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# Differentiation of avian influenza and Newcastle disease viruses in organ samples from sick and dead chickens using a rapid test kit

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## ABSTRACT

**Introduction.** Avian influenza virus (AIV) and Newcastle disease virus (NDV) pose serious threats to poultry health. Both pathogens are highly contagious and, due to their rapid spread, can lead to significant economic losses. Overlapping clinical signs complicate field differentiation of these diseases and delay response measures to isolate affected poultry. Rapid disease detection is critical for ensuring a timely response.

**Objective.** To develop a user-friendly test kit for detection of AIV and paramyxoviruses in organ samples from sick and dead chickens during disease outbreaks in commercial poultry operations.

**Materials and methods.** To differentiate AIV and paramyxoviruses in pathological samples collected from sick and dead birds, 96-well plates coated with fetuin and anti-NDV IgY in designated wells were used. The results obtained were compared with those from polymerase chain reaction (PCR) and virus titration in chicken embryos.

**Results.** The developed method for pathogen detection is based on distinct virus-binding principles: influenza virus binds to a receptor analog, while paramyxoviruses bind to NDV specific antibodies. Previous studies using hundreds of strains have demonstrated that influenza A virus of various subtypes binds to the sialoglycosyl residues of bovine fetal serum protein – fetuin. In contrast, none of the paramyxovirus isolates tested bound to this sialoglycoprotein. For paramyxovirus capture, immunoglobulins isolated from the egg yolks of chickens immunized against NDV were utilized. Binding was performed in 96-well plates using a test-kit analogous to enzyme-linked immunosorbent assay (ELISA).

**Conclusion.** The developed method enables the identification and differentiation of AIV and NDV in organ tissue homogenates from infected chickens within a few hours, representing a significant step toward preventing the spread and facilitating the eradication of dangerous disease outbreaks.

**Keywords:** Newcastle disease virus (NDV), avian influenza virus (AIV), differential diagnosis

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# Тест-система для дифференциации вирусов гриппа птиц и ньюкаслской болезни в органах больных и павших кур

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## РЕЗЮМЕ

**Введение.** Грипп птиц и ньюкаслская болезнь представляют собой серьезную угрозу для здоровья птиц. Оба заболевания характеризуются высокой контагиозностью и в условиях быстрого распространения могут привести к серьезным убыткам. Признаки болезней часто схожи, что затрудняет быструю диагностику и принятие экстренных мер по изоляции больных особей. Оперативное распознавание заболеваний является критически важным для своевременного реагирования.

**Цель исследования**. Целью работы является разработка простой тест-системы для детекции вируса гриппа птиц и парамиксовирусов в органах больных и павших птиц при возникновении вспышек заболеваний в птицеводческих хозяйствах.

**Материалы и методы.** Для дифференциации вирусов гриппа птиц и парамиксовирусов в патологическом материале, полученном от больной и павшей птицы, применяли 96-луночные планшеты, покрытые раствором фетуина и анти-ВНБ IgY в соответствующих лунках. Полученные данные сопоставляли с результатами, полученными методами полимеразной цепной реакции и титрования на куриных эмбрионах.

**Результаты.** Разработанный метод выявления возбудителей основан на разных принципах связывания вирусов. Вирус гриппа связывается с рецепторным аналогом, а парамиксовирусы – с антителами к вирусу ньюкаслской болезни. Ранее на примере сотен штаммов было показано, что вирус гриппа А разных субтипов связывается с сиалогликозильными остатками сывороточного белка эмбриона коровы – фетуина. В то же время ни один из исследованных при проведении работы изолятов парамиксовирусов не связывался с данным сиалогликопротеином. Для связывания парамиксовирусов использовали иммуноглобулины, выделенные из яичного желтка кур, иммунизированных против ньюкаслской болезни. Связывание проводили на 96-луночных плашках в системе, аналогичной иммуноферментному анализу.

**Заключение.** Разработанный способ выявления вирусов в гомогенатах тканей органов инфицированных кур позволяет за несколько часов идентифицировать и дифференцировать вирусы гриппа птиц и ньюкаслской болезни, что является важным шагом в предотвращении распространения и ликвидации очагов опасных болезней.

