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ELISA DIAGNOSTIC OF METAPNEUMOVIRUS AND REOVIRUS INFECTIONS IN POULTRY

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Summary. The import of high-yielding poultry breeds has caused the proliferation of viral diseases which previously did not occur at Ukrainian poultry enterprises, such as avian metapneumovirus (AMPV) and avian reovirus (ARV) infections. Most pathogens cause immunosuppression in poultry (including ARV infection) and are frequently diagnosed as co-infections with other infectious agents which are capable of causing pathological changes in poultry (especially if aided by stress). AMPV and ARV co-infection cannot be excluded; these diseases are manifested in respiratory and intestinal disorders, their subclinical, latent, and associated forms present a challenge for laboratory diagnostic as well as for the implementation of prophylactic measures. The objective of the present work was to conduct the epizootological survey of AMPV and ARV infections incidence at poultry enterprises of different forms of property and to produce an antigen suitable for the development of an ELISA test-system for the determination of antibodies against AMPV in blood serums of turkeys and hens. The pathological material used in the experiments (heads, tracheas, and lungs from ill or deceased poultry) for the isolation of pneumovirus was collected from 60-day-old turkeys in 2009. The pathological material for virology research was preserved at – 20 °C. Serology and virology research was conducted using standard methods. AMPV and ARV incidence monitoring was conducted at poultry enterprises of Kharkiv region among turkeys of 'Big-6' and 'Big-8' breeds imported from Germany and Hungary (aged 4–480 days), among turkeys of 'Large White' breed (aged 70 days), and hens of 'Birkivska Barvysta' and 'Hisex Brown' (aged 250 and 180 days correspondingly). On average, 78.0% of the examined turkeys with antibody titers 1:3,776–1:27,869, 100% of the 'Birkivska Barvysta" hens with antibody titers 1:4,997–1:10,414 and 0% of the 'Hisex Brown' hens were AMPV-positive. No hens were ARV-positive. At the Luhansk National Agrarian University (Kharkiv) the development of a domestic ELISA test-system for the diagnostic of AMPV in turkeys and hens is carried out.

Keywords: epizootiology, metapneumovirus, reovirus, infection, poultry, purification and concentration, ELISA

Introduction. Some pathogens can cause diseases regardless of the environmental factors; however, the majority of diseases occur under specific conditions. Stress frequently plays the role of such a condition. The pathogens of the first group mentioned cause immunosuppression in poultry, and are frequently diagnosed as co-infections. Avian metapneumovirus (AMPV) and avian reovirus (ARV) co-infections cannot be excluded; these infections cause respiratory and gastric disorders.

AMPV infection is a respiratory disease manifested in nasal discharge, sneezing, labored breathing accompanied by rales, inflammation of the upper airways and infraorbital sinuses. This disease in turkeys is frequently called rhinotracheitis (TRT) and in broiler chickens — 'swollen head syndrome' (SHS). In recent literature, the disease of the upper airways of turkeys and hens is frequently called avian metapneumovirus (AMPV) (Borisova and Starov, 2006).

ARV infection is a highly contagious disease of young poultry and synanthropic birds of all breeds and species, which takes latent and persistent forms and is manifested by respiratory and intestinal disorders. For the first time in Ukraine ARV was registered and described by V. V. Herman in 1972. Immunological properties of more than 10 reovirus strains, isolated from different poultry species have been investigated (Abdil'aziz, 1990; German and German, 2002; Aliev, 2005).

For the first time the viral etiology or rhinotracheitis (TRT) in Ukraine was demonstrated in turkeys at poultry enterprises in Donetsk, Chernivtsi and Kharkiv regions in 2008. Clinical examination of 'Big-6' turkeys aged 30–70 days revealed the following symptoms in diseased individuals: inactivity, drowsiness, swelling of the head, intermaxillary space, and infraorbital sinuses, labored breathing, mucus outflow from the nasal passages. Mortality varied from 5% to 14%. Samples of pathological material were collected from the diseased poultry; the causative agent was identified using PCR and isolated using virology methods, it belongs to the genus *Metapneumovirus*, subtype APV/B (Nalyvaiko et al., 2011).

