PRACTICAL GUIDE
"ASSESSMENT OF EMERGING DISEASES"

Developed under Activity 1.4 "Joint research of emerging diseases in cross-border area: sampling and analysis"

of the international project
"SMS-RO-UA: 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 VI-A NEXT Romania–Ukraine Programme 2021–2027

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INTRODUCTION

This Practical Guide has been developed in fulfilment of service agreement No. 173 dated October 2026, concluded with Odesa State Agrarian University under Activity 1.4 "Joint research of emerging diseases in cross-border area: sampling and analysis" of the international project "SMS-RO-UA: 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 VI-A NEXT Romania–Ukraine Programme 2021–2027. The document is based on an analysis of the diseases studied within the project in the "animal–water–soil–human" chain. It contains practical recommendations on sampling, laboratory analysis, and the application of unified approaches and accurate research methods for the relevant diseases.

To emerging infectious diseases (EID) belong infectious diseases that have appeared recently or are characterised by resistance to medicines, as well as those whose incidence is rising rapidly and is likely to continue rising in the future. Taking into consideration their uncertainty and unpredictability, these diseases can cause high mortality and significantly affect social stability and economic development, since people are unable to respond to them immediately and take specific preventive or control measures. Emerging infectious diseases have therefore become a serious public health problem worldwide [1]. The emergence of infectious diseases is driven by specific factors. With proper epidemiological investigation and laboratory surveillance, the causes of outbreaks can be identified, since most new infections (even those resistant to antimicrobials) usually arise in a particular place and then spread to new areas [2]. However, a casual attitude towards vaccination and the use of antibiotics, together with insufficient funding for epidemiological surveillance and control

programmes, has intensified the spread of many diseases [3]. Climate change and natural disasters play a role in the emergence of many infectious diseases [4]. The spread of these diseases is also influenced by rapid urbanisation [5] and cross-species transmission resulting from increased interaction between wildlife, livestock and the human population [6]. The indirect route of transmission of zoonotic pathogens through the food chain is the contamination of food with waste of animal and human origin. Most organisms associated with zoonotic outbreaks of foodborne illness are not new pathogens, but every outbreak of infectious disease should raise the question of whether food (and water) may be the way of transmission [7]. The rapidly expanding scale and globalisation of the food market, although controlled by food safety systems, are vulnerable, because disruption of these processes can lead to scattered outbreaks that are difficult to trace [8]. International travel and migration increase every year, and the number of displaced persons has reached its highest level since the Second World War, at almost 65 million people [9]. Tourism sets new records every year [10]. Global travel is not limited to people: the successful spread of disease vectors through global trade is of particular concern [12].

Emerging and re-emerging infectious diseases in various socio-ecological settings pose unprecedented challenges for global public health and socio-economic development. The One Health concept is based on a systemic, transdisciplinary approach and covers the interaction between humans and animals in given socio-ecological conditions. It can comprehensively take into account the human-animal-environment interaction that underlies zoonotic diseases. The One Health approach is therefore effective in controlling and eliminating the factors that contribute to the emergence of infectious diseases [11]. By synthesising international studies and norms, data from international sources [13], national legislation [14, 15, 16], scientific

programmes and the results of practical case analyses, gaps are identified in countries' capacity to implement the One Health approach, and opportunities are outlined for strengthening biosurveillance, laboratory systems, biosafety and inter-agency cooperation [17]. The Lower Danube region, and in particular the border area between Romania and Ukraine, is a zone of great epidemiological importance at both the regional and the European level. This is due to a combination of geographical, ecological, climatic and anthropogenic factors that favour the persistence and circulation of emerging and re-emerging pathogens and, under certain conditions, their spread. The area includes extensive wetland ecosystems, diverse natural and semi-natural habitats, large inland water bodies, flood-prone territories, agricultural land and scattered rural settlements. In such conditions, contacts between people, domestic animals, wildlife and the environment are frequent and complex. The administrative border between Romania and Ukraine is not an obstacle to the movement of wild birds, certain mammal species or vectors, or to the spread of environmental factors involved in pathogen transmission. In this context, health risk assessment cannot be effective if it is limited to the local or national level; a coordinated, integrated and unified approach covering the entire cross-border territory is needed instead.

The Guide is addressed to specialists who take decisions and carry out supervision in the fields of animal health, food safety and public health: national and regional authorities (in particular veterinary and sanitary services), sectoral agencies, laboratories, research and educational institutions, veterinary clinics, healthcare institutions, as well as civil society organisations, farmers and animal owners in the Romania–Ukraine cross-border region.

1. THE "ANIMAL–WATER–SOIL–HUMAN" CHAIN IN THE CONTEXT OF FOOD SAFETY

The interdependence of the health of humans, animals and the environment or ecosystem in many respects, including food safety, requires that problems in any of these sectors be addressed not in isolation but through a broader systems approach in which all sectors are considered part of the solution [18].

The One Health concept is an expanding area of professional global health protection that has emerged from the recognition of growing interconnections and overlaps – economic, cultural and physical – at the interface of human, animal and ecosystem health (Figure 1).

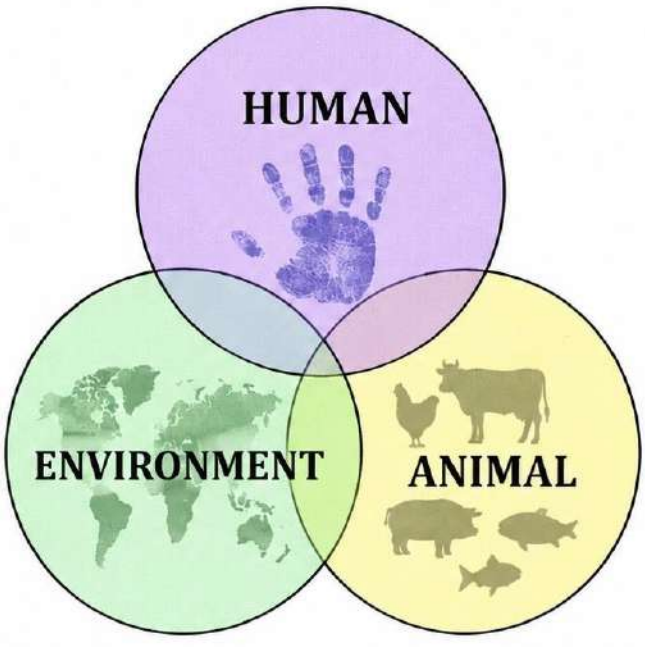

Figure 1. The One Health triad: the interrelationship of humans, animals and the environment [19]

The One Health concept seeks to shift the paradigm from the current "individual" and "disease-oriented" approach focused on treatment to a "systemic" or "societal"

approach focused on prevention. One Health is a creative way of viewing the health of humans, animals and ecosystems as a joint effort of medical professionals and environmental scientists working together in a shared and synergistic way (Figure 2).

One Health provides a framework for addressing food safety problems in a transdisciplinary way, in which solutions come from both within and outside the individual disciplines, creating new perspectives for resolving these complex global challenges.

As the world population grows from 7 billion today to 9.1 billion by 2050 [9], food security, food safety and adequate nutrition will become increasingly important. Over the past few decades, 75% of new infections in humans of all kinds have been of zoonotic origin, meaning that they can be transmitted from animals to humans, and approximately 30% of all new infections that have emerged worldwide over the past 60 years involve pathogens that are usually transmitted through food.

Since many known foodborne pathogens are zoonotic and may be found in wildlife and the environment, eliminating these sources and developing control measures aimed at reducing the risk of human infection at the environmental level is a critical element. However, mitigating the consequences of wildlife contamination is a complex task. There are no cost-effective mechanisms for preventing direct contact [20].