**Ключевые слова:** вирус ньюкаслской болезни, вирус гриппа птиц, дифференциальная диагностика

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## INTRODUCTION

Newcastle disease (ND) ranks among the most economically significant infectious avian diseases worldwide and occurs on all continents except Antarctica. Newcastle disease virus (NDV) is an enveloped, non-segmented single-stranded negative-sense RNA virus belonging to the *Paramyxoviridae* family [1]. NDV strains are classified by chick pathogenicity into apathogenic, lentogenic, mesogenic, and velogenic pathotypes. Velogenic NDV strains cause either neurotropic or viscerotropic infections, depending on strain tropism. Infected birds exhibit fever, anorexia, depression, lethargy, somnolence, and respiratory distress with dyspnea. In viscerotropic NDV infection, the gastrointestinal tract bears the primary brunt of pathology. Lesions include splenic and hepatic necrosis along with hemorrhagic intestinal ulcers. Droppings become liquid and green-tinged. Viscous gray mucus discharge from the nares, poultry sneezes with swallowing efforts. Severe conjunctivitis may occur. Muscle tremor, cervical torticollis, and opisthotonus frequently precede death in neurotropic NDV infections. The most characteristic clinical picture of infection with NDV, which distinguishes it from other highly pathogenic avian viruses is severe lymphoid necrosis Mortality can reach 100% [2, 3]. Velogenic NDV belongs to *Orthoavulavirus javaense* species (OAVJ, formerly AOAV-1) within the genus *Orthoavulavirus*, comprising over two dozen genotypes. Since NDV discovery in the 1930s, four panzootics have occurred, with the ongoing fourth driven by genotypes V, VI, VII, VIII. In recent years, numerous ND outbreaks caused by genotype VII (subgenotype VII.1.1) have been reported in Africa and Eurasia [4]. Russia has notified dozens of Newcastle disease outbreaks, and numerous additional cases likely remain unregistered [5]. Wild birds introduce NDV into backyard poultry flocks by freely feeding alongside chickens in rural areas [6]. ND control measures cover vaccination and quarantine.

High pathogenicity avian influenza (HPAI) inflicts massive economic losses on poultry industries while posing serious zoonotic risks to animal and human health [7]. AIV H5 subtypes have triggered multiple outbreaks among North American mammals, including foxes, seals, raccoons, and others. Moreover, AIV has infected dairy cows with high viral loads shed in milk. The risk of interspecies AIV transmission and human adaptation has increased substantially [8].

H5 and H7 AIV subtypes have driven numerous outbreaks in wild birds and poultry, resulting in over 422 million poultry deaths since 2005 from culling and direct mortality. They have caused 2,634 human cases worldwide, including more than 1,000 deaths [9]. Migratory wild birds spread AIV globally, driving three major waves of influenza outbreaks across multiple continents. The third wave, which started in 2020, is still going on.

Early detection and the timely registration of ND and AI outbreaks are essential prerequisites for effective disease containment and preventing further viral spread. In recent

years, a range of rapid confirmatory tests for these diseases has become available. Immunochromatographic tests (ICT) using gold nanoparticle-labeled antibodies have been developed for detection of both AIV and NDV antigens. This method provides high sensitivity with results readable by naked eye within 15 minutes [10, 11]. Colloidal gold strip (CGS) immunochromatographic tests have been developed to detect AIV/H5 in field conditions [12]. ICTs have been developed for detecting AIV subtypes H9 [13], H7 (targeting A/H7N9 monoclonal antibodies) [14, 15], and H6 in field samples [16]. Strip-based immunochromatographic methods excel for outbreak control due to their low cost, rapid results, and high field sensitivity. However, these tests rely on monoclonal antibodies targeting specific subtypes, rendering them unsuitable for sudden outbreaks of unknown etiology.

We have previously reported a 96-well plate-based assay capable of detecting and differentiating AIV and avian paramyxoviruses [17]. Following cultivation in chicken embryos, the viruses were detected in the allantoic fluid. However, for rapid on-site diagnostics in small poultry farms, there is a growing demand for methods that eliminate the need for preliminary virus propagation.

The objective of this study was to develop a single-plate methodology capable of detecting various subtypes of avian influenza virus and paramyxovirus in samples collected from the organs of both sick and dead birds.

## MATERIALS AND METHODS

*Reagents and solutions.* The following materials were used in the work: MycoKill AB (PAA Laboratories GmbH, Austria); horseradish peroxidase (#P8375, Sigma-Aldrich, USA); peroxidase-conjugated anti-mouse or chicken IgG; fetuin (#F3004, Sigma-Aldrich, USA).