Latent and subclinical forms of infectious diseases, as well as their ability to cause co-infections present a challenge for laboratory diagnostic, implementation of prophylactic and anti-epizootic measures.

To control the immunity stress in immunized poultry against the infections in question certain laboratories of veterinary medicine used 'IDEXX' (USA) ELISA test-kits, which were registered in Ukraine and cost up to 8–10 thousand UAH. Domestically produced test-kits at that time were absent.

However, in 2012 at the Poultry Research Institute of the National Academy of Agrarian Sciences of Ukraine (PRI NAAS), using a domestic virus strain isolated from broiler chickens, an ELISA test system was developed (Riabinin, Nikolaenko and Nalyvaiko, 2012).

Considering the advanced nature of this method, the development of a domestic ELISA test system for the determination of antibodies against AMPV in hen and turkey blood serums is urgent.

The rapidity of the reaction, reproducibility of the results and their automated recording, a potential for standardization make ELISA the most effective, easy to use, and economically sound method for surveying the incidence of the diseases in question at poultry enterprises.

The aim of the work was to conduct an epizootological monitoring of AMPV and ARV infections at some poultry enterprises, to obtain an antigen for the development of an ELISA test system for the determination of antibodies against AMPV in turkey and hen blood serums.

Material and methods. The pathological material used in the experiments (heads, tracheas, and lungs from ill or deceased poultry) for the isolation of pneumovirus was collected from 60-day-old turkeys in 2009 at poultry enterprises in Chernivtsi region. The pathological material for virology research was preserved at – 20 °C.

Indication and identification of the causative agent in the pathologic material were conducted at the Laboratory of the diagnostic of poultry viral diseases FGBI 'ARRIAH' (Vladimir, Russia).

For the determination and quantification of antibodies against AMPV in blood serum 'IDEXX' test-kits (USA) were used. For the determination of ARV infection, a domestic ELISA test-kit developed by the PRI NAAS was used.

Incubatory eggs and chickens: intact hen and turkey embryos; chickens obtained from hens without antibodies against AMPV infection.

Cell cultures. A primary cell culture of chick embryo fibroblasts (CEF).

Nutrient mediums, serums, and solutions: MEM (Minimum Essential Medium) or DMEM (Dulbecco's Modified Eagle's Medium) liquid growth medium with L-glutamine; liquid growth medium No. 199 with L-glutamine; liquid growth medium DMEM/F12 with HEPES; cow embryos blood serum; 0.25% trypsin solution 'USBIO'; 0.02% Versen solution; Hank's solution 'Hyclone', produced by 'Biolot'.

Virology research. Reproduction and the main biological properties of the isolated pneumovirus were studies using quail, hen, and turkey embryos: changes in the embryos and in the chorioallantoic membrane were recorded. The infectious titer of the virus was determined according to Reed and Muench (1938) and expressed in lg $TCID_{50}$ in 1.0 cm^3 (tissue culture infectious dose).

Purification and concentration of the virus. The virus was concentrated using PC-6 and MSE centrifuges.

Results. In 2016–2017 epizootological monitoring of the incidence of AMPV and ARV was conducted at poultry enterprises of Kharkov region among turkeys of 'Big-6' and 'Big-8' breeds imported from Germany and Hungary (aged 4–480 days), among turkeys of 'Large White' breed aged 70 days, parent flocks of 'Birkivska Barvysta' and 'Hisex brown' hens aged 250 and 189 days respectively. 206 samples of hen and turkey blood serums from 5 poultry enterprises were examined using serologic assays. On average, 78.0% of the examined turkeys with antibody titers 1:3,776–1:27,869, 100% of the 'Birkivska barvysta' hens with antibody titers 1:4,997–1:10,414 and 0% of the 'Hisex Brown' hens were AMPV-positive (IDEXX). No hens were positive towards ARV PRI NAAS) (Table 1).

