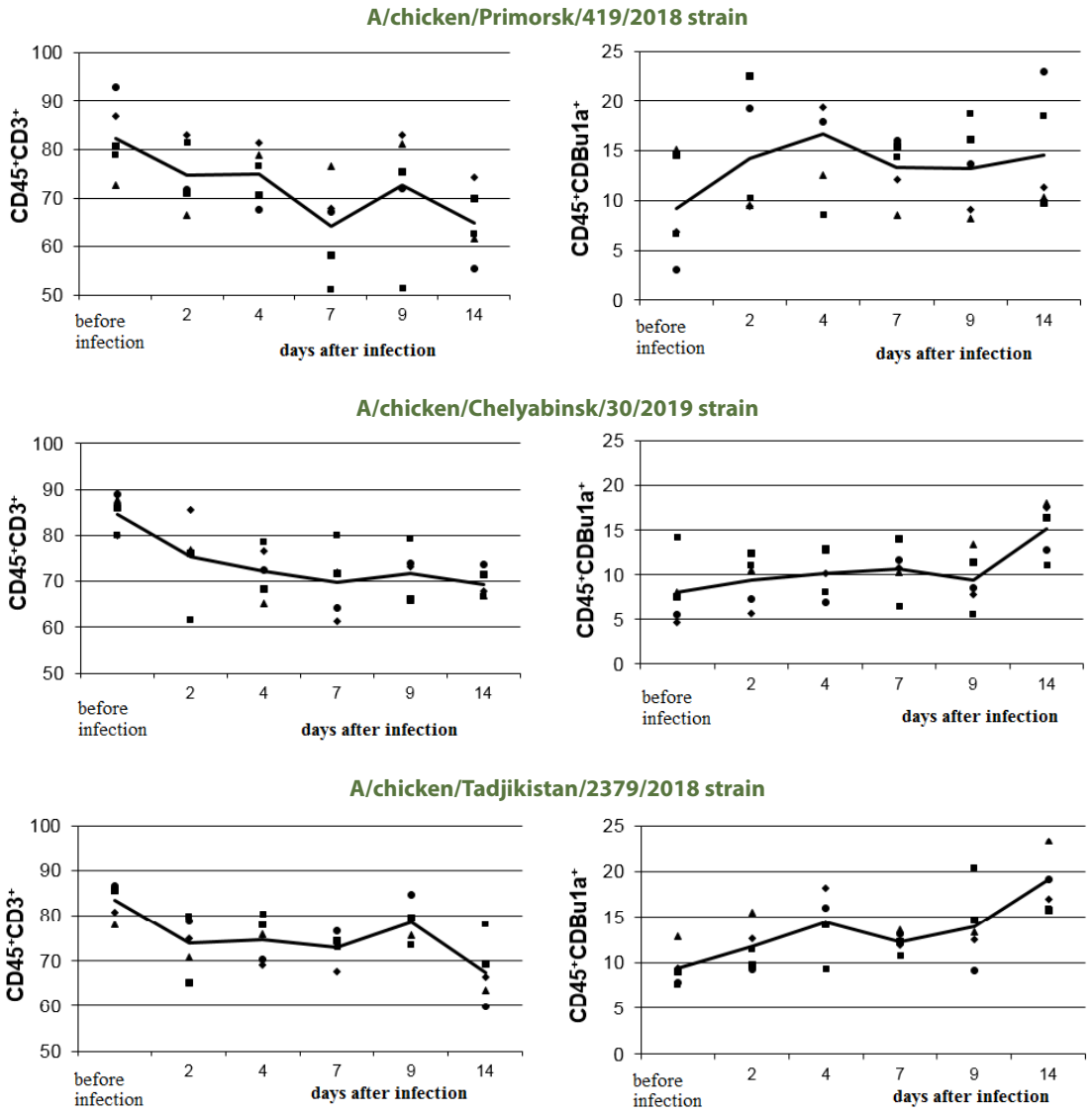

Fig. 3. Dynamics of T- and B-lymphocyte levels in chicken peripheral blood after infection with three H9N2 avian influenza virus isolates.
Solid line – arithmetic mean for group of 5 chickens;
individual symbols – the percentage of cells for each chicken in the group

in cytotoxic ($CD8^+$) T-cell proportion in blood of chickens infected with A/H9N2 avian influenza virus on day 5–7 after infection was also reported by M. Dai et al. [22].