Solutions:

– phosphate-buffered saline – 0.02 M, pH 7.2 (PBS);
– PBS supplemented with 0.1 mg/mL kanamycin, 0.4 mg/mL gentamicin, 0.01 mg/mL nystatin and 2% MycoKill AB solution;
– washing solution – 0.01% Tween 80 in PBS;
– blocking solution – 0.1% bovine serum albumin solution (BSA; 1 mg/mL) in PBS;
– reaction buffer – 0.02% Tween 80 and 0.1% BSA in PBS;
– substrate solution – 1 mg of 3,3′,5,5′-tetramethylbenzidine (TMB) + 10 µL 30% $H_2O_2$ in 10 mL 0.05 M sodium acetate buffer (pH ~5.0–5.5);
– stop solution – 3% sulfuric acid in water;
– horseradish peroxidase (HRP)-labeled synthesis solutions;
– freshly prepared 0.2 M $NaIO_4$ solution in water;
– 1 M and 0.1 M sodium carbonate buffers, pH 9.3;
– freshly prepared $NaBH_4$ solution in water (5 mg/mL);
– 1 M Tris buffer, pH 6.0;
– 0.1 M Tris buffer, pH 7.2.

*Animals.* Embryonated chicken eggs (CEs) were delivered from "Ptichnoye" poultry farm (Moscow), chickens – from "Tomilinskaya" poultry farm (Moscow Oblast). Live viruses were handled in biosafety level 3 facility. All tests were carried out in accordance with the standard governing the maintenance and care of laboratory animals GOST 33215-2014[1].

*Viruses.* Apathogenic AIV strains and paramyxoviruses were isolated from fecal samples during extended monitoring of avian influenza in a mallard population. Fresh feces collected on the shores of Moscow ponds [18] were suspended in two volumes of PBS supplemented with antibiotics (0.4 mg/mL gentamicin, 0.1 mg/mL kanamycin, 0.01 mg/mL nystatin) and 2% MycoKill AB. After centrifugation for 10 minutes at 4,000 rpm, 10-day CEs were infected with the supernatant. Infected allantoic fluid (IAF) was harvested at 48 hours, hemagglutinin (HA)-positive samples were then subjected to three additional passages.

The highly pathogenic NDV/Chicken/Moscow/6081/2022 strain was isolated from the kidneys of dead chickens [18]. For this purpose, the tissue was homogenized with fine glass powder. PBS supplemented with 0.1 mg/mL kanamycin, 0.4 mg/mL gentamicin, 0.01 mg/mL nystatin, and 2% MycoKill AB solution was then added. The mixture was centrifuged, and the supernatant was inoculated into CEs. Embryos were examined for mortality twice daily; IAF was harvested from dead CEs. The obtained virus was cloned and sequenced [19].

Highly virulent AIV A/chicken/Kurgan/3/2005 (H5N1) and A/FPV/Rostock/34 (H7N1) strains were kindly provided by S. S. Yamnikova, Dr. Sci (Biology), D. I. Ivanovskiy Institute of Virology, Moscow.

*Production of NDV-specific immunoglobulins in eggs*. Laying hens were infected with apathogenic NDV/Duck/Moscow/3639/2008 strain by adding $10^9$ $EID_{50}$ virus per chicken to the drinking bowl. After 2 weeks chickens were infected with highly pathogenic NDV/chicken/Moscow/6081/2022 strain. After 2 weeks eggs of immunized chickens were collected [20].

*Extraction and purification of egg yolk immunoglobulins (IgY).* Yolks from four eggs were separated from the whites. The chalazae were carefully removed, and the yolks were washed twice with cold distilled water before being transferred to a plastic container containing 40 mL of phosphate-buffered saline (PBS, pH 7.4). The mixture was homogenized thoroughly, brought to a final volume of 250 mL with water, and the pH was adjusted to 4.2 using 1 M hydrochloric acid. The preparation was then frozen at –30 °C. After 20 hours, the suspension was thawed and centrifuged at 10,000 g for 30 minutes. Activated carbon powder (1 g) was added to the supernatant. The mixture was stirred for 30 minutes and then filtered through filter paper [20]. Ammonium sulfate was added to the filtrate to achieve 25% saturation. The mixture was then held at 4 °C for 2 hours. The mixture was centrifuged at 10,000 g for 30 minutes. The resulting precipitate was dissolved in 10 mL of PBS, aliquoted, and stored at –20 °C.

*Production of mouse antibodies.* Mice were infected intranasally with 50 µL of IAF containing $10^7$ $EID_{50}$ of AIV or NDV. Mice were re-infected in 2 weeks. After another 2 weeks, total blood was collected from mice and serum was separated.

