

PROTOCOL OF STEP-BY-STEP INSTRUCTIONS FOR IDENTIFYING THE SOURCE OF AVIAN INFLUENZA PATHOGEN AND PREVENTING ITS SPREAD FOR JOINT PREVENTIVE AND LIQUIDATION MEASURES (REFERENCE DOCUMENT)

Developed under Activity 1.5. “Increasing awareness on emerging diseases and
the prevention of cross-border epidemics”
of the international project

“Development of screening and monitoring systems for emerging diseases and
prevention of cross-border epidemics in the current context” ROUA00136,
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PREAMBLE

This Protocol of step-by-step instructions for identifying the source of avian influenza and preventing its spread for joint preventive and liquidation measures (hereinafter – the Protocol) was developed by PI "Educational and research institute of biotechnology and biosafety", Agreement No. 151 dated September 22, 2026 (hereinafter – the Agreement), concluded with Odesa State Agrarian University (OSAU) as Partner 3 (PP3) of the international project “Development of screening and monitoring systems for emerging diseases and prevention of cross-border epidemics in the current context” ROUA00136, co-financed by the EU under the Interreg NEXT Romania – Ukraine 2021–2027 Programme (hereinafter – the Project).

The document was prepared in the framework of Activity 1.5. “Increasing awareness on emerging diseases and the prevention of cross-border epidemics” (Deliverable D.1.5.1.).

The purpose of this Protocol is to provide veterinary medicine specialists, medical practitioners, local authorities, sectoral agencies, research institutions, and other interested parties with step-by-step practical instructions for:

- identifying sources and vectors of the avian influenza virus in the chain "wild migratory bird – environmental objects (water, soil, air, surfaces) – domestic poultry – human";
- assessing pathogen transmission routes (airborne, alimentary, contact, fecal-oral) and risk factors in a cross-border context, in particular with regard to seasonal migrations of wild waterfowl;
- conducting joint ornithological, epizootiological, virological, and environmental monitoring studies in line with the "One Health" principles;
- harmonizing approaches to surveillance, early detection, notification, and control of avian influenza between Ukraine and Romania in the Lower Danube and Black Sea coastal area, which are key hubs on migratory bird flyways.

Preparation of this Protocol draws on:

- results of joint field studies and laboratory analyses carried out under the Project, in particular monitoring of environmental objects (water, soil, litter, equipment surfaces, air) as a source of viral circulation;

- the Joint Technical Specification (TOR) agreed by the partners, which defines unified methods for sample collection (from wild and domestic birds, as well as from environmental objects), delivery, preparation, and testing;
- the combined use of molecular-genetic (RT-PCR), virological (virus isolation in embryonated chicken eggs), and serological methods (HI, ELISA) as the reference diagnostic approach;
- experience and findings from previous international projects and scientific activity;
- guidance documents of the World Health Organization (WHO), the World Organisation for Animal Health (WOAH), the Food and Agriculture Organization of the United Nations (FAO), EFSA, and the current regulatory framework of Ukraine and the EU.

This Protocol was prepared with due regard to, and is consistent with, the following documents:

- Regulation (EU) 2016/429 of the European Parliament and of the Council of 9 March 2016 on transmissible animal diseases ("Animal Health Law") — highly pathogenic avian influenza (HPAI) is classified as a category A disease subject to immediate notification and mandatory eradication measures;
- Commission Delegated Regulation (EU) 2020/687 of 17 December 2019 on rules for the prevention and control of certain listed diseases, including measures to be taken upon confirmation of an outbreak of category A avian influenza;
- Commission Delegated Regulation (EU) 2020/689 of 17 December 2019 on rules for surveillance, eradication programmes, and disease-free status;
- Commission Implementing Regulation (EU) 2020/2002 on rules for Union notification and reporting of listed diseases;
- Law of Ukraine No. 1206-IX of 04.02.2021 "On Veterinary Medicine and Animal Welfare";
- Law of Ukraine No. 4718-IX of 16.12.2025 "On Amendments to Certain Laws of Ukraine Regarding the Alignment of Regulation in the Fields of Veterinary Medicine, Animal Welfare, and Feed with the Acts of European Union Law";
- Law of Ukraine No. 2573-IX of 06.09.2022 "On the Public Health System";

- Resolution of the Cabinet of Ministers of Ukraine No. 517 of 21.05.2021 "On the State Emergency Anti-Epizootic Commission under the Cabinet of Ministers of Ukraine";
- Order of the Ministry of Agrarian Policy and Food of Ukraine "On Approval of the Instruction on the Prevention and Eradication of Avian Influenza," registered with the Ministry of Justice of Ukraine on 08.11.2011 under No. 1277/20015;
- Methodological Recommendations of the State Service of Ukraine on Food Safety and Consumer Protection (2024) on preventing and averting the introduction of highly pathogenic avian influenza into Ukrainian holdings;
- Strategy for Ensuring Biological Safety and Biological Protection under the "One Health" Principle through 2025 (Order of the Cabinet of Ministers of Ukraine No. 1416-r of 27.11.2019);
- European Veterinary Big Data Strategy 2022–2027;
- WOAHP Terrestrial Animal Health Code, section "Infection with avian influenza viruses."

Implementation of the SMS-RO-UA project supports the delivery of the EU Strategy for the Danube Region (EUSDR) as it relates to environmental protection and public health, and is consistent with the goals of the EU Biodiversity Strategy for 2030 and the European Green Deal. The Danube Delta and the adjacent wetlands are one of the key hubs on the flyways of migratory waterfowl between Europe, Asia, and Africa, which creates an elevated risk of introduction and circulation of highly pathogenic avian influenza virus strains in the region and calls for a joint cross-border approach to surveillance and response.

RELEVANCE

Avian influenza is one of the most dangerous and socially significant viral infections in the world, posing a serious threat to poultry farming, national economies, and public health systems. The need for stronger monitoring and control of avian influenza stems from its wide geographic spread, the high mutation rate of the virus, substantial economic losses, and the potential for human pandemics.

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The causative agent is an influenza A virus of the family Orthomyxoviridae, characterized by high genetic variability and a capacity for antigenic drift and shift. These properties drive the continuous emergence of new viral variants that can differ in pathogenicity, virulence, and cross-species adaptability [1]. The highly pathogenic H5 and H7 strains pose a particular danger, as they can cause mass illness and death in both domestic and wild birds.

Since the early 2000s, outbreaks of highly pathogenic avian influenza have been recorded in Europe, Asia, Africa, and the Americas. According to the World Organisation for Animal Health (WOAH), the disease continues to spread actively among wild and domestic birds, which points to gaps in current control measures and a need to strengthen epizootic surveillance systems. Substantial economic losses stem from reduced poultry productivity, forced culling of infected flocks, quarantine costs, disinfection, and restrictions on international trade in poultry products [2].

The zoonotic potential of the virus is another important aspect of its relevance — that is, the ability of certain avian influenza strains to shift host and move from birds to mammals and humans. Strains such as H5N1 and H7N9, among others, are known to be able to infect humans, causing severe respiratory illness with high fatality rates. According to the World Health Organization (WHO), most human infections have resulted from direct contact with sick birds or a contaminated environment. At the same time, the ongoing circulation of the virus among animals creates conditions for its adaptation to the human population, which could eventually trigger a new pandemic [3].

Wild migratory birds play a substantial role in spreading the infection, as they are a natural reservoir of the virus. Migration routes facilitate cross-border transmission of the pathogen and the emergence of new disease foci in different regions of the world. Climate change, urbanization, growing international trade, and the intensification of poultry farming create favorable conditions for further spread of the virus and complicate control of the epizootic situation [4].