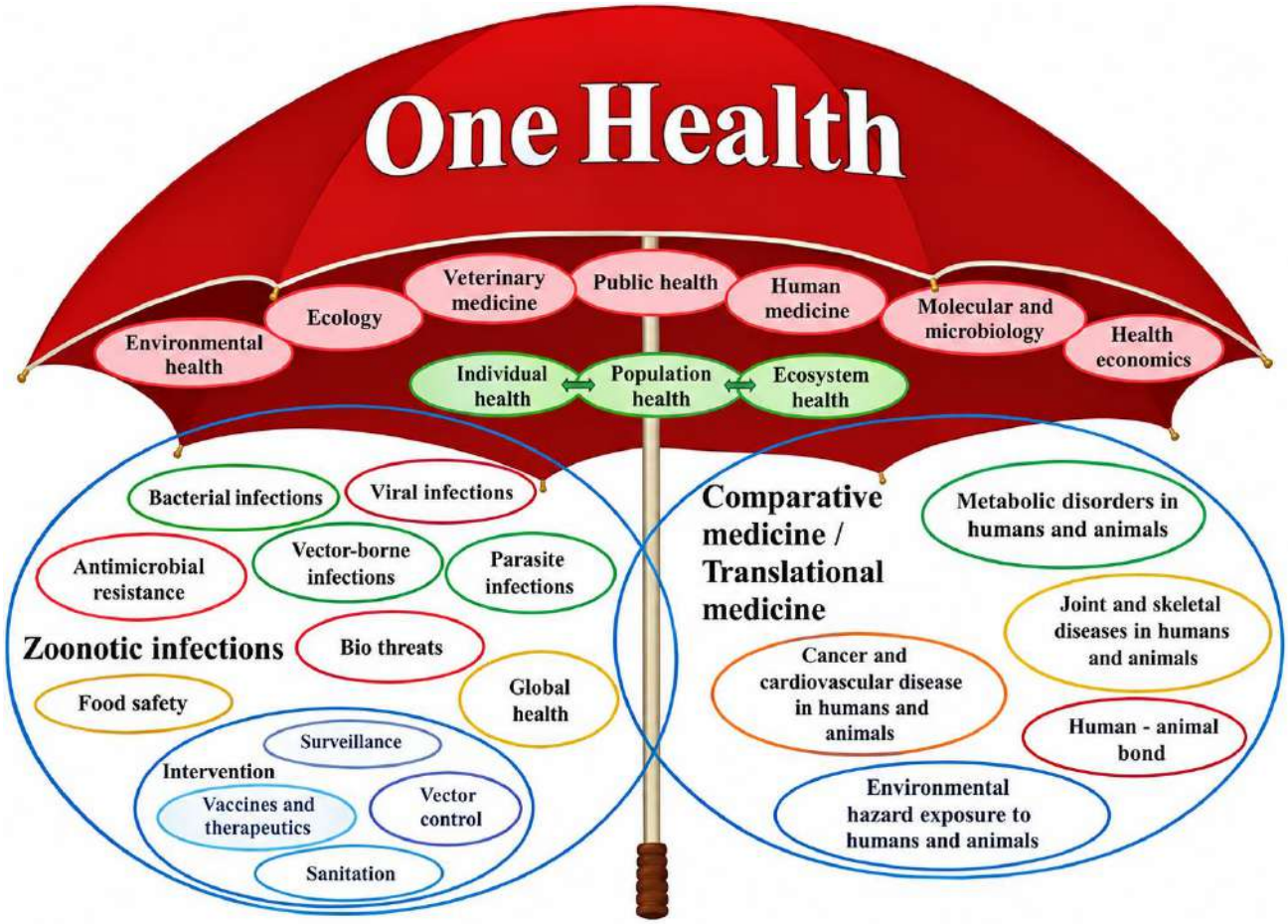

Figure 2. The One Health concept shows the interrelationships and interdependence of different disciplines

The role of animals. Animals and wildlife are regarded as a direct threat to food safety. The rapid growth of the world population brings with it a rapid growth in demand for higher agricultural productivity and quality [20]. In particular, there is an unprecedented increase in demand for animal protein and products of animal origin. In Asia, daily consumption of animal protein has more than tripled over the last fifty years, especially among higher-income populations [20]. This growth in demand has ultimately led to the global biomass of farm animals exceeding that of humans, which has inevitably changed the methods of modern farming [21]. One of the areas in which

livestock production plays a vital role in animal and human health is zoonoses – infectious diseases transmitted between humans and animals. Zoonotic transmission can occur through direct or indirect contact with animals and their environment; consumption of products of animal origin or water contaminated by animals; and through intermediate species such as insect vectors. Although zoonotic pathogens often require a coordinated interaction of environmental, pathogen and host factors to overcome the barriers to animal-to-human transmission, they are able to adapt and cause infections in humans [22, 23], and they are believed to account for approximately two thirds of recent major outbreaks of infectious diseases.

Farm animals such as poultry, pigs and cattle are the main reservoirs and sources of human infection with these pathogens (Figure 3). In addition, the contaminated agricultural environment is also an important transmission route, for example soil contaminated with B. anthracis or Clostridium spp. [24]. However, most such pathogens, such as Campylobacter spp., Listeria spp. and Shigella spp., act as opportunistic microorganisms, colonising the intestines of farm animals, often without significant clinical signs [25].

Livestock production also has other secondary environmental impacts, including ecosystem destruction and environmental pollution from livestock waste and emissions [26].