*Detection of the influenza virus in IAF*. 96-well plates were coated with 5 µ/mL fetuin solution, washed with water and blocked. Two-fold serial dilutions of IAF, starting from a 1:1 dilution, were prepared in the wells and incubated at 4 °C for 2 hours. Following washing, a solution of HRP-labeled fetuin (Fet-HRP) conjugate in reaction buffer was added to the wells, and the plates were incubated at 4 °C for 1 hour. After a final wash, the color reaction was developed with TMB. Absorbance was measured at 450 nm using a Multiskan FC spectrophotometer (Thermo Fisher Scientific, USA).

*Detection of viruses on plates coated for differential diagnosis.* To detect AIV and NDV, a 96-well plate was coated

[1] https://docs.cntd.ru/document/1200127789 (in Russ.)

| Coating | Fetuin | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Detection | Fet-HRP | | | | | | | | | | | |
| Dilution | H3N6 | H3N6 | H3N6 | H3N8 | H4N6 | H4N6 | NDV | NDV | APMV-4 | APMV-4 | APMV-4 | APMV-4 |
| IAF | 5163 | 519 | 5172 | 5908 | 4781 | 4771 | 3639 | 3604 | 4096 | 3575 | 5268 | 4696 |
| 1:1 | 0.99 | 0.95 | 0.98 | 1.14 | 0.95 | 0.93 | 0.09 | 0.09 | 0.10 | 0.11 | 0.20 | 0.15 |
| 1:2 | 0.97 | 0.97 | 0.91 | 1.12 | 0.92 | 0.91 | 0.09 | 0.09 | 0.09 | 0.09 | 0.15 | 0.12 |
| 1:4 | 0.9 | 0.93 | 0.93 | 1.02 | 0.88 | 0.75 | 0.08 | 0.09 | 0.09 | 0.10 | 0.12 | 0.10 |
| 1:8 | 0.86 | 0.83 | 0.87 | 1.02 | 0.80 | 0.68 | 0.07 | 0.09 | 0.08 | 0.08 | 0.10 | 0.09 |
| 1:16 | 0.68 | 0.71 | 0.77 | 0.93 | 0.67 | 0.51 | 0.08 | 0.08 | 0.09 | 0.08 | 0.08 | 0.09 |
| 1:32 | 0.51 | 0.59 | 0.62 | 0.82 | 0.50 | 0.35 | 0.09 | 0.09 | 0.08 | 0.08 | 0.08 | 0.08 |
| 1:64 | 0.38 | 0.45 | 0.49 | 0.71 | 0.34 | 0.27 | 0.07 | 0.08 | 0.09 | 0.08 | 0.08 | 0.08 |
| 1:128 | 0.24 | 0.30 | 0.38 | 0.50 | 0.22 | 0.15 | 0.09 | 0.09 | 0.09 | 0.08 | 0.08 | 0.08 |

*Fig. 1. Representative results showing the binding of HA-positive allantoic fluid samples to conjugated fetuin. Optical density was measured at 450 nm*

| Coating | Fetuin | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Detection | Murine sera | | | | | | | | | | | |
| Serum dilution | anti-H1N1 | anti-H3N2 | anti-H3N8 | anti-H4N6 | anti-H5N2 | anti-H5N3 | anti-H6N2 | anti-H7N1 | anti-H11N6 | anti-H11N9 | anti-H14N6 | Normal serum |
| Control 1:2 | 0.34 | 0.35 | 0.35 | 0.43 | 0.38 | 0.41 | 0.23 | 0.17 | 0.24 | 0.44 | 0.22 | 0.24 |
| 1:2 | 0.70 | 1.48 | 1.64 | 0.72 | 0.76 | 0.98 | 1.06 | 0.60 | 0.55 | 0.73 | 0.62 | 0.44 |
| 1:4 | 0.55 | 1.51 | 1.57 | 0.63 | 0.77 | 0.94 | 1.18 | 0.55 | 0.49 | 0.62 | 0.53 | 0.36 |
| 1:8 | 0.46 | 1.42 | 1.51 | 0.55 | 0.59 | 0.82 | 0.97 | 0.51 | 0.37 | 0.59 | 0.48 | 0.27 |
| 1:16 | 0.38 | 1.31 | 1.44 | 0.59 | 0.42 | 0.69 | 0.84 | 0.46 | 0.41 | 0.55 | 0.31 | 0.22 |
| 1:32 | 0.36 | 1.26 | 1.47 | 0.35 | 0.37 | 0.61 | 0.59 | 0.34 | 0.38 | 0.48 | 0.34 | 0.20 |
| 1:64 | 0.29 | 1.18 | 1.42 | 0.25 | 0.42 | 0.41 | 0.35 | 0.36 | 0.29 | 0.43 | 0.22 | 0.15 |
| 1:128 | 0.28 | 1.08 | 1.37 | 0.27 | 0.36 | 0.34 | 0.25 | 0.32 | 0.22 | 0.37 | 0.19 | 0.11 |