For Ukraine, avian influenza also remains a highly relevant problem because of the country's location along seasonal wild-bird migration routes. Cases were recorded in the country in the 1950s and 1970s, and have recurred cyclically since autumn 2005, underscoring the need for continuous monitoring, improved laboratory diagnostics, and

stronger biosecurity in both private and industrial holdings. Modern molecular diagnostic methods are particularly important, as they allow rapid identification of virus strains and timely response to epizootic threats.

Studying avian influenza is also important in the context of the One Health concept, which calls for integrating the efforts of veterinary medicine, human medicine, and ecology to combat infectious disease. Effective control of avian influenza is only possible through cross-sectoral cooperation, information exchange, and coordinated action among international organizations, government bodies, and research institutions.

GENERAL CHARACTERISTICS OF THE AVIAN INFLUENZA PATHOGEN

Avian influenza is caused by an influenza A virus (Influenza A virus), belonging to the family Orthomyxoviridae and genus Alphainfluenzavirus. The virus is highly contagious, shows considerable genetic variability, and can infect a wide range of bird species and, in some cases, mammals and humans [2].

The virions are spherical or filamentous and are enveloped in a lipoprotein membrane, on the surface of which sit two glycoproteins — hemagglutinin (H) and neuraminidase (N). These surface antigens define the virus subtype. To date, 18 hemagglutinin types (H1–H18) and 11 neuraminidase types (N1–N11) have been identified, and their various combinations give rise to numerous influenza A serotypes, including H5N1, H5N8, H7N9, and others [5]. This diversity complicates both human influenza control (prevention and treatment) and effective control in poultry farming.

The viral genome consists of a single-stranded, segmented, negative-sense RNA made up of eight segments. This structure gives the virus a high capacity for genetic recombination and mutation, resulting in new variants with increased pathogenicity or adaptation to new host species [6].

The main natural reservoir of the virus is wild waterfowl, particularly ducks, geese, and swans, in which the infection is often asymptomatic. In domestic poultry, especially chickens and turkeys, highly pathogenic strains can cause a severe acute or

peracute course of disease with high mortality. Based on pathogenicity in birds, the viruses are divided into low pathogenic (LPAI) and highly pathogenic (HPAI) forms. The highly pathogenic H5 and H7 strains pose the greatest danger, as they can cause mass epizootics [3, 7].

The avian influenza virus is unstable when exposed to high temperatures, ultraviolet radiation, and most disinfectants, but survives well at low temperatures and in humid environments. It can remain viable for a long time in water and organic substrates, which facilitates its spread among birds [8].

The virus is transmitted mainly by airborne, alimentary, and contact routes. The source of infection is sick or recovered birds, which shed the virus through respiratory secretions and excrement. Wild migratory birds, along with contaminated feed, water, equipment, and vehicles, play a significant role in spreading the infection.

Of particular concern is the ability of some viral strains to cross the species barrier and infect humans. Although human-to-human transmission remains limited, the continuous circulation of the virus among animals creates conditions for the emergence of new pandemic variants. This is why avian influenza is regarded as one of today's potential global biological threats [7].

In summary, the avian influenza pathogen is characterized by high variability, a broad host range, and substantial epizootic and epidemiological potential, which calls for continuous monitoring, improved diagnostic methods, and effective preventive measures.

IMPACT OF AVIAN INFLUENZA ON HUMAN HEALTH

Avian influenza is not only a veterinary concern but also a serious medical and biological problem, since certain viral strains can infect humans and cause severe respiratory illness. The highly pathogenic strains H5N1, H7N9, H5N6, and several other influenza A variants pose the greatest danger to people, owing to their high fatality rates and substantial pandemic potential [3].

Human infection most often occurs through direct contact with infected domestic or wild birds, or through contact with contaminated secretions, feathers, feed, water, or

surfaces. People at particular risk include poultry farm workers, veterinary specialists, slaughterhouse workers, and others who come into contact with sick animals [9, 10].

Once inside the human body, the virus primarily affects the respiratory epithelium, causing acute respiratory illness. Clinical presentation can range from mild symptoms to severe viral pneumonia, acute respiratory distress syndrome, and multi-organ failure. The most common symptoms are fever, cough, sore throat, shortness of breath, muscle pain, weakness, and lower respiratory tract involvement. In severe cases, the disease is accompanied by respiratory failure and high mortality [13].

According to the WHO, the fatality rate for infection with some strains, particularly H5N1, can exceed 50%, considerably higher than for seasonal influenza. At the same time, most human infections remain isolated, and sustained human-to-human transmission is currently limited. However, the continuous circulation of the virus among birds creates conditions for mutation and genetic reassortment, which could give rise to new strains with an increased capacity for human-to-human transmission [6].

An important aspect of avian influenza's impact on human health is its pandemic potential. Researchers believe that recombination between animal- and human-origin viruses can give rise to new pandemics. Similar processes have occurred before in the history of influenza, notably during the 1918 "Spanish flu" pandemic and the 2009 "swine flu" (H1N1) pandemic [1].

Beyond the direct impact on human health, avian influenza outbreaks carry substantial socioeconomic consequences for the population. Mass culling of poultry, quarantine restrictions, and the threat of further spread negatively affect food security, the economy, and public mental wellbeing. This underscores the need for stronger epidemiological surveillance, the development of early-response systems, and the implementation of a cross-disciplinary One Health approach.

As the World Health Organization notes, avian influenza viruses circulate mainly among birds, but some can infect people and cause severe illness [3].

The CDC likewise points out that people with close or prolonged contact with infected birds face an elevated risk of infection [9].

In summary, avian influenza poses a serious potential threat to human health, since certain strains can cause severe illness and create conditions for new pandemics. This underscores the need for ongoing viral monitoring, improved preventive measures, and international cooperation in biosecurity and public health.

EPIDEMIOLOGY AND EPIZOOTOLOGY OF AVIAN INFLUENZA

Avian influenza is a highly contagious viral disease of birds with substantial epizootic and epidemiological potential. It is present in many countries around the world and poses a serious threat to commercial poultry production, wildlife, and human health. The highly pathogenic influenza A strains, particularly H5 and H7, pose a particular danger, as they can spread rapidly among bird populations and cause mass mortality [2].

Epizootology of avian influenza. The main natural reservoir of the virus is wild waterfowl — ducks, geese, swans, gulls, and other wetland bird species. In most cases, the infection is asymptomatic in wild birds, which helps the virus persist and spread in nature over the long term [5].

Transmission between birds occurs mainly by airborne, alimentary, and contact routes. The virus is shed by infected birds through respiratory secretions, saliva, and feces. Contaminated water, feed, equipment, vehicles, staff clothing, and poultry slaughter products all play a significant role in spreading the infection. In commercial operations, high stocking density and poor biosecurity practices favor rapid disease spread [8].

Seasonal migration of wild birds is central to the epizootology of avian influenza. Migration routes facilitate cross-border transmission of the virus and the emergence of new disease foci. This is why countries located along major bird migration routes, including Ukraine, fall into elevated-risk zones for introduction of the infection.

Epidemiology of avian influenza in humans. The epidemiological significance of avian influenza stems from the ability of certain strains to infect humans. Human infection most often results from close contact with infected birds or a contaminated environment. The main risk groups are poultry farm workers, veterinary specialists, live-bird market workers, and people involved in outbreak response [3].

At present, human-to-human transmission is limited, but the continuous circulation of the pathogen among animals creates a risk of mutation and genetic reassortment. This could lead to the emergence of new pandemic strains adapted for efficient transmission among people [6].

