UDC 619:578.832.1:636.592

DOI 10.29326/2304-196X-2018-3-26-46-51

RESULTS OF EXPERIMENTAL INFECTION OF TURKEYS WITH A/DUCK/ALTAI/469/14 H5N1 STRAIN OF AVIAN INFLUENZA VIRUS

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SUMMARY

The data on experimental infection of 6-week-old Big-6 cross turkeys with an epidemic A/duck/Altai/469/14 H5N1 clade 2.3.2.1c strain of avian influenza virus are presented. The characteristics of the infection process in birds inoculated intranasally at a dose of 5.0 lg EID_{50}/0.5 cm^3 are described with an indication of the incubation period and the mean time of death. The pathomorphological changes at the tissue and cellular level are shown based on histological and immunohistochemical studies of fragments of respiratory, digestive, cardiovascular, nervous, excretory, lymphoid and muscular systems of experimental birds. The testing was carried out using paired preparations of paraffin-embedded tissue sections from experimentally infected and healthy turkeys. One sample was subjected to histological staining using hematoxylin and eosin dyes, and its duplicate was subjected to immunohistochemical assay using a preparation of polyclonal antibodies as primary antibodies against the ribonucleoprotein of avian influenza virus. The results of histological and immunohistochemical studies are photodocumented and presented in the paper. Inflammatory and necrotic lesions of varying severity are detected in the preparations of the trachea, lung, muscular stomach, glandular stomach, small intestine, large intestine, pancreas, brain, cerebellum, heart, kidneys, liver and spleen of turkeys. Immunohistochemical analysis showed the greatest distribution of the influenza virus antigen in the cerebral endothelium, cerebellar Purkinje neurons, acinar cells of the pancreas and in myocardiocytes of the heart. In the course of the experiment it was established that A/duck/Altai/469/14 H5N1 caused a generalized form of infection in turkeys with clinical and pathologic lesions characteristic of highly pathogenic avian influenza.

Key words: avian influenza, H5N1, turkey, experimental infection, histology, immunohistochemistry.

INTRODUCTION

Highly pathogenic avian influenza (HPAI) associated with Asian genetic lineage virus (A/goose/Guangdong/1/1996 H5N1) has demonstrated an epidemic pattern of spread and proved itself to be capable of causing disease in different bird species, as well as in mammals. Isolates underwent significant variations and antigenic modifications during their circulation within the bird population, and that resulted in the emergence of 10 main genetic groups of the virus (0–9) and multiple sublineages [6, 12].

Genetic clade 2.2 isolates closely related to the viruses recovered from Qinghai Lake waterfowl caused the outbreaks of the disease in domestic and wild birds in the Russian Federation between 2005 and 2007. In 2008, clade 2.3.2 avian influenza A/H5N1 virus isolates were recovered in the Primorsky Krai. This genetic subgroup spread in China and Vietnam and gave rise to several virus sublineages [5, 7]. In the Russian Federation, clade 2.3.2 A/H5N1 isolates caused HPAI outbreaks in the wild bird population in the Republic of Tyva in 2009–2010.

In 2014, a new reassortant of HPAI sublineage 2.3.2.1c was newly detected during acute infection outbreaks in backyard poultry in the Altai Krai. In 2015, the disease outbreaks caused by clade 2.3.2.1c A/H5N1 virus were reported in wild birds in the Siberian and Southern Federal Districts of Russia [5].

Globally, the isolates of that genetic subgroup spread in the countries of Southeast Asia, West Africa and Middle East in the period from 2014 until present day; sporadic HPAI A/H5N1 outbreaks were registered in Western Europe [6, 7]. Therewith, in 2017 and during the first half of 2018, the co-circulation of A/H5N1, A/H5N2, A/H5N3, A/

H5N6 and A/H5N8 virus strains as well as of the low pathogenic subtypes of avian influenza virus occurred in China, Nigeria, Cameroon, India, Iran, Laos and Vietnam [5]. Such epidemic situation poses the risk of virus reassortment and mutation that may result in significant changes in the biological properties of the virus.

Russia is still at risk of A/H5N1 virus re-introduction to the country from endemic areas along the East-African and Central- and East-Asian flyways of wild birds, and that is a matter of concern to veterinary services and poultry farm owners.

Turkey meat production is an actively developing poultry industry sector in this country. In 2017, meat product commercial production volume was estimated at 222 thousand tons in carcass weight, it is expected to increase to 485 thousand tons by 2020 [1]. However, this bird species is highly susceptible to A/H5 virus [4, 8, 11, 13]. For instance, more than 500 thousand turkeys were destroyed in the Rostov Oblast during the HPAI A/H5N8 outbreaks at the end of 2016 and at the beginning of 2017 [5].