*Fig. 2. Representative results showing the binding of A/duck/Moscow/5908/2021-containing allantoic fluid to sera from mice immunized with different influenza virus subtypes. Optical density was measured at 450 nm*

as follows. Rows 1 and 12 were not coated and served as negative controls. Rows 2–4 were coated with fetuin, while rows 5 and 6 were coated with anti-NDV IgY to serve as a negative control for AIV. Rows 7 and 8 were coated with fetuin to serve as a negative control for NDV, and rows 9–11 were coated with anti-NDV IgY. The plate, coated as described above, was then blocked with BSA solution. Then, 100 µL of a virus-containing solution at a uniform concentration was added to each well of the plate (the same solution was used for all 12 wells of a given row). Thus, the plate design enabled the analysis of up to 8 different samples. After incubation for 2 hours at +4 °C, the plate was washed and Fet-HRP conjugate was added to rows 1–6; anti-NDV IgY-HRP in a reaction buffer was added to rows 7–12 and incubated for 1 hour at +4 °C. After a final wash, the color reaction was developed with TMB. Absorbance was measured at 450 nm using a Multiskan FC spectrophotometer (Thermo Fisher Scientific, USA). AIV was detected in rows 1–6, and NDV was detected in rows 7–12.

*Detection of viruses in chicken tissues.* 45 Leghorn chickens of the same age and weight were used for the experiment. The poultry were divided into equal groups: 10 chicks per experimental group and 5 chicks per control group. Two groups were infected with AIV (A/chicken/Kurgan/3/2005 H5N1 and A/FPV/Rostock/34 H7N1 strains, respectively), and one group was infected with NDV (NDV/Chicken/Moscow/6081/2022 strain). Each experimental group had a matched control group. The poultry were kept in separate cages in different rooms depending on the group. A total of 10 chickens per site were used. To assess viral pathogenicity, the poultry were deprived of water overnight prior to the experiment. The next day, 10 mL of water containing $10^8$ $EID_{50}$ of the viruses was placed in the drinkers and introduced into the cage with the birds. The control group received regular water.

Organs (kidneys, lungs, intestines) were extracted from dead or euthanized chickens, homogenized in a double volume of PBS, and centrifuged for 10 minutes at 5,000 rpm. The supernatant was used for further analysis. The organs of control poultry were prepared in the same way. Aliquots of the supernatants were retained for virus typing by polymerase chain reaction (PCR) and titration in CE. The remaining material was then added to 12 wells of a plate prepared as described above.

| Coating | anti-NDV IgY | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Detection | anti-NDV IgY-HRP | | | | | | | | | | | |
| IAF dilution | H3N1 3554 | H6N2 4031 | H11N9 6454 | NDV 6081 | NDV 3639 | NDV 3604 | NDV LaSota | APMV-4 5268 | APMV-4 4572 | APMV-4 4096 | APMV-4 3579 | Virus-free control |
| 1:1 | 0.22 | 0.21 | 0.19 | 1.22 | 1.35 | 1.11 | 1.03 | 0.19 | 0.16 | 0.16 | 0.15 | 0.14 |
| 1:2 | 0.19 | 0.16 | 0.16 | 1.11 | 1.23 | 1.06 | 0.89 | 0.15 | 0.15 | 0.15 | 0.15 | 0.14 |
| 1:4 | 0.17 | 0.15 | 0.15 | 1.02 | 1.10 | 0.97 | 0.83 | 0.15 | 0.15 | 0.16 | 0.14 | 0.15 |
| 1:8 | 0.17 | 0.15 | 0.15 | 0.56 | 1.00 | 0.82 | 0.70 | 0.15 | 0.15 | 0.15 | 0.15 | 0.15 |
| 1:16 | 0.18 | 0.15 | 0.16 | 0.36 | 0.74 | 0.61 | 0.53 | 0.15 | 0.15 | 0.15 | 0.14 | 0.15 |
| 1:32 | 0.17 | 0.15 | 0.15 | 0.28 | 0.53 | 0.44 | 0.44 | 0.14 | 0.15 | 0.15 | 0.16 | 0.15 |
| 1:64 | 0.16 | 0.15 | 0.14 | 0.22 | 0.38 | 0.31 | 0.37 | 0.13 | 0.14 | 0.15 | 0.15 | 0.14 |
| 1:128 | 0.17 | 0.16 | 0.16 | 0.18 | 0.27 | 0.19 | 0.30 | 0.12 | 0.13 | 0.13 | 0.14 | 0.15 |