According to the World Health Organization, human infections with avian influenza viruses have followed close contact with infected birds or exposure to a contaminated environment [7]. The CDC likewise notes that wild waterfowl are the natural reservoir of avian influenza A viruses [11].

In recent years, the strains H5N1 and H7N9 have posed the greatest epidemiological risk to humans, causing severe illness with high fatality rates. Human infections have been recorded mainly in countries across Asia, Africa, and the Middle East, where people have close contact with domestic poultry.

The importance of epizootic and epidemiological monitoring. The high variability of the virus and its capacity for rapid spread make continuous monitoring of circulating strains among wild and domestic birds essential. Key components of prevention include:

- monitoring migration and the health status of wild birds;
- maintaining biosecurity on poultry farms;
- regular laboratory testing;
- quarantine measures;
- timely detection and elimination of outbreaks;
- international exchange of epizootic information.

In summary, the epidemiology and epizootology of avian influenza are shaped by complex transmission mechanisms, a wide range of reservoir hosts, and substantial potential for cross-species transmission. This calls for global epizootic surveillance and cross-sectoral cooperation within the One Health framework.

ROUTES OF INFECTION (TRANSMISSION) FOR AVIAN INFLUENZA

Avian influenza is an extremely contagious viral disease that spreads quickly among wild and domestic bird populations. Its high transmissibility results from the virus's environmental stability, the large number of transmission routes, and the substantial amount of virus shed by infected animals. The source of infection is sick, recovered, or asymptotically infected birds, which shed the virus through feces, saliva, respiratory secretions, and eye discharge [8].

The main transmission routes for the virus include:

a. Contact transmission

Direct contact between infected and healthy birds is one of the most important transmission mechanisms. Under crowded housing conditions, the virus spreads quickly from bird to bird through:

touch;

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- shared housing;
- shared feeders and waterers;
- contact with secretions from sick birds.

The infection spreads especially quickly on commercial poultry farms, where high stocking density creates favorable conditions for mass infection [4].

b. Airborne (droplet) transmission

The virus can be transmitted through the air along with dust, mucus droplets, and aerosols. Infected birds shed the virus when they:

- breathe;
- cough;
- sneeze;
- flap their wings, stirring up contaminated dust.

In enclosed spaces with poor ventilation, risk of airborne infection rises considerably, that is why large poultry operations are high-risk zones for virus spread[2].

c. Fecal-oral transmission

The avian influenza virus is characterized by fecal-oral transmission. Infected birds shed large amounts of virus in droppings, which contaminate:

- water;
- feed;
- litter;
- equipment;
- surfaces in poultry housing.

Healthy birds become infected by ingesting contaminated feed or water. This route is particularly important among waterfowl, since the virus survives well in aquatic environments [5].

d. Waterborne transmission

Water is one of the key factors in spreading the virus among wild birds. The virus can remain viable in cold water for extended periods. Contamination of bodies of water with droppings from infected birds creates a persistent source of infection for other animals [5].

e. Mechanical transmission via objects and personnel



Mechanical carriers play an important role in spreading the virus. The pathogen can be carried by:

- vehicles;
- cages used for holding or transporting birds;
- equipment;
- protective clothing;
- workers' footwear;
- feed containers;
- poultry-handling equipment.

Failure to follow sanitary rules and inadequate disinfection are among the leading causes of virus introduction into holdings.

f. The role of wild migratory birds

Wild waterfowl and migratory birds are the natural reservoir of influenza A viruses. In most cases they carry the infection without clinical signs, which allows the pathogen to spread over long distances during seasonal migration without being detected.

Bird migration routes play a major role in the global spread of the highly pathogenic H5N1 and H5N8 strains between continents.

g. Transmission from birds to humans

Human infection occurs mainly as a result of:

- contact with sick birds;
- inhaling contaminated dust;
- contact with feces;
- slaughtering and processing carcasses;
- time spent at live-bird markets.

The groups at highest risk of infection are:

- poultry farm workers;
- veterinarians;
- slaughterhouse workers;
- people involved in outbreak response.

The World Health Organization notes that most human infections with highly pathogenic avian influenza have resulted from direct or indirect contact with infected birds [3].

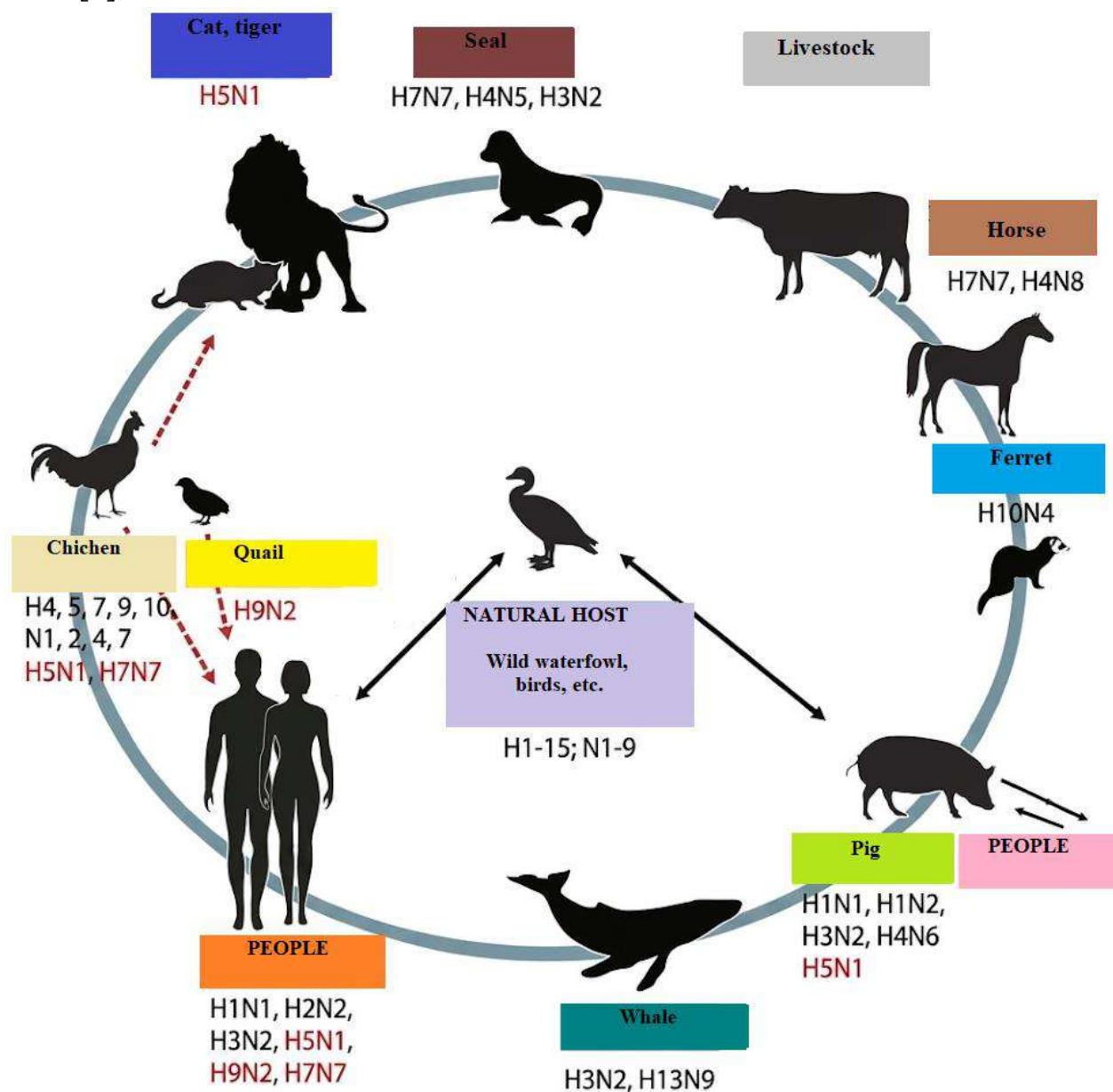


Figure 1 — Cross-species transmission of influenza viruses

The virus's ability to cross the species barrier is a particular concern (Figure 1). Documented cases of infection include:

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- pigs;
- cats;
- dogs;
- minks;
- marine mammals.