The studies of HPAI peculiarities in turkeys caused by A/duck/Altai/469/14 H5N1, isolate of A/H5N1 current genetic subgroup 2.3.2.1c, are of scientific and practical interest.

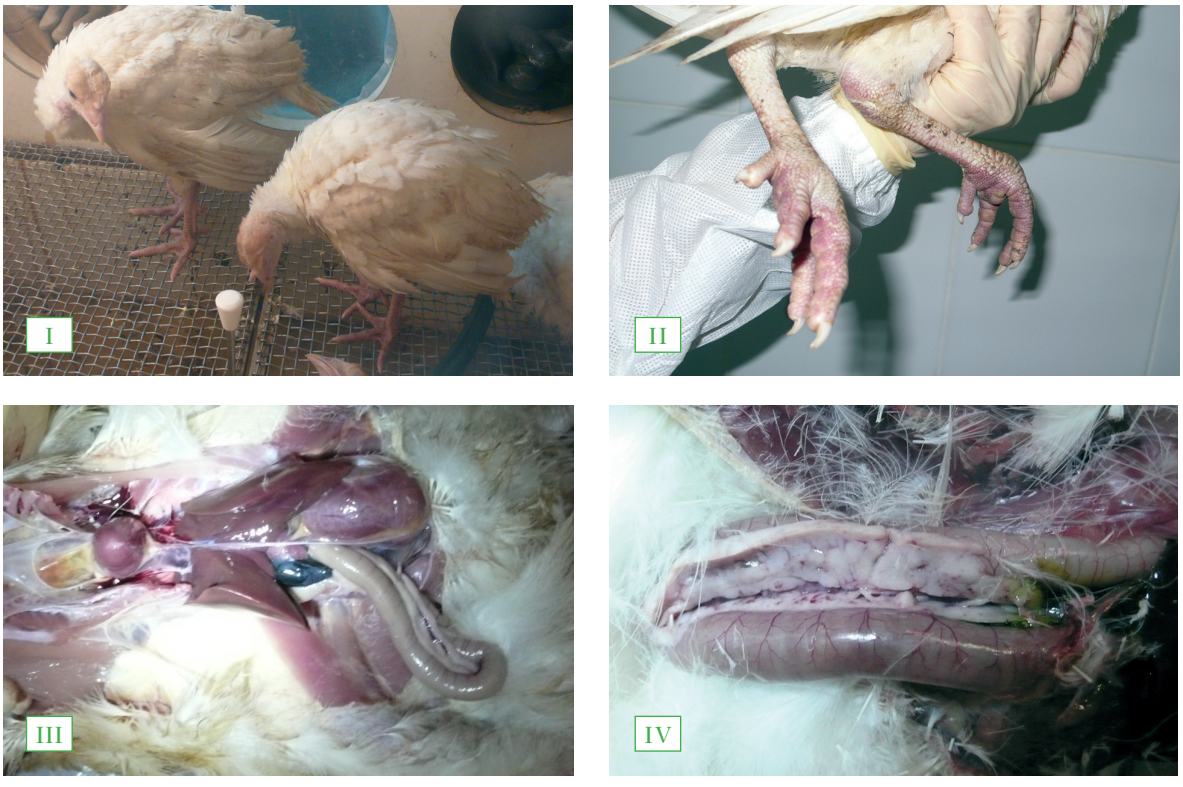
MATERIALS AND METHODS

Virus. Avian influenza A/duck/Altai/469/14 (H5N1) virus isolate recovered from the biological material samples collected from domestic ducks was used in the experiment. The samples were submitted to the FGBI "ARRIAH" in 2014. Virus isolation was performed in 10-day-old chicken embryonated eggs free from pathogenic microflora (SPF CEE). Suspension (10–20%) was prepared from the biological material based on pH 7.2–7.4 phosphate buffer solution; 0.2 cm^3 of suspension was inoculated to CEE allantoic cavity. Extraembryonic fluid (EEF) was collected from addled eggs with the embryos that had died after 24 hours of incubation or later for subsequent tests. The turkeys were infected with the virus material of passage 2 in 10-day-old SPF CEEs.