In order to create a domestic ELISA test system for the determination of antibodies against AMPV in hen and turkey blood serums, first, its main components were produced.

Table 1 — The results of the serology research conducted using ELISA (n = 206)

Poultry breed	Number of samples	Age, days	ELISA antibody titers (M ± m) towards:	
			AMPV	ARV
Hyperimmune chicken serum (control)	1	1	1:4,963	1:6,400
Negative serum (control)	1	1	0	0
Turkeys				
'Big-6' (Enterprise 1)	40	4	1:3,776–1:14,404 (1:7,505 ± 698)	—
		22–25	1:68–1:594 (1:363 ± 70)	—
'Big-8' (Enterprise 2)	17	25–30	1:78–1:740 (1:394 ± 69)	—
	20	480	1:4,865–1:27,869 (1:16,564 ± 1,599)	—
'Large White' (Enterprise 3)	80	70	1:0–1:9,547 (1:1,298 ± 192)	—
Hens				
'Birkivska Barvysta' (Enterprise 4)	24	250	1:4,997–1:10,414 (1:7,162 ± 633)	1:1,402 ± 83
'Hisex Brown' (Enterprise 5)	25	189	1:87–1:958 (1:453 ± 54)	1:127 ± 190

Production of the antigen. The accumulation of AMPV was accomplished by reproduction in CEF cell culture. The development of characteristic syncytium took place after 3 passages, in 48–72 h after inoculation. The infectious titer of PVT-09/B strain grown in CEF cell culture amounted to 4.33 lg $TCID_{50}/cm^3$.

Purification and concentration of the antigen. 1,200 ml of the obtained antigen were purified and concentrated using an original method: PVT-09/B AMPV strain seed of an established infectious titer was frozen to – 20 °C with subsequent thawing to + 4 °C twice.

1st step. Preliminary virus purification — the cellular debris was removed from the culture liquid by centrifugation at 1,000 g and + 4 °C, the supernatant was collected.

2nd step. Virus concentration — the purified virus seed was mixed with PEG 6000 (polyethyleneglycol) (6% by volume) and incubated at + 4 °C, and then centrifugated at 12,000 g and + 4 °C. The obtained sediments were resuspended in TSE buffer (Tris/Saline/EDTA buffer pH 7.6).

3rd step. Final virus purification — the samples were centrifugated through 20–30% sucrose solution at 76,000 g and + 4 °C. The obtained sediment was resuspended in TSE buffer (10 ml). The obtained antigen was used to produce hyperimmune chicken blood serums and to sensitize ELISA test plates.

Negative and positive serums production. Hyperimmunization of chickens and turkey with TRT antigen yielded positive chicken blood serums with antibody titers 1:3,200, turkey 1:6,400 (IDEXX).

Negative serums were obtained from healthy 30-day-old chickens grown in a sterile environment. The antibodies in the negative serums were absent.

Conclusions. According to the results of the immunosurvey, conducted using ELISA test systems, at poultry enterprises in eastern regions of Ukraine AMPV infection is prevalent among turkeys and hens of different breeds and age. Meanwhile, ARV infection, in its latent and persistent forms (not manifested in specific symptoms) is prevalent in domestic populations of hens, with antibody titers 1:1,402 ± 83. The most susceptible turkey breed towards AMPV is 'Big-6' (an imported breed), and the least — 'Large White' (36%). 100% of the examined 'Birkivska Barvysta' hens were virus carriers, with antibody titers 1:10,414 (as determined by ELISA). Technological parameters for the purification and concentration of AMPV antigen were developed; positive chicken blood serums with antibody titers 1:3,200, turkey 1:6,400 (as determined by ELISA) were obtained.

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