Pigs are considered “intermediate hosts,” since they can be simultaneously infected with human and avian influenza strains, creating conditions for genetic reassortment and the emergence of new pandemic viral variants [6].

Factors contributing to the spread of the virus. The spread of avian influenza is driven by:

- intensive commercial poultry farming;
- the global poultry trade;
- migration of wild birds;
- poor biosecurity;
- climate change;
- informal live-bird markets;
- insufficient veterinary oversight.

In summary, avian influenza is characterized by a complex system of transmission routes that enables its rapid spread among animals and creates a risk of human infection. This calls for strict adherence to veterinary and sanitary measures, continuous monitoring, and implementation of One Health principles to prevent epizootics and potential pandemics.

IDENTIFYING THE SOURCE OF THE AVIAN INFLUENZA PATHOGEN

Identifying the avian influenza pathogen is essential for timely disease detection, control of epizootic outbreaks, and prevention of highly pathogenic strains spreading among birds and people. Modern diagnosis relies on a combination of clinical, pathological, virological, serological, and molecular-genetic methods.

Diagnosis of avian influenza includes:

- clinical diagnosis;



- pathological examination;
- virus isolation;
- serological methods;
- molecular-genetic methods;
- rapid antigen-detection tests.

Clinical diagnosis.

The first step in identification is assessing clinical signs and the epizootic situation.

The main clinical signs of influenza include:

- lethargy;
- reduced appetite;
- a sharp drop in egg production;
- cyanosis of the comb and wattles;
- head swelling;
- coughing and sneezing;
- nasal discharge;
- diarrhea;
- neurological disorders;
- sudden death.

Highly pathogenic strains are associated with very high mortality, which can reach 90–100% [2].

Epizootiological factors taken into account include:

- contact with wild birds;
- seasonal migration;
- movement of birds;
- biosecurity breaches;
- outbreaks recorded in the region.

Laboratory diagnosis of avian influenza. Laboratory identification uses the following sample types:

- tracheal swabs;
- cloacal swabs;
- blood;
- lungs;
- trachea;
- spleen;
- brain;

- intestine;
- feces;
- embryonic tissue.

Samples are collected during the acute phase of illness, under strict biosecurity precautions.

Virological identification methods. The classical diagnostic approach is virus isolation in embryonated chicken eggs. In the standard method, 9–11-day-old chicken embryos are inoculated with pathological material.

After inoculation, the following are assessed:

- embryo mortality;
- hemagglutinating activity;
- changes in the chorioallantoic membrane.
- This method is considered the “gold standard” of virological diagnosis [14].
- The virus can also be isolated and cultured in cell cultures, including:
- Madin-Darby canine kidney (MDCK) cells;
- chicken embryo cells;
- primary fibroblast cultures.
- Culturing in cell lines makes it possible to:
- determine cytopathic effect;
- study viral replication;
- assess the virus’s sensitivity to antiviral drugs.

Serological methods. Serological testing is used to detect viral antibodies or antigens.

The hemagglutination inhibition (HI) test is one of the most widely used methods for serotyping the virus. It is based on the virus’s ability to agglutinate erythrocytes, and the loss of this ability in the presence of specific antibodies.

The HI test can be used to determine the hemagglutinin subtype, assess the immune response, and confirm infection.

Enzyme-linked immunosorbent assay (ELISA) is used for:

- mass screening;
- antibody detection;
- monitoring vaccinated flocks.

Its advantages include high sensitivity, speed, and the ability to automate monitoring and diagnosis.

Molecular-genetic diagnostic methods. Reverse-transcription polymerase chain reaction (RT-PCR) is the main modern method for identifying the avian influenza virus.

The method allows:

- detection of viral RNA;
- subtype determination;
- assessment of pathogen pathogenicity;
- rapid results.

The genes most often targeted are those encoding hemagglutinin (HA), neuraminidase (NA), and the matrix protein (M).

The advantages of PCR-based influenza diagnosis include high sensitivity and specificity, fast turnaround, and the possibility of early diagnosis.

Genome sequencing is used to:

- determine strain origin;
- monitor viral evolution;
- identify pandemic risks.
- analyze mutational changes;

Sequencing makes it possible to track the emergence of new viral variants and assess cross-species adaptation.

Immunofluorescence assay. This method relies on fluorescently labeled antibodies to detect viral antigens in cells.

Immunochromatographic (rapid) tests. Rapid tests allow field detection of viral antigens.

The drawbacks of these methods are lower sensitivity and the risk of false-negative results. For this reason, positive results require confirmation by PCR.

Avian influenza must be differentiated from:

- Newcastle disease;
- infectious bronchitis;
- infectious laryngotracheitis;
- pasteurellosis;
- mycoplasmosis;
- poisoning.

Differentiation relies on laboratory testing using specific molecular-genetic and serological assays.

Biosafety in laboratory diagnosis. Work with highly pathogenic strains requires:

- BSL-3-level laboratories;



- personal protective equipment;
- specialized ventilation systems;
- rigorous disinfection.

The WHO emphasizes that timely and accurate identification of the avian influenza virus makes it possible to:

- quickly contain an outbreak;
- determine strain pathogenicity;
- assess risks to humans;
- carry out effective epizootic monitoring;
- forecast pandemic threats;
- monitor vaccination effectiveness.

In summary, modern identification of the avian influenza pathogen relies on a combination of classical and molecular-genetic methods. RT-PCR and genome sequencing are currently the most informative and accurate tools, allowing rapid detection of new viral variants and control of their spread.

Environmental sampling for avian influenza is an important part of epizootic monitoring and the biosecurity system, since the virus can persist in the external environment for a long time and serve as a source of infection for domestic and wild birds, as well as humans. Environmental monitoring allows timely detection of viral circulation, assessment of spread risk, and prevention of outbreaks.

The importance of environmental testing. The avian influenza virus is shed into the environment through feces, respiratory secretions, saliva, and eye discharge from infected birds. In the external environment, the virus can contaminate water, soil, feed, litter, surfaces, equipment, vehicles, and staff clothing.

Testing these objects is important for establishing the source of infection and the routes of its spread [8].

Main environmental objects tested for avian influenza.

Water. Water plays a key role in the circulation and transmission of avian influenza viruses among waterfowl, which serve as the natural reservoir of the infection. Epizootiological monitoring covers natural bodies of water, drinking troughs, wastewater, and water-supply reservoirs. Bodies of water located along wild bird migration routes are of particular importance, since they can serve as sites where the pathogen accumulates and persists.

According to Webster et al. [5], influenza A viruses can persist for a long time in aquatic environments, which facilitates transmission between birds. At low ambient

temperatures, the infectious agent can remain viable in water for several weeks or even months.

Soil and litter. Soil and litter in poultry housing areas are important objects of environmental surveillance. Because of the accumulation of droppings, these substrates can contain substantial amounts of viral particles and serve as a source of further spread. Samples are collected in poultry houses, outdoor runs, waste storage and disposal sites, and quarantine facilities.