The experimental infection of turkeys. Six-week-old Big-6 cross turkeys non-immune to avian influenza virus were divided into 3 groups and tested: group 1 (10 birds) – for pathological process, group 2 (17 birds) – for pathomorphological changes; group 3 (6 birds) served as control group. The turkeys were kept in the isolated chambers with free access to feed and water. Group 1 and 2 birds were experimentally infected with the virus at a dose of 5.0 lg EID_{50}/0.5 cm^3 intranasally (0.25 cm^3 per nare). SPF EEC extraembryonic fluid was administered to control group birds in the same way. The experiment lasted for 5 days during which the birds were clinically observed daily, the dead ones were necropsied.

Histological and immunohistochemical assays. During the experiment, samples (0.5 cm^3) were collected from the following internal organs of dead, euthanized for diagnostic purposes and AIV-uninfected turkeys: trachea, lung, gizzard, proventriculus, small intestine, large intestine, pancreas, brain, cerebellum, heart, kidneys, liver, spleen and skeletal muscles. The preparations were fixed in alcohol/formol mixture according to Shaffer; after 48 hours, the material was processed and embedded into paraffin blocks. The paired sections (5 μm thick) of turkey organ tissues were prepared using Microm HM340E rotary microtome. One of the preparations was stained with he-

Fig. 1. Clinical signs and postmortem lesions in experimental group 1 turkeys
I – rumpleness of plumage, drooping wings (paresis); II – foot cyanosis;
III – hydropericarditis; IV – pancreatic haemorrhages.