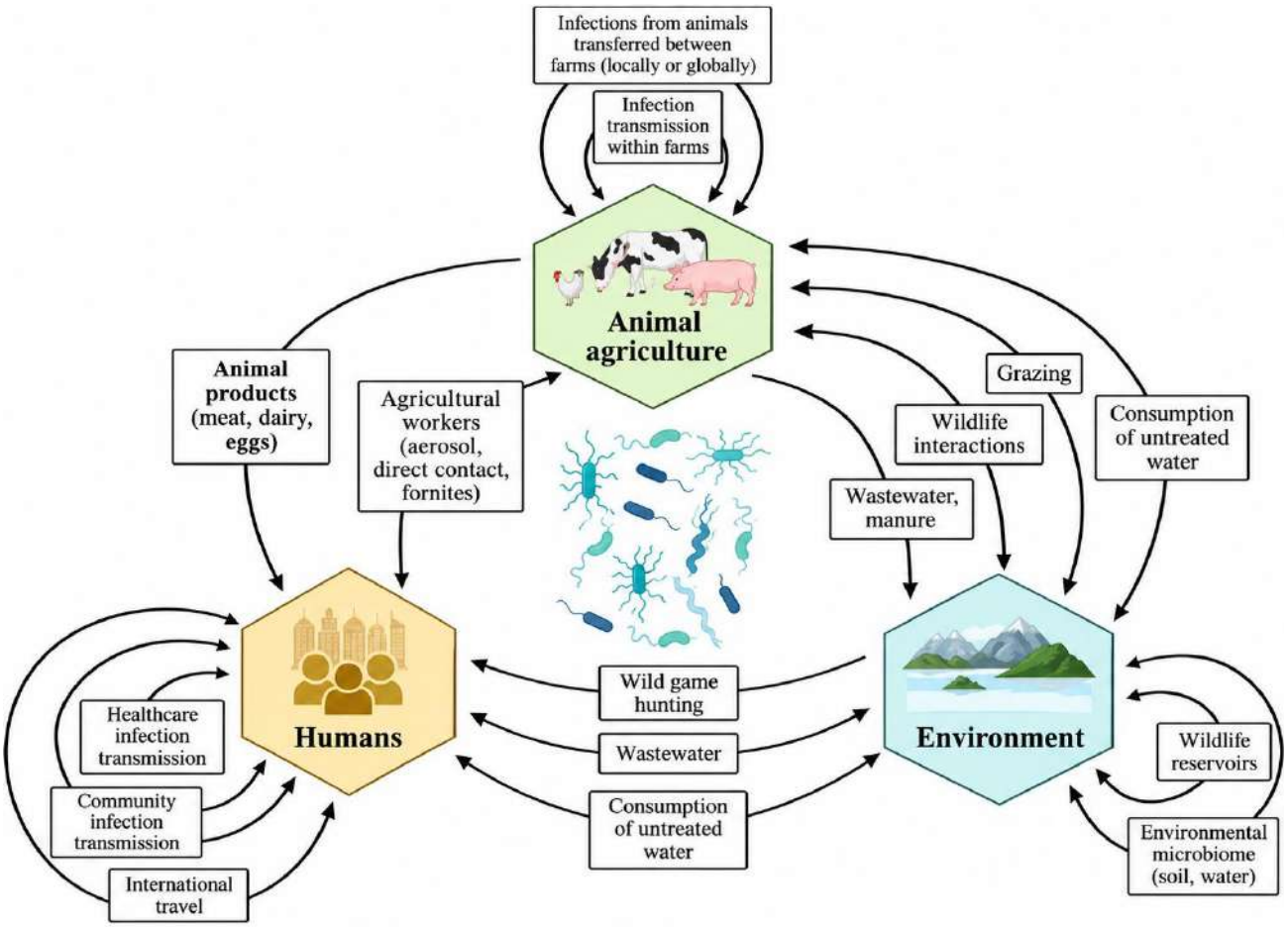

Figure 3. Typical transmission routes of bacterial zoonoses in livestock production

**Arrows indicate common routes of bacterial transmission between the three main groups: farm animals, humans and the environment*

For example, the conversion of natural biocoenoses to agricultural use causes ecological changes that may increase the diversity of hosts of zoonotic diseases. Livestock production also generates enormous quantities of waste, including manure, urine, greenhouse gases and surplus feed [27]. Wastewater containing chemicals, pathogens and nutrients often enters and pollutes the environment, which is not only a mechanism for the spread of antimicrobial resistance, but may also have broader negative consequences for environmental health. Excess nitrogen and phosphorus from

agricultural runoff causes eutrophication in adjacent water bodies, posing significant risks to the health of animals in aquatic ecosystems and of those who depend on these ecosystems [28].

The role of water. Most waterborne diseases are spread by pathogenic microorganisms transmitted through contaminated fresh water. These diseases can be transmitted during bathing, washing or drinking water, and through eating food that has come into contact with contaminated water. Infection usually occurs through drinking contaminated water, using such water in food preparation, or eating food contaminated with these pathogens [29]. The overall picture of water-related diseases is complex for a number of reasons. With the emergence of new waterborne infectious diseases and the re-emergence of older known ones, the picture of water-related human health problems has become increasingly comprehensive over recent decades. There are few data on some diseases associated with water, hygiene or sanitation, such as cholera, salmonellosis or shigellosis [30]. It is becoming difficult to determine precisely the relative importance of the aquatic components of a local ecosystem, even where water plays a substantial role in disease ecology [31]. Contaminated water can carry pathogens of animal origin directly onto crops (for example, during irrigation or other operations such as diluting pesticides or nutrients). This transmission route has probably been studied better than any other. Numerous studies have been published on the catchments that supply water for agricultural needs in areas (or near areas) where specialised crops are grown [32, 33]. Another microbiological aspect of water used in agriculture that researchers have studied is the entry of microorganisms into the atmosphere as a result of mechanical processes. Bacteria attached to water droplets can be released from water bodies and become aerosolised. Droplets formed, for example,

when bubbles burst or water breaks up are carried into the atmosphere by wind; many small droplets are able to travel considerable distances [34].

Given the potential ability of microbial pathogens to enter water resources, reach humans and cause infections, it is important to properly understand the factors and principles that influence the emergence, survival/persistence and transfer of pathogens into water resources from agricultural areas [35]. The possibility of microbial pathogens entering the human body and causing infection through water contamination depends on the following prerequisites: the ability of an infectious dose of the shed pathogen to reach water resources, or the presence of intermediate hosts in which the pathogen can multiply to form an infectious dose in those water resources [36]. Various types of pathogens have been detected in accessible natural, drinking, recreational, treated and waste waters. Pathogens enter these aquatic environments by various routes, including human activity (for example, swimming), animal activity (for example, bathing), natural processes (for example, precipitation) and other incidental routes (for example, deposition of airborne particles) [37]. The main source of pathogens is faecal waste of humans and animals [38]. A growing body of research points to the ubiquitous presence of pathogens and their ability to spread through interconnected aquatic environments [39]. Classical waterborne pathogens are widespread in various water bodies, especially in large numbers in freshwater bodies. However, the quantitative content of new and unknown pathogens in aquatic environments remains unknown owing to limitations in detection methods [40].

The role of soil. The vast biodiversity of soil is crucial in regulating the impact of pathogens and pests on microorganisms, plants and animals, including humans [41]. Soil types and their physical and chemical characteristics, especially those related to soil texture, clay and organic matter content, porosity, surface area, density, pore

volume and pore/particle size distribution, significantly influence the overall fate of microbial species in the soil environment and the likelihood of their penetration into groundwater [42]. Although soils can act as a filter/barrier, attenuating or preventing the transport of microorganisms through the soil and thereby minimising pathogenic contamination of groundwater sources, leaching of pathogens through soil into groundwater has been demonstrated [43]. Microorganisms are thought to be capable of considerable migration in soil, which may occur by convection/advection and dispersion [44]. In advection, microorganisms are carried by the flow of water, and their movement is therefore determined by the velocity of the water. Hydrodynamic dispersion, which includes two distinct processes (molecular diffusion and mechanical mixing), is the spreading of microorganisms as they move along the water path as a result of both microscopic and macroscopic interactions [44]. High-quality soil structure ensures optimal circulation of air, water and nutrients in soil spaces, thereby promoting the formation of microbial populations in soil pores. Small pore size makes it difficult for microbial cells to access soil moisture, dissolved organic matter and, probably, other nutrients [35]. Increased survival of bacteria in organic soils and a faster decline of E. coli populations in more oligotrophic soils have been reported. In general, finer-textured soils (for example, clay), which have a better capacity to retain moisture and nutrients, support bacterial survival better than coarser-textured (sandy) soils, which consist of unbound particles and retain organic matter poorly [45]. Studies show that moisture is a critical condition affecting the viability and survival of enteric bacteria in the soil environment [46].

Soil is a reservoir for a multitude of microorganisms, including bacteria, fungi, protozoa and viruses, which can be pathogenic to humans and animals. Human and animal pathogens in soil can be divided into four broad groups reflecting the degree of

their residence in soil: permanent, periodic, transient and incidental [35]. Permanent pathogens are soil inhabitants that spend their entire life cycle in the soil and occasionally become infectious to humans and animals. Examples are organisms such as Clostridium botulinum or Clostridium tetani, which produce a neurotoxin upon ingestion of contaminated food or through contaminated wounds, respectively. Many zoonotic pathogens either live in the soil, or their vectors live in or spend part of their life cycle in the soil [47]. Periodic pathogens are soil organisms that need the soil environment to complete part of their life cycle. For example, Bacillus anthracis, the causative agent of anthrax in humans and livestock, is frequently found in soil and can survive for a long time in the form of endospores [48]. Transient organisms are those that occur naturally in soil, often through contact of their hosts with the soil, but do not need soil to complete their life cycle. For example, the protozoan parasite Giardia lamblia can enter the soil with the urine and faeces of rodents, but does not need soil to survive. Finally, incidental pathogens can enter the soil from anthropogenic sources, as a result of human and animal activity or by other routes, but survive in the soil for only a relatively short time. For example, pathogens enter the soil through the application of improperly treated raw manure, contaminated irrigation water or runoff, especially when farmland is in close proximity to pastures or livestock feedlots [35]. Traditional and local farming typically involves the use of organic fertilisers and diversified crop rotations, at least in part to reduce crop losses from harmful organisms [49, 50]. Many of these practices are used today in organic farming, in some cases being reintegrated into conventional farming systems both to maintain and to enhance the resilience of agroecosystems (Figure 4). Many management practices have both co-benefits and trade-offs. Some of them, applied to improve soil fertility or structure, also affect, both negatively and positively, the soil's capacity to regulate harmful organisms [51].