Contamination of litter is considered an important factor in within-farm virus transmission, particularly under conditions of high stocking density and inadequate biosecurity.

Surfaces and equipment. Avian influenza viruses can persist for some time on various surfaces and objects in the production environment. Objects subject to monitoring include metal and plastic surfaces, cages, feeders, transport containers, ventilation system components, and other equipment.

Surface swabs are regularly collected to assess the effectiveness of sanitary measures and disinfection. The results make it possible to identify contamination hotspots in time and prevent mechanical spread of the pathogen.

Air and dust. In enclosed production facilities, the virus can spread through aerosols and dust particles. This transmission route is particularly relevant during outbreaks of highly pathogenic avian influenza, when the concentration of virus in the environment rises considerably.

Air sampling relies on aerosol samplers, filtration systems, and dust-particle settling methods. Air monitoring makes it possible to assess the risk of infection spreading within a holding and to take timely anti-epizootic measures.

Methods for testing environmental objects.

Environmental samples are collected in line with international recommendations and veterinary-sanitary requirements. Sterile swabs, biological sample containers, special filters, and equipment for water and air sampling are used for this purpose.

After collection, material is transported to the laboratory under cold-chain conditions and biosafety requirements. WOAHA emphasizes that environmental testing is an important part of the avian influenza surveillance system.

The main laboratory method for detecting avian influenza viruses in environmental samples is reverse-transcription PCR (RT-PCR). This technology can detect viral RNA even at low concentrations in a sample, determine its subtype, and assess the level of environmental contamination (Figure 2).

The advantages of RT-PCR include high sensitivity, specificity, and speed of results, which is why the method is considered the “gold standard” for laboratory diagnosis of avian influenza. According to the CDC [11], RT-PCR remains the priority method for detecting avian influenza viruses in both clinical and environmental samples.

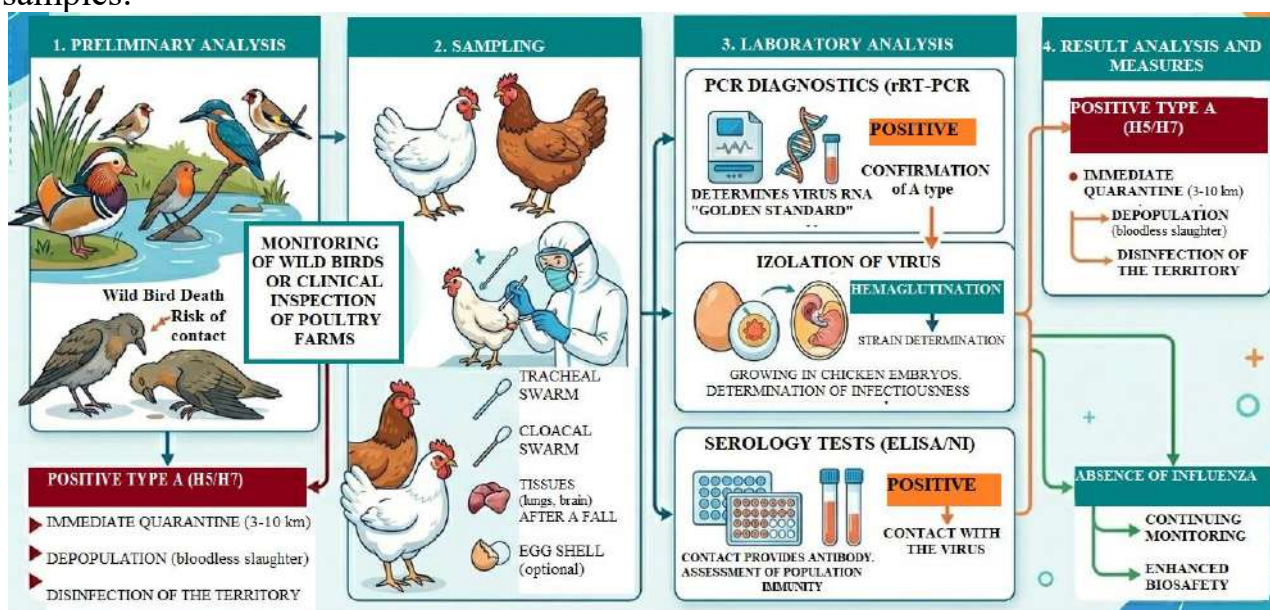


Figure 2 – Scheme of avian influenza monitoring and diagnosis (algorithm and methods for poultry holdings)

Classical virological methods are used to confirm the presence of a viable virus in environmental samples. These involve isolating the pathogen by inoculating embryonated chicken eggs or culturing it in susceptible cell lines. Such studies make it possible not only to detect the virus, but also to assess its infectivity and potential for further spread.

Virological methods are particularly important for confirming the epizootiological role of contaminated environmental objects and determining the potential danger of the strains detected.

Alongside molecular and virological testing, immunological diagnostic methods are also widely used, including ELISA, immunofluorescence methods, and various rapid antigen tests.

These methods are used mainly for initial screening of large numbers of samples. Their advantages are relative simplicity, speed, and suitability for field use. However, positive results usually require confirmation by more sensitive laboratory methods, particularly RT-PCR.

Factors affecting viral survival in the environment. How long the avian influenza virus persists in the environment depends on a range of physical and chemical factors. The most important are temperature, humidity, pH, ultraviolet radiation intensity, and the presence of organic matter.

The most favorable conditions for virus survival are low temperatures, high humidity, and the presence of organic substrates such as droppings, litter, or silt. In aquatic environments and organic waste, the virus can remain viable for a long time, facilitating its spread among susceptible animals.

Conversely, higher temperatures, exposure to solar ultraviolet radiation, and effective disinfectants quickly inactivate the virus. According to Alexander [2], proper disinfection is one of the most effective measures for controlling viral circulation on holdings.

The epizootiological value of environmental testing. Monitoring environmental objects is an important part of the avian influenza surveillance system. The resulting data make it possible to detect hidden viral circulation in time, forecast the risk of new outbreaks, and evaluate the effectiveness of anti-epizootic measures already taken.

In addition, environmental monitoring results are used to assess the level of biosecurity at poultry enterprises and prevent the spread of infection between holdings. Regular monitoring is particularly important for bodies of water along wild-bird migration routes, live-bird markets, commercial poultry farms, logistics centers, and transport hubs, all of which can play a role in spreading the pathogen.

Biosafety requirements during testing. Working with environmental samples potentially contaminated with the avian influenza virus requires strict adherence to biosafety requirements. Personnel must use personal protective equipment, including protective clothing, gloves, respirators, and eye protection. Strict disinfection of equipment, work surfaces, and vehicles is also essential.

Laboratory testing must be carried out in facilities with an appropriate level of biosafety, using procedures that minimize the risk of generating infectious aerosols and

infecting personnel. The World Health Organization emphasizes that environmental contamination can play an important role in transmitting avian influenza viruses to people and animals.

In summary, environmental testing is an integral part of the modern avian influenza surveillance system. Comprehensive monitoring of water, soil, air, production equipment, and other environmental components makes it possible to detect viral circulation in time, establish routes of spread, and assess the effectiveness of preventive measures. The resulting findings are important for preventing large-scale epizootics among birds and reducing the risk of potential epidemic threats to the population.