Analysis of changes in $CD4^+/CD8^+$ ratio showed that it decreased by 1.9; 2.6 and 2.3 times in chickens of group 1, 2 and 3, respectively, on day 4 after infection due to decrease in relative $CD4^+$ counts and increase in a $CD8\alpha^+$ T-lymphocytes (Fig. 2). $CD4^+/CD8^+$ ratio increased again by day 14 after infection but it remained averagely 1.3–1.6 times lower than the initial one.

Yang Y. et al. [28] and Dai M. et al. [22] also demonstrated that viral infections in chickens induced immune suppression manifested, among others, by decrease in $CD4^+/CD8^+$ ratio in blood T-lymphocytes. On the contrary, vaccination gave rise to immune response activation and reverse dynamics towards to increase in $CD4^+/CD8^+$ ratio [15, 22].

Xue M. et al. [23] and Yang S. et al. [24] believed that an increase in the $CD4^+/CD8^+$ ratio after immunization and a decrease in the $CD4^+/CD8^+$ ratio after infection suggested that immune response enhanced after vaccination and immunity was suppressed in case of viral infection. Vaccination induced a pronounced humoral immune response and $CD4^+$ T-cell-mediated response.

Studies performed by L. Fu et al. [25] and M. Dai et al. [26] showed that the virus infection mainly stimulated $CD8^+$ T-cell response, and immunization stimulated $CD4^+$ T-cell response. High level of antibodies to A/H9N2 avian influenza virus and an increase in cytotoxic $CD8^+$ T-cell proportion play an important role in anti-virus protection [29, 30].

Figure 3 shows dynamics of relative T- and B-lymphocyte counts in infected chicken blood. Decrease in T-lymphocyte counts averagely by 15–20% was reported in all three groups that was indicative of insufficient cell-mediated immunity. Increase in relative B-lymphocyte counts by 5–10% depending on the group was reported as early as by day 14 after infection. Together with an increase in

the T-helper proportion, this demonstrated the activation of the immune response in infected chickens.

The function of B-lymphocytes responsible for humoral immunity is to transform B-cells into plasma cells secreting immunoglobulins having specific activity against the invaded antigen. Assessment of the dynamics of the relative B-lymphocyte counts in the blood of infected chickens revealed that they increased.

Chicken sera were HI tested before and on day 14 after infection. Results of tests for specific antibodies to A/H9N2 avian influenza virus are given in the Table.

Mean HI antibody titre in chickens of all groups was higher than 6 $\log_2$ on day 14 after infection. High anti-AIV antibody level was indicative of pronounced post-infection humoral immune response development.

Dai M. et al. [22] during comparative analysis of the key factors of immune protection of chickens infected with the A/H9N2 virus and SPF chickens immunized with an inactivated vaccine concluded that the lack of $CD8^+$ T-cells was a key cause of immunodeficiency and infection of poultry in vaccinated flocks.

Table
Results of HI tests of chicken sera for specific antibodies to A/H9N2 avian influenza virus

Isolate	Before infection		On day 14 after infection	
	Total number of samples/ positive samples	Mean antibody titre, $\log_2$	Total number of samples/ positive samples	Mean antibody titre, $\log_2$
A/chicken/ Tadjikistan/2379/2018	15/0	0	15/15	8.7 ± 0.3
A/chicken/ Primorsk/419/2018	15/0	0	15/15	8.1 ± 0.4
A/chicken/ Chelyabinsk/30/2019	15/0	0	15/15	6.9 ± 0.4

CONCLUSION

Key factors of immune response of chickens infected with various A/H9N2 avian influenza viruses were examined. Quantitative analysis of peripheral blood lymphocyte subpopulations in chickens infected with three A/H9N2 avian influenza virus isolates revealed the following changes caused by the virus infection: decrease in relative T-lymphocyte counts in blood, significant changes in T-lymphocyte subpopulation composition towards to decrease of $CD4^+$cell proportion and increase in $CD8^+$ cell proportion and, as a result, decrease in $CD4^+/CD8^+$ ratio. Assessment of the dynamics of T- and B-lymphocyte levels in blood of infected chickens showed decrease in relative T-lymphocyte counts and increase in relative B-lymphocyte counts. HI tests demonstrated pronounced humoral immune response. No significant differences both in humoral and cell immune responses were detected in chickens infected by three different low pathogenic A/H9N2 avian influenza virus isolates.

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Received 27.08.2021
Revised 08.10.2021
Accepted 18.10.2021

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