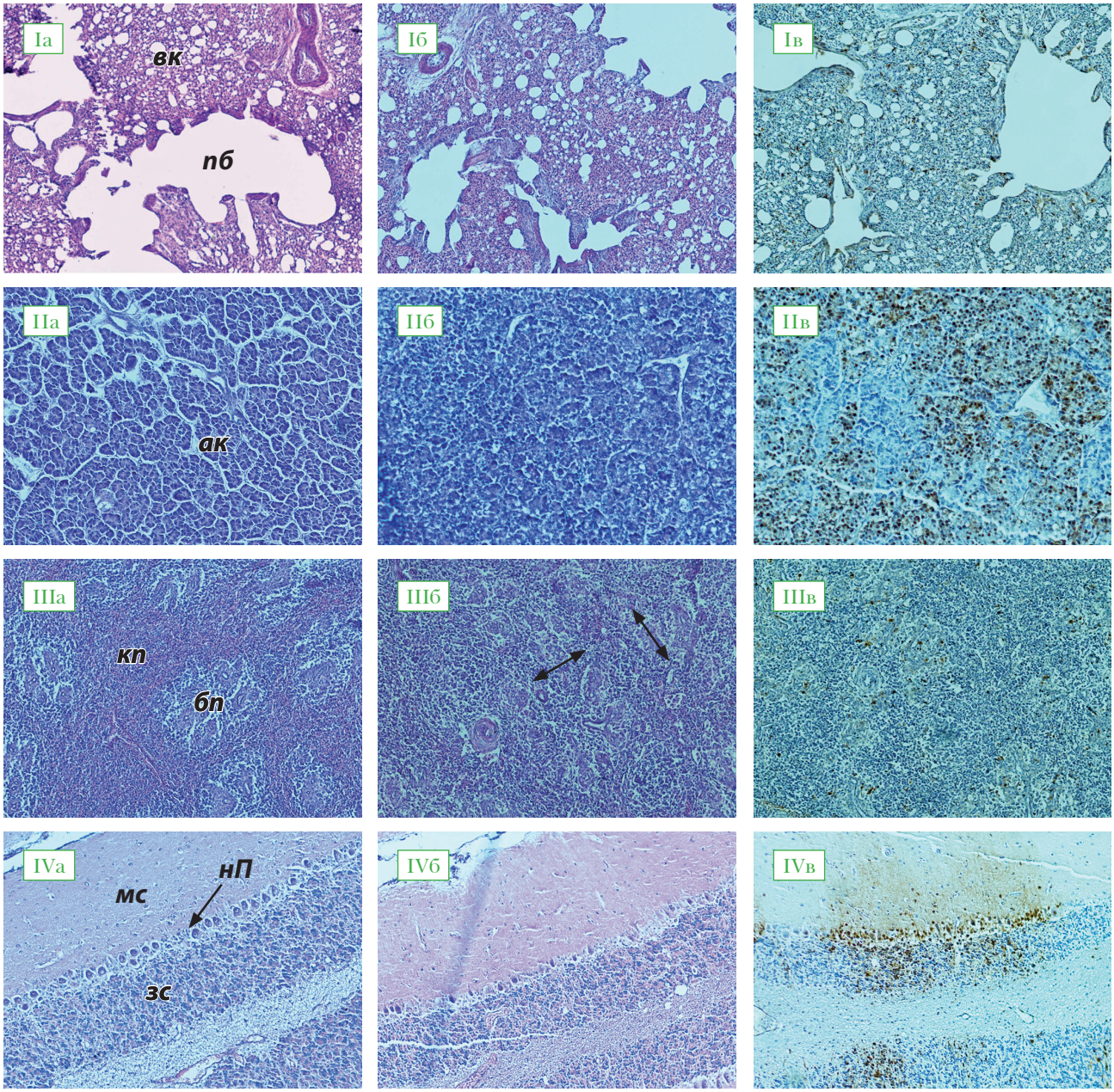


Fig. 2. Microphotographs of turkey tissue preparations (day 3 of the experiment, 100-fold magnification)

I – lungs: пб – parabronchus; вк – air capillaries;
II – pancreas: ак – acinar cells;
III – spleen: бп – white pulp; кп – red pulp;
↕ – area of necrosis;
IV – cerebellum: мс – molecular layer;
зс – glandular layer; нП –Purkinje neurons;
а – control group, H-E stain;
б – experimental group, H-E stain;
в – IHC staining with DAB-hematoxylin.

matoxylin-eosin (H-E), the other one was subjected to immunohistochemistry (IHC). The samples of sections were prepared for IHC staining according to the previously described procedure with some modifications [2].

The assay was performed according to the protocol for Reveal Biotin-Free Polyvalent DAB kit (Spring Bioscience, USA) for IHC tissue testing. Anti-type A-avian influenza virus-rNP rabbit immunoglobulin G, purified serum IgG fracture, was used as a primary antibody.

The results of IHC assay were scored as follows:
– no virus antigen (Ag) was detected;
+ single foci of virus Ag were detected;
++ several foci of virus Ag aggregation were detected;
+++ multiple foci of virus Ag aggregation were detected.

RESULTS AND DISCUSSION

In order to study the pathogenesis of the infection caused by avian influenza A/duck/Altai/469/14 (H5N1) virus in turkeys, the birds were observed for clinical signs daily. An ordinal number was assigned to each experimental group 1 bird in order to identify the disease pattern and to subsequently estimate mean death time (MDT) [8]. In the course of the experiment, one bird from control group (group 3) was euthanized every day for diagnosis, necropsied, and its internal organs were collected for subsequent comparative assessment.

Table
Results of IHC tests performed on the tissues of turkeys infected with avian influenza A/duck/Altai/469/14 (H5N1) virus

Type of tissue	Localization of AIV antigens	Concentration of AIV antigens (IHC)
Respiratory tract		
Trachea	Submucosal haemorrhage foci	+
Lungs	Walls of parabronchi and air capillaries (Fig. 2, *Iв*)	++
Digestive system		
Proventriculus	Intestinal gland epithelium	+
Pancreas	Acinar cells (Fig. 2, *IIв*)	+++
Small intestine	Villus epithelium, the lumen of the crypts of Lieberkühn	++
Liver	Hepatocytes, vascular endothelium	+
Excretory system		
Kidneys	Distal canaliculus epithelium	+
Cardiovascular system		
Heart	Cardiomyocytes	++
Lymphoid organs		
Spleen	Lymphatic vessel endothelium (Fig. 2, *IIIв*)	++
Nervous system		
Cerebellum	Purkinje cells (Fig. 2, *IVв*)	+++
Brain	Vascular endothelium	++
Skeletal muscles	No diffused antigens were observed	–

The turkeys developed the clinical signs of the disease in 2 days post virus inoculation. The birds were depressed, some of them would stand with their wings drooped (paresis). Half of the turkeys had nasal discharge, dyspnea, white and yellow diarrhea (Fig. 1, *I*).

In three days after the beginning of the experiment, the test birds demonstrated pronounced circulatory system disorders: neck and foot cyanosis was observed in eight out of ten birds, including those that had died (Fig. 1, *II*).

Seven out of ten turkeys developed nervous system disorders of varying severity such as incoordination, tremor, paresis, eye nystagmus in 3–4 days post infection.

The experiment completed in 5 days after the inoculation of turkeys with avian influenza A/duck/Altai/469/14 (H5N1) virus resulting in the death of all the test birds. The infection incubation period was 2 days; the estimated MDT was 3.8 days. The following pathological lesions were detected at the necropsy of the birds that had died and of those euthanized for diagnosis: splenomegaly, haemorrhagic and exudative diathesis of many internal organs, myocardiodystrophy and hydropericarditis, pulmonary engorgement, nephritis, macrofocal pancreatic haemorrhages, gastric and intestinal catarrhal inflammation (Fig. 1, *III–IV*).