The role of humans. More than half of all known human pathogens are of animal origin [53], and all but one of the 17 Category A pathogens of the US National Institutes of Health that are capable of causing severe disease and mortality in humans are zoonoses [54]. However, the entire list of all known pathogens is probably only a small fraction of the total number of potential pathogens that exist in nature [55].

The threat posed by these unknown, not yet emerged pathogens calls for the strengthening and sustainable development of a pathogen detection network, based on One Health principles, in order to prevent a global large-scale pandemic caused by a zoonotic agent.

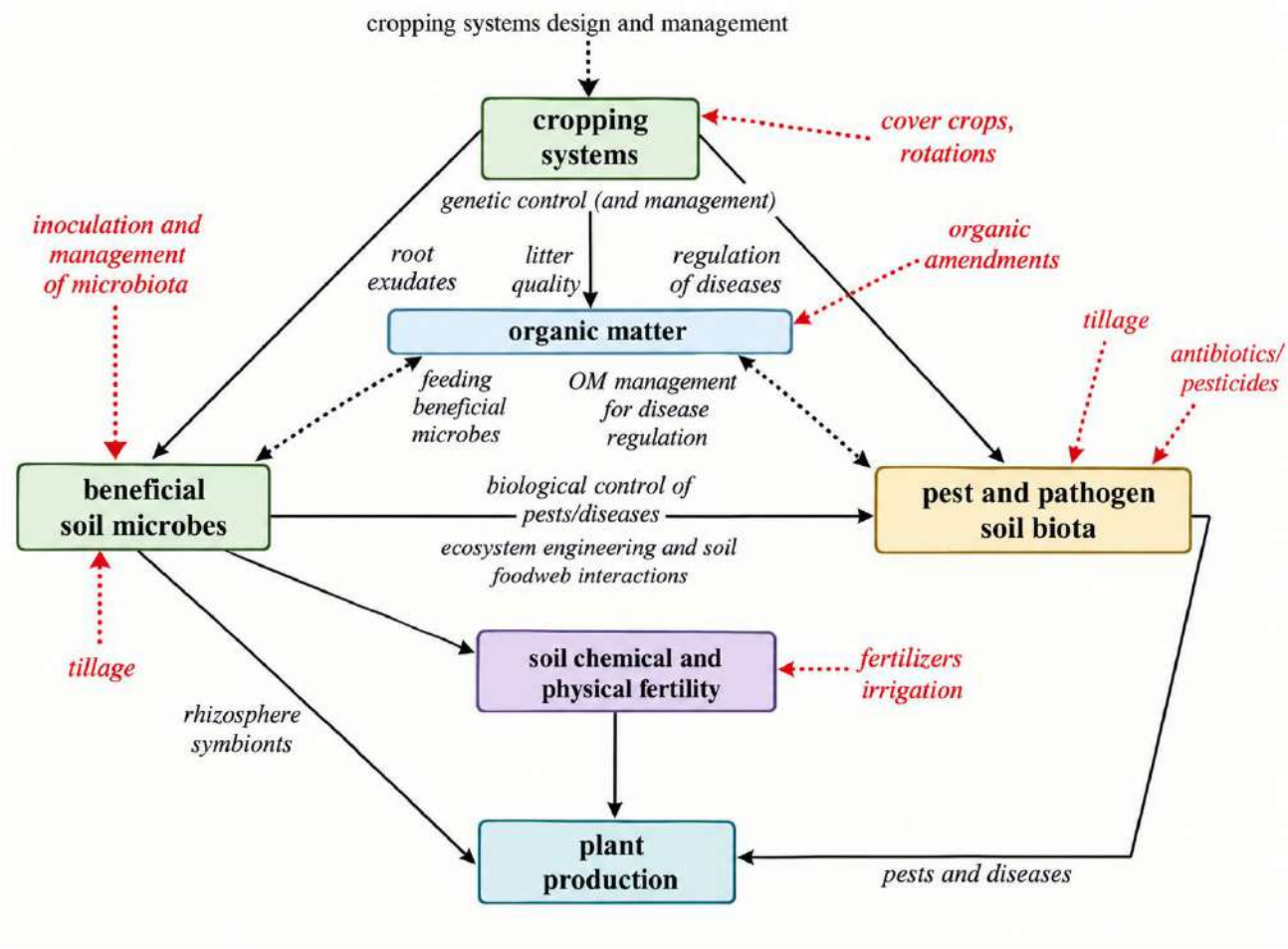

Figure 4. Entry points of beneficial and pathogenic soil microorganisms, and the impact of farming practices on crop yields. Modified from [52].

Over time, changes in population density, mobility, lifestyle, behaviour and food choices have influenced the dynamics of zoonotic disease emergence and become drivers of pathogen transmission [56]. As populations grow, the effect of urbanisation, combined with rising incomes, has also led to increased demand for meat products from domestic and wild animals. This development has significantly expanded both our consumption of animal protein and our need for land to raise these animals and their feed, leading to the enormous transformation of previously remote and rarely visited regions into areas of even more intensive livestock and agricultural production [57]. The main challenge of rapid zoonosis detection at the system level is how best to create reliable and resilient epidemiological surveillance networks capable of detecting rare and isolated cases of disease at the local level and linking these observations to highly qualified public health laboratory staff [58]. The question is whether a highly centralised and concentrated network in national or regional reference institutions or government ministries is most likely to succeed, or a distributed network of local surveillance teams managed by partners, with basic laboratory capacity to confirm/exclude a diagnosis on site. The key issues that influence the determination of which of these approaches is most suitable for a particular country relate to the sustainability of funding, training needs and the level of laboratory methods required for pathogen detection.

For integrated approaches to function effectively in the control system of the "animal–water–soil–human" chain in the context of food safety, long-term, sustainable funding and investment in human capital, infrastructure, laboratory and communication technologies are needed to establish local or regional epidemiological surveillance

centres. A key first step in this process is outreach to local residents and tourists about the threats of infection with local diseases, their clinical manifestations and preventive measures. Within the project, the spread of the following diseases was studied in the cross-border region under investigation: African swine fever, avian influenza, West Nile fever, leptospirosis and toxoplasmosis.

Transmission routes of the African swine fever agent. African swine fever virus is transmitted by various routes, which accounts for the disease's high capacity to spread among both domestic pigs and wild boar populations. The biology of the virus, its persistence in the environment and its high concentration in the blood and tissues of infected animals create numerous mechanisms of transmission [59].

Direct contact route. The most effective transmission route is direct contact of healthy animals with sick or infected pigs. The virus is present in high concentrations in the blood, saliva, nasal discharge, faeces, urine and other body fluids of infected animals. Transmission can occur during co-housing of pigs, during transport, or through contact via damaged skin or mucous membranes [60, 61, 62].