*Fig. 3. Detection of NDV on a plate coated with anti-NDV IgY, and treated with anti-NDV IgY-HRP. Optical density was measured at 450 nm*

*NDV detection with anti-NDV IgY and HRP conjugated anti-NDV IgY*. A 96-well plate was coated with a 5 µg/mL solution of purified anti-NDV IgY. The plate was then washed with water and blocked with a BSA solution for 1 hour. Test supernatants from the homogenized samples were then added to the wells (100 µL per well) and incubated at 4 °C for 2 hours. The plate was washed, anti-NDC IgY-HRP conjugate in a reaction buffer was added and incubated for 1 hour at +4 °C. After a final wash, the color reaction was developed with TMB. Absorbance was measured at 450 nm using a Multiskan FC spectrophotometer (Thermo Fisher Scientific, USA).

*Synthesis of HRP-labeled conjugates*. The synthesis Fet-HRP was described in detail by M. N. Matrosovich and A. S. Gambaryan [21]. Conjugation of HRP with anti-NDV IgY was performed similarly. A freshly prepared solution of 0.2 M $NaIO_4$ in water was added to HRP dissolved in bidistilled water. The mixture was then incubated in the dark at room temperature for 20 minutes. The reaction mixture was desalted on a Sephadex G-25 column. Solutions of fetuin or immune IgY in sodium carbonate buffer (pH 9.3) were then added, and the mixture was incubated in the dark for 4 hours. 5 mg/mL of freshly prepared $NaBH_4$ solution in water was added and incubated for 30 minutes on ice. The pH was adjusted to neutral using 1 M Tris buffer (pH 6.0) on ice. Chromatography was performed on a Sephacryl S-200 column. Fractions containing HRP were collected, pooled, aliquoted, and stored at –20 °C.

*Detection of viruses by PCR*. The presence of AIV and NDV RNA in the samples was determined by real-time reverse transcription polymerase chain reaction (RT-PCR) with fluorescence detection, including an internal control. RNA was extracted from biological samples using a commercial magnetic particle-based kit for nucleic acid isolation via sorption "DNA/RNA-M-FLEX-FAKTOR" (VET FAKTOR, Russia). Commercial kits for detection of AIV and NDV RNA were used respectively – "PCR-INFLUENZA-A-FAKTOR" and "PCR-NEWCASTLE-FAKTOR" (VET FAKTOR, Russia). The matrix for RT-PCR was RNA samples extracted from the test material (feces, fragments of organs and tissues).

*Ethics*. Animal experiments were conducted in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 1986). The study design was approved by the Ethics Committee of Chumakov Federal Scientific Center for Research and Development of Immune-and-Biological Products of Russian Academy of Sciences (Resolution No. 4 dated 02.12.2014). All measures were taken to minimize animal suffering.

## RESULTS

During the monitoring of migratory birds, we isolated dozens of AIV strains and paramyxoviruses [22]. In addition, during the investigation of the outbreak in Moscow Oblast NDV was isolated from chickens [18]. Final virus identification was based on complete or partial genome sequencing. However, at the initial stage, AIV was differentiated from NDV and avian paramyxovirus serotype 4 (APMV-4) using solid-phase ELISA. The materials presented herein describe the protocols employed for virus detection and identification.

***Differentiation of AIV from paramyxoviruses.*** AIV detection relied on viral hemagglutinin binding to sialoglycosyl residues on fetuin [21]. IAF was incubated in wells of plated coated with fetuin. Following sorption and washing, plates were incubated with Fet-HRP conjugate solution and developed via chromogenic reaction. Final identification was confirmed by partial/complete genome sequencing of the virus isolates. All AIV samples were positive in this test, while all paramyxoviruses were negative (Fig. 1).

All viruses were isolated from duck feces in Moscow. AIV isolates were identified by subtype, while paramyxoviruses were classified by serotype. The second line contains the strains identified by the laboratory number. Eight wells of each row contain double IAF dilutions.