CLINICAL MANIFESTATIONS OF AVIAN INFLUENZA

The clinical course of avian influenza is shaped by several factors, chief among them the pathogenicity of the viral strain, the species and age of the birds affected, immune status, housing conditions, and the presence of concurrent infections. Based on virulence, avian influenza viruses are divided into low pathogenic (LPAI) and highly pathogenic (HPAI) forms. Low pathogenic variants generally cause mild respiratory disturbances or run an asymptomatic course, whereas highly pathogenic strains cause severe systemic disease with high mortality and substantial economic losses [2].

Incubation period

The incubation period usually lasts from a few hours to seven days, depending on the biological properties of the virus, infectious dose, route of infection, and species of bird involved. Highly pathogenic strains are associated with rapid onset of clinical signs, which can appear as early as 24–48 hours after infection.

Main clinical signs in birds

The initial signs of disease are usually nonspecific. Affected birds show lethargy, reduced activity, drowsiness, loss of appetite, ruffled feathers, and increased thirst. They become less mobile, tend to stay in groups, and respond weakly to external stimuli.

Since the respiratory tract is the virus's primary target, the disease is often accompanied by coughing, sneezing, wheezing, and difficulty breathing. Nasal discharge, shortness of breath, and inflammation of the nasal sinuses may also occur. In severe cases, birds breathe through an open beak, and cyanosis of the mucous membranes and tracheal swelling develop. According to Swayne and Halvorson [8], respiratory distress is one of the most typical signs of highly pathogenic avian influenza.

In many cases the infection also affects the digestive tract. Diarrhea, watery or greenish droppings, dehydration, and digestive disturbances are commonly seen. In ducks and other waterfowl, gastrointestinal signs may predominate over respiratory ones.

When the infection becomes generalized and the central nervous system is affected, neurological signs of varying severity appear. Sick birds may show tremor, convulsions, loss of coordination, twisted neck, limb paralysis, circling movements, and opisthotonus. These signs are particularly characteristic of highly pathogenic H5N1 strains.

Highly pathogenic avian influenza is typically associated with marked changes in the vascular system. Affected birds show cyanosis of the comb and wattles, head swelling, hemorrhages on mucous membranes and the skin of the limbs, and subcutaneous edema. Generalized vascular damage leads to severe intoxication and a sepsis-like state. Alexander [2] notes that highly pathogenic avian influenza viruses can cause systemic disease accompanied by hemorrhages and edema in various organs and tissues.

In laying hens, one of the characteristic manifestations of infection is a sharp decline in productivity. This includes a marked drop in egg production, misshapen or thin-shelled eggs, reduced egg size, and increased embryo mortality. In some cases, deteriorating reproductive indicators are the first sign of disease in a flock.

Highly pathogenic avian influenza can also run a peracute course, in which clinical signs are essentially absent or barely noticeable. In such cases, the only sign of disease is sudden mass mortality. Mortality in some outbreaks can reach 90–100% within a few days [4].

Differences in disease course among bird species. Susceptibility to the virus and the nature of clinical signs vary considerably by species. Chickens and turkeys are among the most susceptible species and, when infected with highly pathogenic strains, usually develop acute disease with high mortality. Ducks and geese often carry the infection with few or no clinical signs, but play an important role in maintaining and spreading the virus. In wild waterfowl, the disease is mostly asymptomatic or subclinical. According to the CDC (2024), wild waterfowl are frequently carriers of avian influenza viruses without visible signs of illness.

Pathological changes

Postmortem examination of birds that have died of avian influenza reveals characteristic changes indicating a systemic infection. The most common findings are multiple hemorrhages in internal organs, pulmonary edema, tracheal inflammation, splenomegaly, foci of tissue necrosis, serofibrinous lesions of serous membranes, and congestion of internal organs (Figures 3,4). Highly pathogenic forms of avian influenza are characterized by generalized involvement of most organs and systems, resulting from viremia and severe vascular disturbances. Clinical manifestations of avian influenza in humans

Human infection with avian influenza viruses is accompanied by clinical signs ranging in severity from mild respiratory symptoms to severe systemic disease with a high risk of death. The course of the disease largely depends on the viral subtype, infectious dose, route of infection, and individual characteristics of the person infected.

The initial signs of illness usually include a sudden rise in body temperature, general weakness, chills, headache, and pronounced myalgia. Most patients show signs of respiratory tract involvement, including a dry cough, sore throat, difficulty breathing, and shortness of breath. A characteristic feature of some avian influenza strains is conjunctivitis, which may precede or accompany other clinical signs.

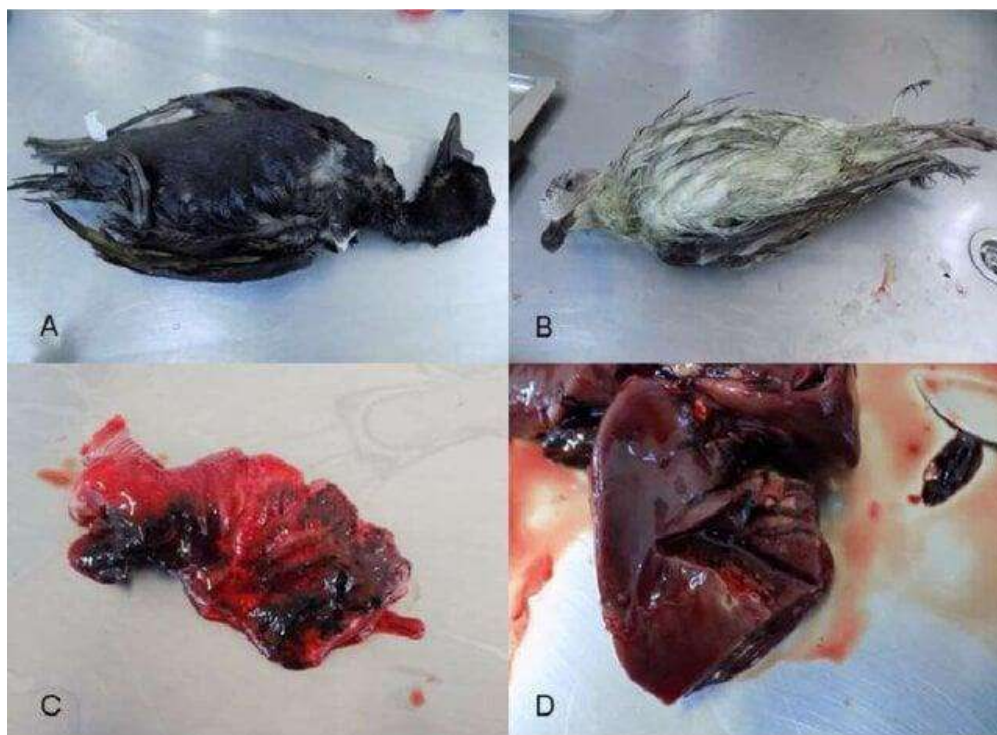


Figure 3 – Pathological changes in avian influenza in wild birds [16].