Transmission through infected carcasses and biological material. Carcasses of dead pigs and wild boar are an important source of infection. The virus remains viable for a long time in the tissues, blood and bone marrow of dead animals, especially at low temperatures. Contact of susceptible animals with carcasses or their remains can lead to infection even a long time after the death of the infected animal [63].

Alimentary route. Infection is possible through the consumption of feed or food waste contaminated with the ASF virus. Waste containing insufficiently heat-treated pork or products from infected animals is particularly dangerous. This mechanism is considered one of the main causes of interregional and international spread of the disease [64].

Mechanical transmission through environmental objects. Contaminated objects and environmental items (fomites) play an important role in the spread of the virus. They include vehicles, equipment, tools, protective clothing, staff footwear, feed containers, hunting equipment and other items that have been in contact with infected animals or their excretions [61, 62]. Inadequate disinfection of such objects significantly increases the risk of introducing the virus into disease-free farms.

Transmission through pig products. The virus persists for a long time in pork, meat products, sausages, smoked products and frozen meat. Illegal movement or sale of contaminated products is one of the most important factors in the cross-border spread of ASF [65]. For this reason, control of the movement of products of animal origin is one of the priority preventive measures.

Transmission through wild boar. Wild boar populations play a key role in maintaining virus circulation under natural conditions. Infection is transmitted both through direct contact between animals and through contact with infected carcasses. The movement of infected boar contributes to the spread of the virus over considerable distances and the formation of new foci of infection [66].

Vector-borne transmission. In ASF-endemic areas of Africa, the vector-borne transmission route involving soft ticks of the genus Ornithodoros is important. Ticks can become infected while feeding on infected animals, retain the virus for a long time and pass it on to subsequent generations and other animals [67]. For most European countries, including Ukraine, this mechanism is not of significant epizootiological importance.

The role of humans in the spread of ASF. Humans are not susceptible to ASF and do not become ill with African swine fever; however, human activity is considered one of the main factors in the spread of the disease. Failure to observe biosecurity

requirements, illegal transport of animals and pig products, use of contaminated feed, and improper disposal of carcasses and food waste significantly increase the risk of new outbreaks [66].

Thus, the spread of ASF occurs through a complex of interrelated transmission mechanisms, of which the most important are direct contact between animals, contaminated pig products, contaminated environmental objects, wild boar populations and the anthropogenic factor. Establishing the specific transmission route during an epizootiological investigation is one of the key tasks for determining the source of infection and implementing effective control measures.

Routes of infection (transmission) of avian influenza. Avian influenza is a highly contagious viral disease that spreads rapidly among populations of wild and domestic birds. The virus's high transmissibility is due to its persistence in the external environment, the many routes of spread and the substantial shedding of the agent by infected animals. The source of infection is sick, recovered or asymptomatically infected birds that shed the virus in faeces, saliva, respiratory mucus and eye secretions [66].

The main routes of virus transmission include:

1. Contact transmission route

Direct contact between infected and healthy birds is one of the most important mechanisms of spread of infection. Where birds are kept at high density, the virus is quickly transmitted from one individual to another through:

- touch;
- co-housing;
- use of shared feeders and drinkers;

– contact with the discharges of sick birds.

The infection spreads particularly quickly in industrial poultry farms, where the high concentration of birds creates favourable conditions for mass infection [67].

2. Aerogenic (airborne) route

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

– breathing;
– coughing;
– sneezing;
– flapping their wings, which raises contaminated dust.

In enclosed premises with inadequate ventilation, the risk of aerogenic infection increases significantly. For this reason, large poultry factories are zones of increased risk of virus spread [65].

3. Faecal–oral mechanism

The influenza virus is characterised by a faecal–oral mechanism of transmission. Infected birds shed a considerable amount of virus in their droppings, which contaminate:

– water;
– feed;
– litter;
– equipment;
– surfaces of premises.

Healthy birds become infected by ingesting contaminated feed or water. This route is especially important among waterfowl, since the virus persists well in the aquatic environment [60].

4. Transmission through water

Water is one of the key factors in the spread of the virus among wild birds. In cold water the virus can remain viable for a long time. Contamination of water bodies with droppings of infected birds creates a permanent source of infection for other animals [60].

5. Mechanical transmission through objects and personnel

Mechanical carriers play an important role in the spread of the virus. The agent can be carried by:

- vehicles;
- cages used for keeping and transporting birds;
- equipment;
- protective clothing;
- workers' footwear;
- feed containers;
- poultry care equipment.

Failure to observe sanitary and hygienic rules and inadequate disinfection are among the main reasons for the introduction of the virus into farms.

6. The role of wild migratory birds

Wild waterfowl and migratory birds are the natural reservoir of influenza A virus. In most cases they carry the infection without clinical signs, which contributes to the hidden spread of the agent over long distances during seasonal migrations.

Bird migration routes are of great importance in the global spread of highly pathogenic H5N1 and H5N8 strains between continents.

7. Transmission from birds to humans

Human infection occurs mainly as a result of:

- contact with sick birds;
- inhalation of contaminated dust;
- contact with faeces;
- slaughter and processing of carcasses;
- visits to live poultry markets.

The highest risk of infection is faced by:

- poultry farm workers;
- veterinarians;
- slaughterhouse workers;
- persons involved in the elimination of outbreaks.

The World Health Organization notes that most cases of human infection with highly pathogenic avian influenza have resulted from direct and indirect contact with infected birds [68].

The virus's ability to cross the interspecies barrier is of particular concern (Figure 5). Cases of infection have been reported in:

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

Pigs are considered "intermediate hosts" because they can be infected simultaneously with human and avian influenza strains, creating conditions for genetic reassortment and the emergence of new pandemic variants of the virus [61].

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

- intensive industrial poultry farming;
- global trade in poultry;
- migration of wild birds;
- low level of biosecurity;
- climate change;
- spontaneous live poultry markets;
- insufficient veterinary control.