***Subtyping of influenza A virus (AIV) using a combined assay with fetuin murine monoclonal sera against distinct AIV of different subtypes.*** The combination of universal fetuin capture and subtype-specific antibody detection simplifies AIV identification. An example of A/duck/Moscow/5908/2021 subtyping is shown in Figure 2. The influenza virus (H3N8) subtype was subsequently confirmed by sequencing.

IAF containing A/duck/Moscow/5908/2021 virus was added to rows B–H of plates coated with fetuin. Row A served as a negative control and contained no virus. Then, double dilutions of immune murine sera were added to each column. In the last column, normal murine serum was titrated. At the next stage, HRP-conjugated anti-mouse antibodies were incubated with plates. Following washing, plates were developed with TMB to visualize antibody binding.

| Coating | --- | Fetuin | | | anti-NDV IgY | | Fetuin | | anti-NDV IgY | | | --- |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Detection | Fet-HRP | | | | | | anti-NDV IgY-HRP | | | | | |
| IAF AIV H5N1 | 0.19 | 1.11 | 1.26 | 1.21 | 0.37 | 0.29 | 0.15 | 0.15 | 0.14 | 0.15 | 0.15 | 0.12 |
| IAF NDV 3639 | 0.12 | 0.12 | 0.17 | 0.20 | 0.22 | 0.20 | 0.45 | 0.44 | 1.30 | 1.38 | 1.35 | 0.13 |
| Feces NDV 6081 | 0.15 | 0.22 | 0.24 | 0.23 | 0.25 | 0.27 | 0.19 | 0.21 | 0.58 | 0.63 | 0.55 | 0.14 |
| Kidneys (control) | 0.14 | 0.16 | 0.20 | 0.19 | 0.17 | 0.13 | 0.16 | 0.16 | 0.18 | 0.17 | 0.15 | 0.15 |
| Kidneys AIV H5N1 | 0.12 | 0.92 | 0.97 | 1.00 | 0.22 | 0.20 | 0.25 | 0.28 | 0.19 | 0.21 | 0.23 | 0.12 |
| Kidneys AIV H7N1 | 0.14 | 0.62 | 0.71 | 0.68 | 0.17 | 0.13 | 0.16 | 0.16 | 0.18 | 0.17 | 0.18 | 0.15 |
| Kidneys NDV 6081 | 0.17 | 0.22 | 0.19 | 0.21 | 0.40 | 0.37 | 0.24 | 0.26 | 1.10 | 1.00 | 1.02 | 0.14 |
| Kidneys NDV 6081 | 0.18 | 0.23 | 0.18 | 0.22 | 0.33 | 0.39 | 0.29 | 0.26 | 0.98 | 1.04 | 1.00 | 0.17 |

*Fig. 4. Testing of preparations for AIV and NDV: A/chicken/Kurgan/3/2005 (H5N1) infectious allantoic fluid; NDV/Duck/Moscow/3639/2008 infectious allantoic fluid; faecal extract of chicks infected with NDV/Chicken/Moscow/6081/2022; kidney extract of uninfected chick; kidney extract of a chick infected with A/chicken/Kurgan/3/2005; kidney extract of a chick infected with A/FPV/Rostock/34 (H7N1); and kidney extracts of chicks infected with NDV/Chicken/Moscow/6081/2022*

**Table**
**Detection of NDV and AIV from feces and organs of sick chickens using solid-phase analysis, PCR and titration in chicken embryos**

| Method of detection | NDV | | | | | | | AIV |
|---|---|---|---|---|---|---|---|---|
| | IAF | Feces | | | Kidneys | Lungs | Intestine | Kidneys |
| | | Day 3 | Day 5 | Day 7 | | | | |
| Solid-phase ELISA | 6/6 | 5/10 | 0/4 | 0/8 | 10/10 | 2/2 | 4/6 | 5/5 |
| PCR | 6/6 | 10/10 | 4/4 | 6/8 | 4/4 | 5/5 | 6/6 | 5/5 |
| Titration | 6/6 | 7/7 | 2/2 | 1/1 | 4/4* | 1/1 | 2/2 | 3/3* |

* The virus infectivity titer was $10^9$ $EID_{50}$/mL.

***Detection of paramyxoviruses using solid-phase ELISA.*** Chickens were double-immunized with apathogenic NDV/duck/Moscow/3639/2008, then challenged with velogenic NDV/chicken/Moscow/6081/2022 (ch6081, subgenotype VII.1.1); eggs were harvested to purify anti-NDV IgY. IgY was concentrated and purified by 25% ammonium sulfate precipitation, then used to coat 96-well plates. Anti-NDV IgY was also conjugated to HRP. In tests with these antibodies, all NDV-infected allantoic fluids tested positive, while all APMV-4-infected allantoic fluids were negative (Fig. 3).