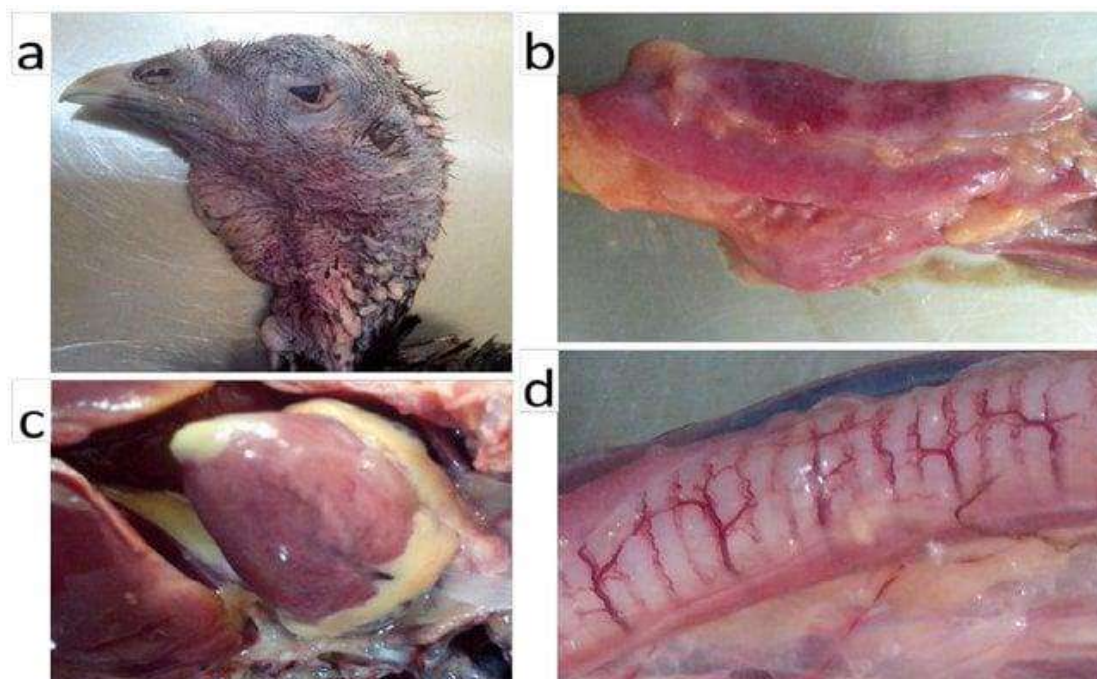


Figure 4 — Pathological changes in avian influenza in turkeys [17].

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As the disease progresses, viral pneumonia can develop, involving severe damage to lung tissue and a rapid decline in respiratory function. In such cases, acute respiratory failure often occurs, requiring intensive care and respiratory support. Severe forms of the disease can be complicated by acute respiratory distress syndrome (ARDS), septic shock, multi-organ failure, and other critical conditions. The high pathogenicity of some strains accounts for the substantial mortality among infected individuals. According to the World Health Organization, human infection with avian influenza viruses can cause severe respiratory illness with high fatality rates.

Differential diagnosis. Because the clinical signs of avian influenza are not disease-specific, establishing a definitive diagnosis requires comprehensive differential diagnosis. In birds, the disease must be distinguished from other infectious and non-infectious conditions presenting with respiratory, neurological, or systemic signs.

Differential diagnosis is primarily conducted against Newcastle disease, which presents with similar respiratory and nervous-system signs and is likewise highly contagious. Infectious bronchitis, infectious laryngotracheitis, bacterial infections such as pasteurellosis, and mycoplasmosis must also be ruled out. The possibility of toxic damage from feed or chemical toxins should also be considered.

Since the clinical and pathological picture of many diseases overlaps considerably, a definitive diagnosis of avian influenza can only be confirmed by laboratory methods, including PCR, virus isolation, and serological testing.

MEASURES FOR THE CONTROL AND PREVENTION OF AVIAN INFLUENZA

Effective control of avian influenza relies on a comprehensive combination of biosecurity measures, epizootic monitoring, and veterinary-sanitary control, in line with the recommendations of the Food and Agriculture Organization of the United Nations (FAO), the World Organisation for Animal Health (WOAH), and Ukraine's national legislation.

One key area of prevention is ensuring bio-isolation of flocks by housing domestic poultry in enclosed buildings or specially equipped enclosures that prevent contact with wild migratory and synanthropic birds. This measure is especially important in areas near bodies of water and along seasonal bird migration routes, which serve as the natural reservoir of type A influenza viruses.

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Strict veterinary biosecurity measures at poultry enterprises are another key component of prevention. This includes setting up sanitary checkpoints for personnel, mandatory changes of protective clothing and footwear, disinfection of hands and vehicles, and restricting access by outsiders to production areas. It is also advisable to prohibit staff at commercial holdings from keeping domestic poultry of their own, which reduces the risk of introducing the pathogen into production.

Particular attention is given to controlling feed and water. Feed should be stored in sealed facilities inaccessible to wild birds and rodents, and only clean drinking water or properly treated water should be used for poultry. Where grain feed is used, heat treatment is advisable to help inactivate potential pathogens.

Epizootic monitoring occupies an important place in the system of anti-epizootic measures. It involves regular serological, virological, and molecular-genetic testing, including PCR, for timely detection of viral circulation in bird populations. Particular attention is paid to holdings located in elevated-risk zones, including those along wild bird migration routes.

To prevent the spread of infection through poultry products, veterinary-sanitary control is applied at every stage of production and sale. Slaughter must take place exclusively at specialized facilities under veterinary supervision, and the sale of meat and eggs without proper accompanying documentation is not permitted.

Raising public and industry awareness of disease signs, transmission routes, and personal protective measures is an important component of prevention. When handling sick or suspect birds, personal protective equipment – gloves, protective clothing, and respirators – should be used.

Once an outbreak of highly pathogenic avian influenza is confirmed, quarantine restrictions are introduced to contain and eliminate the disease focus. Depending on the epizootic situation, protection and surveillance zones are established, movement of birds, products, and vehicles is restricted, and susceptible flocks are culled, followed by safe disposal of biological waste. After the focus is eliminated, thorough cleaning and repeated disinfection of buildings, equipment, and the holding's grounds is carried out.

Consequences of failing to comply with control measures

Ignoring established biosecurity and veterinary-sanitary requirements can lead to rapid spread of infection among susceptible flocks and large-scale epizootic outbreaks. Highly pathogenic strains are extremely contagious and can cause mass mortality within a short period.

The spread of the disease results in substantial economic losses linked to flock destruction, halted production, outbreak-elimination costs, and international trade restrictions. In addition, viral circulation in bird populations raises the risk of human infection and creates potential preconditions for new pandemic variants to emerge through mutation or genetic reassortment.

The spread of avian influenza can also negatively affect a country's food security, through reduced production of poultry meat and eggs, rising prices for animal-derived products, and disruption of the agricultural sector's stability. Additional consequences may include socioeconomic problems linked to reduced farm profitability, job losses, and environmental contamination risks from improper disposal of infected biological material.