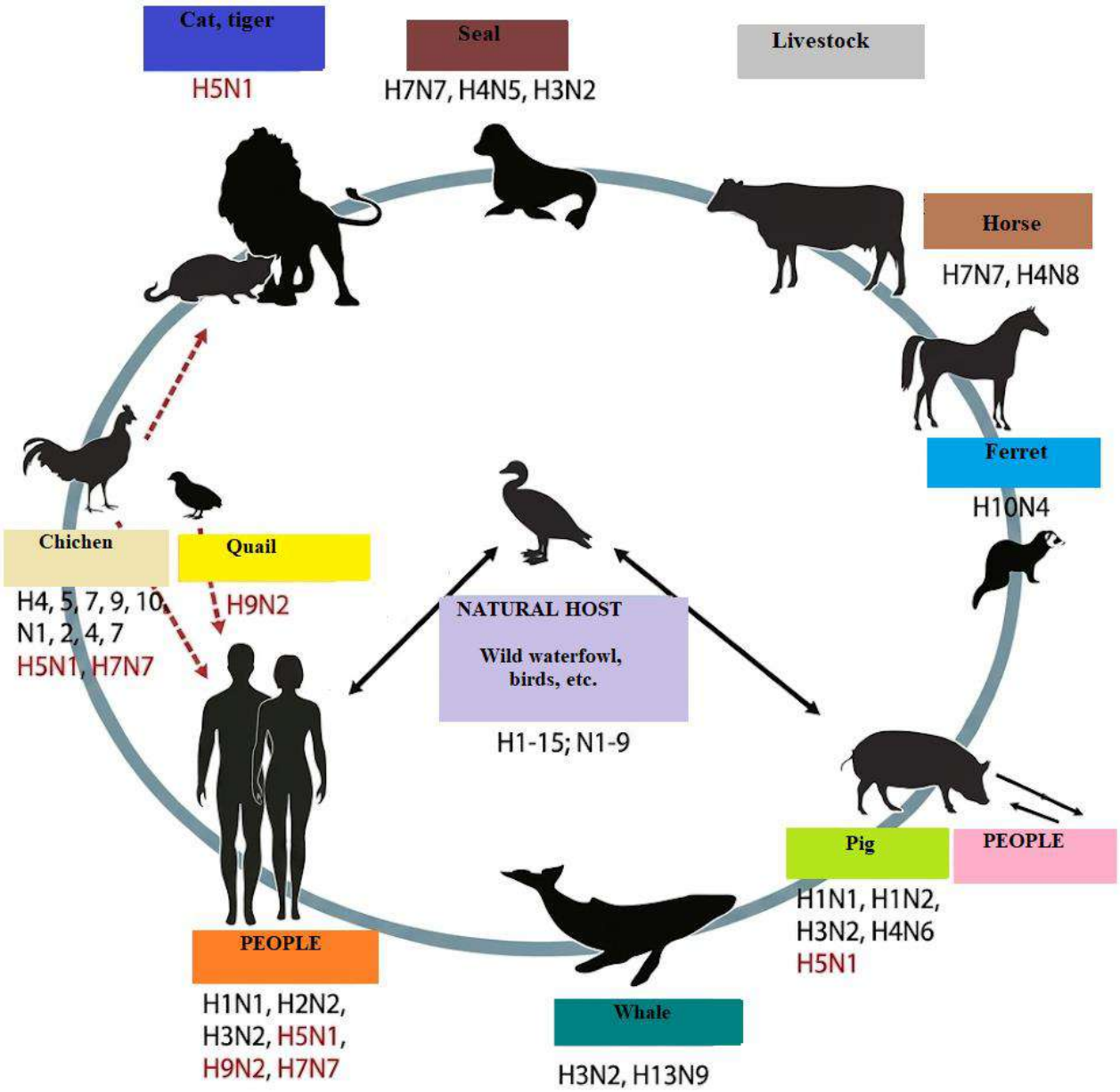

Figure 5. Interspecies transmission of influenza viruses

Thus, avian influenza is characterised by a complex system of transmission routes that ensures its rapid spread among animals and creates a risk of human infection. This necessitates strict observance of veterinary and sanitary measures, continuous

monitoring and implementation of One Health principles to prevent epizootics and potential pandemics.

Routes of infection (transmission) of the leptospirosis agent. Humans can become infected when swimming in stagnant water bodies, fishing, during agricultural work, when caring for sick animals, and when eating food contaminated by rodents. Leptospires can enter the human body through cuts and scratches on the skin, as well as through intact mucous membranes (nose, mouth, eyes). Infection is also possible through drinking raw water from unknown natural sources. Domestic animals (for example, dogs) can become infected by sniffing the scent marks of other animals, by eating contaminated feed or raw meat, or by hunting sick rodents. The infection is transmitted from mother to foetus. According to epidemiological investigations [69], in 2023 leptospirosis infection occurred:

- through contact of damaged areas of skin with contaminated objects and surfaces – 57% of cases;
- in water (swimming in water bodies, fishing) – 38% of cases;
- through consumption of contaminated food – 5% of cases.

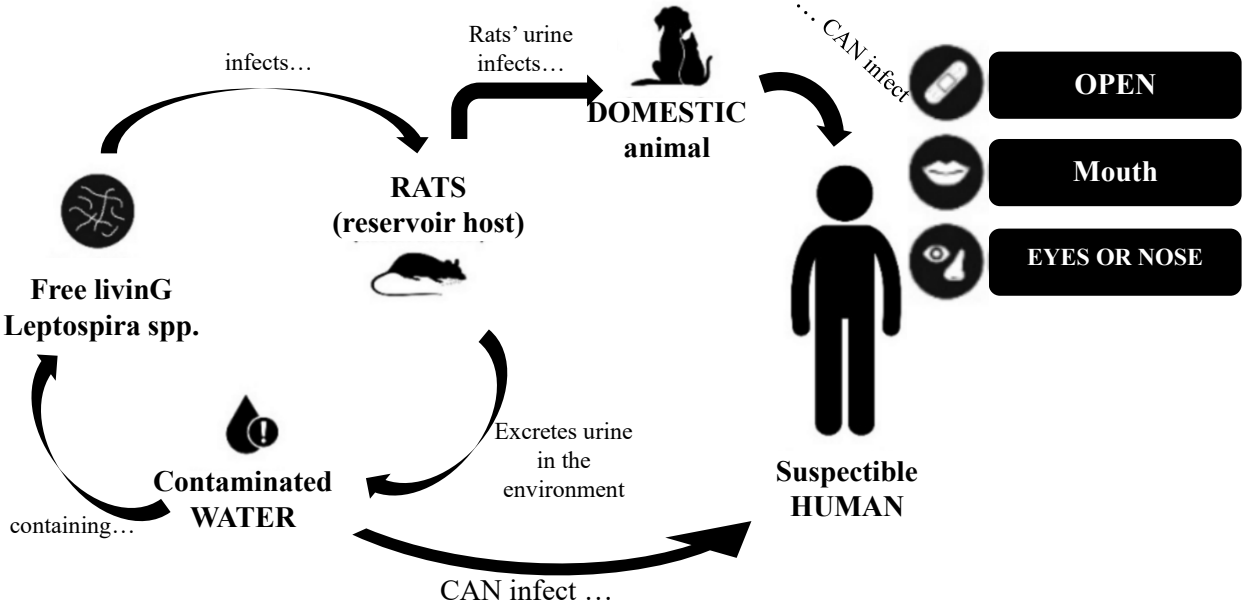

Figure 6. Transmission of Leptospira spp. at points of interaction of humans, animals and the environment, with emphasis on different reservoir hosts [70].

Routes of infection (transmission) of West Nile virus (WNV). The most common route of human infection with West Nile virus is the bite of an infected mosquito of the subfamily Culicinae. Mosquitoes become infected when they feed on the blood of infected birds that carry high levels of West Nile virus. Infected mosquitoes can transmit West Nile virus when feeding on the blood of humans or other animals. Humans, horses and most other mammals do not develop high viraemia and are therefore traditionally considered "dead-end" hosts.

West Nile virus has been detected in dead birds of at least 138 species. Birds of the family Corvidae (crows and blue jays) are particularly susceptible to WNV. People at risk of WNV infection include those who work outdoors during the period of

mosquito activity, such as farmers, foresters, landscapers, gardeners, construction workers and other outdoor workers. WNV is not transmitted from person to person via mosquitoes. However, in 2002, five new modes of transmission were identified, which are described below. The discovery of these modes of transmission refutes the theory that humans are dead-end hosts of the virus.

Blood transfusion. Transmission of West Nile virus has been found to occur through transfusion of red blood cells, plasma and platelets.

Organ transplantation. Four transplant recipients who received organs from a common donor developed fever; three of them developed meningoencephalitis. West Nile virus infection was diagnosed in all four recipients. Serum and plasma samples taken from the organ donor at the time the organs were procured tested positive for West Nile virus by PCR and culture [71].

Breastfeeding. A woman developed meningoencephalitis caused by West Nile virus 11 days after a blood transfusion in the postpartum period from a donor infected with WNV. Specific IgM antibodies to WNV were detected in a cerebrospinal fluid sample. She breastfed her newborn, and a sample of her breast milk taken 16 days after delivery also showed the presence of WNV by PCR and of specific IgG and IgM antibodies to WNV.