Previous studies on experimental infection of certain Galliformes species with AI A/H5N1 virus isolates recovered in Eurasia and Africa in 2007–2012 evidenced in all the cases the fulminant character of the disease with high lethality in chickens, ducks and quails (MDT was 2–6 days) [3, 4, 8–13]. The clinical signs and severe lesions of internal organs are similar to the findings of the present study; however, there are no data on cyanosis of unfeathered skin in the previously published papers.

Samples of the following internal organs were taken from diseased and dead birds of experimental group 2 starting from day 1 post infection: respiratory, digestive, cardiovascular, nervous, excretory, lymphoid and muscular system tissues. At least two birds were used for postmortem lesion examination.

Histological analysis of the tissues taken from experimental group 2 turkeys demonstrated lesions in all the organ systems of the bird body with the most severe of them detected in circulatory, nervous and digestive systems. There were the following tissue lesions: necrosis of various severity and inflammations.

Respiratory tract. Focal haemorrhages associated with RBC infiltration into mucosal and submucosal membranes were detected in tracheas. Moderate atelectasis of air capillaries was observed in lungs (Fig. 2, *Iб*).

Digestive system. The lumen of submucosal intestinal glands in proventriculus was narrowed, lymphocyte pro-

liferation foci were observed in the interlobular space. The foci of inflammatory cell infiltration were detected at the bases of small intestine villi, and epithelial cell vacuolization – in their apical parts. Vast areas of coagulation necrosis of acinar cell epithelium were detected in the pancreas preparation (Fig. 2, *III6*).

Excretory system. Renal tubule dilation and renal corpuscle necrosis with RBC escape were observed in kidneys.

Cardiovascular system. Myocardial dystrophy, the vast areas of cardiomyocyte necrosis, lymphoplasmacytic infiltrate were observed in the heart.

Lymphoid organs. The regions of white and red splenic pulp were almost undistinguishable due to multiple foci of lymphoid cell necrobiosis (Fig. 2, *III6*).

Nervous system. Neuropil vacuolization, perivascular edema were detected in brain tissue. Purkinje neuron necrosis was observed in cerebellum, there were haemorrhagic areas in molecular and granular layers (Fig. 2, *IV6*).

The detected lesions apparently correlated with AIV Ag distribution in the samples. The data on AIV Ag localization and concentration in the tested tissues taken from turkeys are presented in the table.

Distribution of AIV antigens was the highest in cerebral vascular endothelium, cerebellar Purkinje neurons, pancreatic acinar cells and myocardiocytes.

The postmortem lesions detected during the study are comparable to the results of the studies of the previous years on experimental infection of the Galliformes.

In 2015, similar lesions with AIV antigen localized in cerebellum, small intestine and trachea were observed when the virus suspension of A/duck/Altai/469/14 H5N1 isolate was administered intranasally to 8-week-old chicks [2].

Experimental data on infection of 8-week-old chicks with avian influenza A/grebe/Tyva/433/10 (H5N1) virus described by I. V. Bakhchin et al. (2014) showed that the pathogen propagated mainly in the pulmonary tissues and intestinal tract of chickens [3].

K. Bertran et al. (2013) found high Ag concentration and related lesions in nervous, digestive and cardiovascular systems after the inoculation of 8-week-old quails with avian influenza A/grebe/Basque Country/06.03249/2006 (H5N1) virus [9].

High virulence of avian influenza A/H5N1 virus for the Galliformes remains, therefore, an important biological property of the virus throughout the years.

CONCLUSION

Based on the experimental data obtained, it is concluded that avian influenza A/duck/Altai/469/14 (H5N1) virus causes the generalized form of infection in turkeys with clinical signs and postmortem lesions characteristic of highly pathogenic avian influenza.

Immunohistochemical assay showed pathogen accumulation in vascular endothelium, in the cells of internal organs, as well as in the tissues of cerebellum and cerebral hemispheres. Virus antigen distribution was the highest in the foci of necrosis and inflammation in the affected tissues of nervous, cardiovascular and digestive systems.

The results of the present study on infection of turkeys with Asian group AI A/duck/Altai/469/14 (H5N1) virus complement current literature data on the studies of AIV field isolate pathobiological characteristics and are indicative of stable high pathogenicity of clade 2.3.2.1c for this bird species.

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Submitted on 25.07.18
Approved for publication on 16.08.18