AIV isolates were identified by subtype, while paramyxoviruses were classified by serotype. The second line contained the strains identified by the laboratory number. Eight wells of each row contained double IAF dilutions.

***Detection of viruses in organ samples from sick chickens.*** In the three aforementioned tests, AIV or NDV presence in allantoic fluid was confirmed following propagation in embryonated chicken eggs. However, the cultivation and isolation of viruses is not possible in small poultry farms, especially in backyards. Therefore, the possibility of detecting viruses in extracts obtained from organ tissues of infected chickens was investigated. The extracts were added to 12 wells of a 96-well plate prepared as described in section "*Detection of viruses in coated plates for differential diagnosis*" of "Materials and methods".

Figure 4 shows the results of this experiment.

Samples containing AIV (A/chicken/Kurgan/3/2005 IAF, kidney extracts from A/chicken/Kurgan/3/2005 and A/FPV/Rostock/34 – infected chickens) yielded positive signals in fetuin-coated wells detected by Fet-HRP conjugate. Only fetuin coating with Fet-HRP detection reliably detected AIV antigens across all tested strains. Samples containing NDV (NDV/Duck/Moscow/3639/2008 IAF, feces from NDV/Chicken/Moscow/6081/2022 infected chickens, and kidney extracts from NDV/Chicken/Moscow/6081/2022 infected chickens) give a positive signal in anti-NDV IgY-coated wells detected by anti-NDV IgY-HRP. Only IgY anti-NDV coating with IgY-HRP detection reliably detected NDV antigens across all tested strains. Thus, a single reaction differentiates HPAIV (H5N1, H7N9) from NDV in infected poultry.

***Comparison of solid-phase ELISA with PCR and embryonated chicken egg infectivity titration.*** A comparison of the described solid-phase ELISA with PCR and titration in chicken embryos shows that the latter two are much more sensitive (Table). Using these methods, virus was detected in nearly all samples from days 3–7 post-ch6081 infection; solid-phase ELISA detected fecal shedding only at peak disease.

On days 5 and 7, no NDV/AIV was detected in fecal samples by this method; however, the proposed solid-phase ELISA achieved 100% detectability in avian organ tissues. The virus was detected in all kidney samples from chickens that died or were euthanized at the terminal disease stage. Highly pathogenic AIV and NDV accumulate in chicken kidneys at concentrations up to $10^9$ $EID_{50}$/mL; this enables detection by solid-phase ELISA (analytical sensitivity

$10^8$ $EID_{50}$/mL). The developed method exhibits low sensitivity for fecal samples and is unsuitable for preliminary diagnosis at this stage. However, rapid poultry mortality on infected farms enables immediate and reliable cause determination using this test, facilitating timely control measures.

## DISCUSSION

Recently, avian influenza and Newcastle disease have caused major global poultry industry losses. In recent decades, H5 subtype AIV has spread from Southeast Asia to all continents, including the Americas, underscoring the need for effective monitoring and diagnostics for this dangerous pathogen. NDV genotype VII has spread from Africa, with outbreaks reported from Japan to Northwestern Europe [23]. Since stamping out remains the primary containment strategy for HPAI and NDV timely outbreak detection is critically important [24].

The developed method enables simultaneous identification and differentiation of NDV and AIV, facilitating timely disease control measures. Since AIV detection uses a universal receptor analog and NDV detection employs polyclonal IgY from chickens immunized with different strains, the method should detect any poultry-pathogenic AIV/NDV strains responsible for mortality. The method enables on-site preliminary diagnostics. Commercial AIV test kits target specific subtypes, whereas our method simultaneously detects multiple AIV subtypes in feces/pathological material, significantly reducing diagnostic time and enabling timely pathogen control in poultry.

Development of a test kit for differential diagnosis of avian influenza and Newcastle disease represents a critical advance in poultry health management.

Future work will focus on validating this method and testing it in backyard flocks, as well as comparing it with existing methods for identifying these pathogens.

## CONCLUSION

Global circulation of highly pathogenic H5N1 AIV and NDV outbreaks across Africa, Asia, and Europe necessitated development of a simple, rapid pathogen detection method for organs from sick/dead poultry. The developed method identifies and differentiates NDV from multiple AIV subtypes in feces and organ tissues of diseased/dead chickens.

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