Transplacental transmission. In August 2002, a previously healthy 20-year-old woman at 27 weeks of pregnancy was hospitalised with symptoms consistent with myeloid encephalitis caused by West Nile virus, and specific antibodies were detected in her serum and cerebrospinal fluid samples. Five weeks later she gave birth to a live newborn. The infant had serological evidence of West Nile virus infection, bilateral chorioretinitis, and magnetic resonance imaging (MRI) findings indicating severe bilateral white matter loss in the temporal and occipital lobes [71].

Occupational infection. Infection is possible during the autopsy of animal carcasses infected with this disease, and when protective measures are not observed during contact with potentially infected biological objects.

Routes of infection (transmission) of the toxoplasmosis agent. Cats become infected by ingesting sporulated oocysts or meat containing cysts of the parasite, but no more than 50% of cats shed oocysts. The time after which shedding of oocysts in faeces begins is on average 20 days (30 days after ingestion of meat containing cysts). Sporulated oocysts can end up on hands, footwear or clothing contaminated with soil when working with soil in a garden, vegetable plot or flower bed. In dogs they can be found on the coat after walks (especially in dogs that roll on grass, soil, and sometimes in the faeces of other animals).

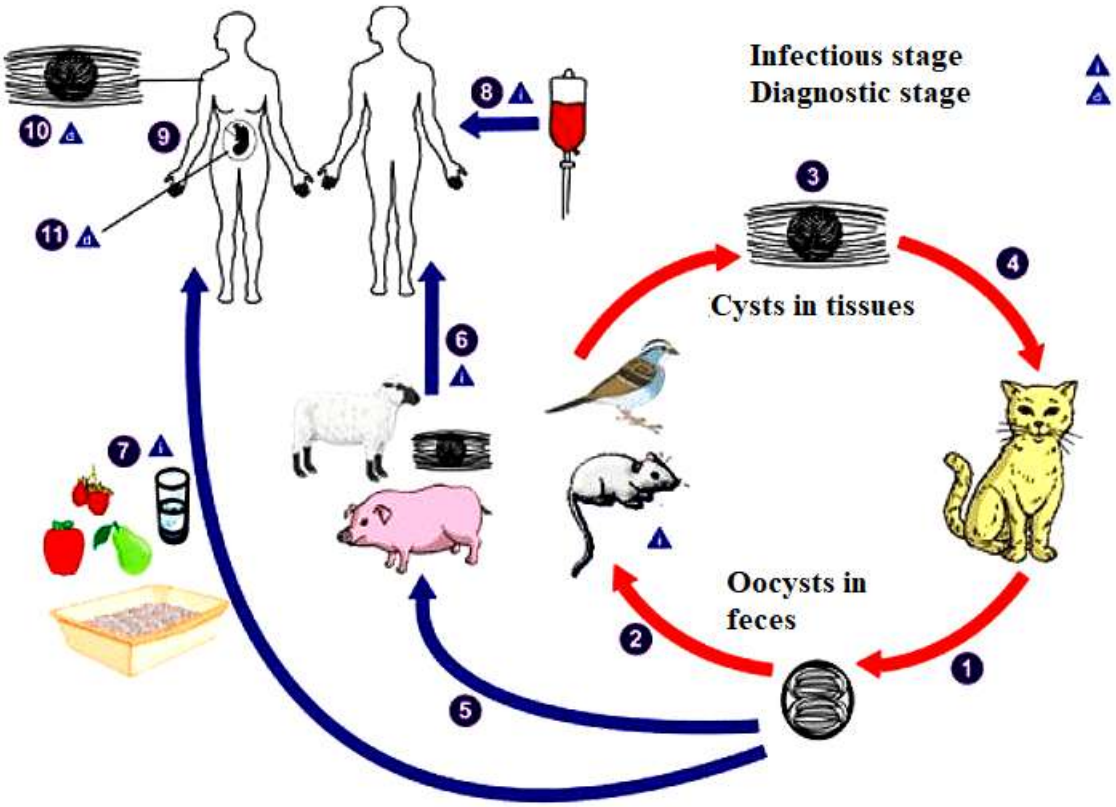

Figure 7. Life cycle of Toxoplasma gondii

Infection of herbivorous animals and birds with toxoplasma occurs mainly through oral ingestion of sporulated oocysts, which remain viable in the environment for a long time. Toxoplasma gondii is transmitted through the consumption of raw and undercooked meat containing tissue cysts with bradyzoites, and through ingestion of oocysts in food, untreated or insufficiently treated drinking water, or surface water during recreational swimming [72]. In addition, ingestion of oocysts may also occur through consumption of contaminated vegetables and fruit, as well as raw or undercooked molluscs [73]. Contact with T. gondii oocysts is also possible through ingestion of soil (for example, when playing in sandpits or gardening). Moreover, studies show that cat ownership is a risk factor for Toxoplasma gondii infection [74].

Humans are an intermediate host and do not shed oocysts; therefore, humans are not a source of transmission and are not considered contagious. Nevertheless, human-to-human transmission may occur through vertical transmission, resulting in congenital toxoplasmosis.

2. UNIFIED SAMPLING PROTOCOLS IN LINE WITH INTERNATIONAL STANDARDS

The protocols in this section have been developed within the "SMS-RO-UA" project as a common basis for sampling by the partners from Ukraine and Romania. They are intended for use by staff of veterinary services, laboratories and research institutions when planning and carrying out sampling, so that results are comparable on both sides of the border.

In line with current recommendations of the WHO, WOAH, FAO and UNEP, effective surveillance of emerging and cross-border diseases must cover not only animals but also environmental objects that may act as reservoirs or factors in the transmission of infectious agents.

The sampling methodology is based on the recommendations of the WOAH Guidelines – Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, thirteenth edition, 2024, and on the requirements of the national veterinary legislation of both countries, as well as EU norms and the EU legal framework:

1. Instruction on the prevention and eradication of avian influenza (approved by Order of the Ministry of Agrarian Policy and Food of Ukraine of 17.10.2011 No. 547, registered with the Ministry of Justice of Ukraine on 8.11.2011 under No. 1277/20015) [75]
2. Instruction on the prevention and control of African swine fever (approved by Order of the Ministry of Agrarian Policy and Food of Ukraine of 07.03.2017 No. 111, registered with the Ministry of Justice of Ukraine on 29.03.2017 under No. 432/30300) [76]
3. Instruction on measures for the prevention and recovery of animals from leptospirosis (approved by the Main Directorate of Veterinary Medicine under the State

Veterinary Inspectorate of the Ministry of Agricultural Products of Ukraine, Order of 15 March 1994 No. 5) [77]

4. Rules for the collection of samples of pathological material, blood, feed and water and their submission for laboratory testing (approved by the State Department of Veterinary Medicine of the Ministry of Agriculture and Food of Ukraine of 15 April 1997 No. 15-14/111) [78]

5. Methodological recommendations on sampling procedures for the implementation of the State Monitoring Plan for residues of veterinary medicinal products and contaminants in live animals and unprocessed food of animal origin (approved by Order of the Chief State Inspector of Veterinary Medicine of Ukraine of 9 February 2017 No. 7) [79]

6. Leptospirosis in humans: guidelines for diagnosis, epidemiological surveillance and control [80]

7. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 13th edition, 2024. World Organisation for Animal Health (WOAH). [81]

8. REGULATION (EU) 2016/429 OF THE EUROPEAN PARLIAMENT AND OF THE COUNCIL of 9 March 2016 on transmissible animal diseases and amending and repealing certain acts in the area of animal health ("Animal Health Law") [82]

9. Government Ordinance No. 42/2004, approved by Law No. 215/2004, on the organisation of veterinary activity and food safety

10. The annual Strategic Programme for surveillance, prevention, control and eradication of animal diseases, approved by an ANSVSA order.

11. Order ANSVSA/MADR No. 20/195 of 5 March 2018 approving the veterinary sanitary norm on biosecurity conditions in pig holdings.