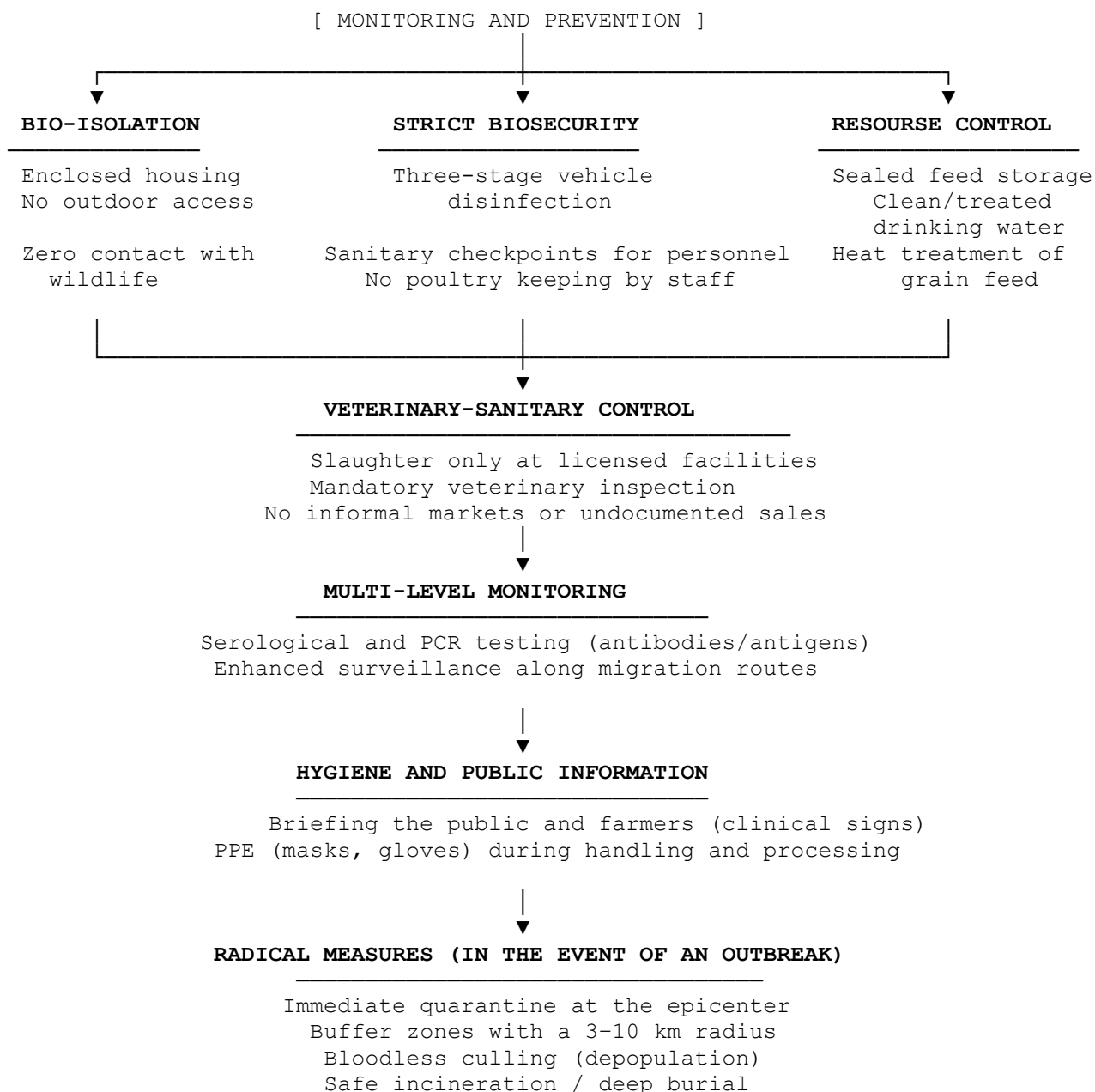
Advantages of complying with biosecurity measures

Comprehensive implementation of preventive and anti-epizootic measures effectively contains viral circulation and prevents its introduction into commercial poultry operations. Applying biosecurity principles helps preserve the economic stability of enterprises, minimizes outbreak-elimination costs, and sustains the sector's export potential.

Beyond the economic benefits, compliance with control measures is also important for protecting public health. Reducing the level of viral circulation in the environment lowers the risk of cross-species transmission and human illness. Avian influenza prevention is therefore an integral part of the One Health concept, which calls for an integrated approach to protecting the health of animals, humans, and the environment.

AVIAN INFLUENZA CONTROL SYSTEM

(In line with the standards of the State Service of Ukraine on Food Safety and Consumer Protection and FAO)



CONCLUSIONS AND OUTLOOK

The transmission chain “wild migratory bird – environmental objects (water, soil, air) – domestic poultry – human” is confirmed by the results of the environmental monitoring carried out under the Protocol. The virus’s ability to persist for extended periods in aquatic environments and organic substrates points to the existence of a hidden environmental reservoir of infection, which substantially extends the scope of surveillance beyond purely clinical observation of domestic poultry.

The combined use of molecular-genetic, virological, and serological methods is the priority approach to diagnosis. The analysis shows that reverse-transcription PCR (RT-PCR), together with classical virus isolation in embryonated chicken eggs, the hemagglutination inhibition test (HI), and ELISA, provides the highest diagnostic reliability at every stage — from initial screening to confirmation of strain pathogenicity. This combination of methods forms the basis of the Protocol as the recommended approach for unified cross-border monitoring.

The lack of a harmonized approach between Ukraine and Romania remains a critical gap, particularly given the shared migration routes of wild waterfowl through the Danube Delta. Before the SMS-RO-UA project, the collection and exchange of epizootiological data by the two countries took place without adequate coordination, which made it impossible to assess cross-border risks of introduction of highly pathogenic strains (H5N1, H5N8, and others) in a timely manner. The Joint TOR and the Protocols developed are a first step toward closing this gap.

Wild migratory waterfowl remain the key, and practically uncontrollable, natural reservoir of the virus. In the case of avian influenza, preventive influence over the reservoir (wild birds) is limited, which increases the importance of bio-isolating domestic flocks and conducting systematic ornithological monitoring along migration routes as the principal tool of containment.

The seasonal nature of epizootic risk reinforces the need for early preventive measures. The highest probability of pathogen introduction is linked to the periods of autumn (August–November) and spring (February–April) waterfowl migration. Campaigns to strengthen biosecurity at poultry holdings and smallholder farms should be rolled out no later than one month before the start of each migration period.

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Implementation of the “One Health” principle in its cross-border dimension remains the central strategic benchmark for the further development of the avian influenza monitoring system. Specific perspectives, taking EU priorities into account, are set out below.

Integration into the EU’s joint epizootic surveillance system. Since highly pathogenic avian influenza is classified under Regulation (EU) 2016/429 as a category A disease subject to mandatory notification within 24 hours of confirmation, the results of the SMS-RO-UA project provide a foundation for connecting laboratories to the EU-level data exchange system.

Development of a GIS platform for predictive migration monitoring. Predictive modeling of virus-introduction risk using artificial intelligence algorithms is consistent with the European Veterinary Big Data Strategy 2022–2027.

Harmonization of Ukraine’s national legislation with EU standards. In the context of Ukraine’s status as an EU candidate country, the approach to joint standardized data collection established in this Protocol is a practical tool for aligning with Regulation (EU) 2016/429 and Commission Delegated Regulation (EU) 2020/687 on the control of category A diseases.

Development of balanced vaccination strategies. Vaccination of domestic poultry against avian influenza remains a matter of debate, owing to the limited cross-protection between viral subtypes and possible trade restrictions on vaccinated flocks. Further research should focus on monitoring the subtypes circulating in the cross-border region, to support evidence-based decisions on the advisability of selective vaccination in elevated-risk zones, in line with WOA and FAO recommendations.

Incorporating the results into educational programs. The materials in this Protocol, together with the Practical Guide to Assessing Emerging Diseases, are recommended for integration into the training programs of veterinary medicine faculties and partner institutions, in line with the goals of the Erasmus+ Programme and the EU Strategy for the Danube Region (EUSDR).

Engaging communities and raising public awareness. The project’s findings underscore the need for systematic public information campaigns, particularly among smallholder farmers, live-bird market workers, and poultry farm employees in the

border zone, with an emphasis on recognizing clinical signs and rules for handling dead wild birds.

Building a cross-border network for laboratory and ornithological surveillance. The SMS-RO-UA project lays the groundwork for a sustainable cross-border network among laboratories and potential new participants from neighboring countries located along the same migration corridor. Such a network is consistent with the priorities of EU macro-regional strategies and could be supported under future Interreg or Horizon Europe programmes..

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