12. Commission Delegated Regulation (EU) 2020/689 of 17 December 2019 supplementing Regulation (EU) 2016/429 as regards rules for surveillance, eradication programmes and disease-free status for certain listed and emerging diseases.

13. Commission Delegated Regulation (EU) 2020/687 of 17 December 2019 supplementing Regulation (EU) 2016/429 as regards rules for the prevention and control of certain listed diseases.

Sampling in the Project was carried out in accordance with the Joint Terms of Reference for analysis (hereinafter – the Joint ToR) agreed by the project partners, which defines the timeframe and stages of the research, requirements for sampling, laboratory analysis methods, assessment procedure and reporting. Transport of blood samples, swabs, pathological material and environmental samples to the laboratory is to be carried out in compliance with biosafety measures and cold-chain conditions, in accordance with the recommendations of the WOAH Guidelines – Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, thirteenth edition, 2024, and national veterinary legislation.

General approaches to sampling of biological material:

Samples taken from live animals for testing:

- whole blood without anticoagulant/serum on clot;
- whole blood with anticoagulant;
- cerebrospinal fluid;
- nasal discharge;
- ocular discharge;
- preputial and vaginal discharge;
- skin or mucosal epithelium and fluid from unopened vesicles, nodules and scabs;

- bucco-pharyngo-oesophageal discharge;
- faeces;
- tracheal and cloacal swabs.

Whole blood samples without anticoagulant, or serum on clot, are collected separately in vacuum containers. The minimum quantity of blood required from each animal is 7.5–9 ml for large animals, 4 ml for medium-sized animals and 1 ml for small animals and birds. In terms of quality, samples must not contain anticoagulants or foreign bodies, and must not be haemolysed or contaminated. After collection, the vacuum container is kept at room temperature to allow serum to be expressed. The blood is allowed to clot, separated and centrifuged for 15 minutes at 3,500 rpm, and the resulting serum is collected. Blood serum obtained from whole blood is stored in a refrigerator or frozen. Freezing of whole blood samples or serum on clot is prohibited. When a sample is transported over long distances, it is advisable to separate the serum from the clot as soon as possible after expression to prevent haemolysis. In the case of a serum neutralisation test, the collected sample is sent sterile in vacuum containers, without extracting the serum from the clot.

Whole blood samples with anticoagulant are collected in a vacuum container with anticoagulant, after which the container is gently tapped under the cap to prevent the additive from sticking to the walls of the tube or its cap. Blood samples are collected separately in vacuum containers with lithium heparin or another anticoagulant such as sodium citrate or EDTA. The minimum quantity of blood collected per animal should be 5 ml for large animals, 3 ml for medium-sized animals and at least 1 ml for small animals and birds. Blood samples with anticoagulant must be collected sterile, must not contain clots and must not be haemolysed. After collection, the vacuum container is

gently shaken with a rotating motion of the wrist or gently inverted vertically 5–6 times. Blood samples are not frozen.

Whole blood samples without anticoagulant/serum on clot and whole blood samples with anticoagulant are identified with legible, conspicuous and durable written labels bearing the sample number corresponding to the numbering in the table of sample/animal records. Blood samples must be transported to the laboratory after serum separation at refrigeration temperature. Samples are placed upright in racks, and the racks in transport boxes of suitable volume. Samples must be transported in such a way as to avoid their tipping over or spilling. The time interval from sampling to receipt by the laboratory must not exceed 72 hours.

Cerebrospinal fluid samples are collected aseptically using a syringe; the required quantity is 4 to 10 ml. The collected samples are identified with legible, conspicuous and durable labels bearing the sample number corresponding to the numbering in the table attached to the analysis request. Samples are transported to the laboratory as quickly as possible and under chilled conditions.

To collect nasal discharge, sterile cotton or gauze swabs are used; they are introduced into the nasal cavities with rotating and advancing movements of moderate intensity, avoiding damage to the mucosa; 3–5 swabs are collected from each animal. Nasal discharge is collected with sterile swabs and then immersed in transport medium. This type of sample is collected during the febrile period of respiratory diseases or in the catarrhal phase of the inflammatory process. After collection of nasal discharge, the swab is placed in a sterile tube with transport medium that meets the preservation requirements for maintaining the viability of the virus suspected to be present in the sample. The collected samples are identified with legible, conspicuous and durable written labels bearing the sample number corresponding to the numbering in the table.

Ocular secretion samples are collected with a swab moistened in sterile physiological saline from the bottom of the conjunctival sac with light but firm movements from the inner corner of the eye to the outer. After sampling, the swab is placed in a sterile tube with transport medium, stored at refrigerator temperature and sent to the laboratory within a maximum of 24 hours. The ocular secretion samples taken are identified with legible, conspicuous and durable labels bearing the sample number corresponding to the numbering in the table.

Preputial and vaginal secretion samples are taken with sterile swabs, which are then immersed in transport medium, or by washing the preputial area. In the case of visible and circumscribed lesions, secretions are taken from them. The preputial and vaginal secretion samples taken are identified with legible, conspicuous and durable labels bearing the sample number corresponding to the numbering in the table. Samples are packed and sent to the laboratory as quickly as possible.

Freshly voided faecal samples are collected using a sampling tool into sterile containers fitted with a stopper, or with rectal or cloacal swabs in the case of sampling from birds. In live birds, in the case of avian influenza, tracheal and cloacal swabs are collected into transport medium. After collection of faecal samples, the swab is placed in a sterile tube with 5 ml of transport medium consisting of physiological phosphate buffer and antibiotic, which is stored at refrigeration temperature and sent to the laboratory on the day of collection. After collection, the vials with faeces are stored at refrigeration temperature and sent to the laboratory on the day of collection. Samples collected with cloacal and tracheal swabs are transported to the laboratory as quickly as possible. If transport does not take place within 24 hours of collection, the samples are immediately frozen and then transported to the laboratory. The samples taken are

identified with legible, conspicuous and durable labels bearing the sample number corresponding to the numbering in the table.

Samples taken from dead/slaughtered animals are as follows:

- carcasses;

- organs.

Sampling from carcasses:

Carcasses are taken from small and medium-sized animals. Carcasses of large young animals can also be taken and sent to the laboratory. The collected carcasses are packed in sealed containers or in double polyethylene bags, between which absorbent material impregnated with disinfectants is placed. If transport does not take place within 24 hours of collection, the samples are immediately frozen and then transported to the laboratory. Carcass samples are identified with legible, conspicuous and durable written labels bearing the sample number corresponding to the numbering in the table.

Skin samples with lesions, lymph nodes, whole organs or organ fragments are taken in quantities of 20–100 g. Organ samples and/or organ fragments are taken using appropriate sterile instruments. The organ samples taken are packed in sterile bags or containers, as appropriate, and transported under chilled conditions to the laboratory as quickly as possible. If transport does not take place within 24 hours of sampling, they are frozen and then transported to the laboratory. A bag or container with a tight lid is used for each organ. The samples taken are identified with legible, conspicuous and durable labels bearing the name of the organ type and the sample number corresponding to the numbering in the table.

Culicoides species. Samples of insects of the genus Culicoides are collected using stationary or mobile traps during the vector activity season, according to a predetermined schedule, at sites representing optimal areas for Culicoides, recording

geographical coordinates – latitude, longitude and altitude above sea level – using GPS devices at all vector collection points; it is not recommended to set traps in the following places: above a water body, directly on the ground, near areas with artificial lighting, in places exposed to strong winds, and in places with industrial smog or smoke.

In terms of quality, the sample (catch) must be fresh.

Method of submission: plastic cups with screw caps, with labels indicating: locality, site (river, forest, mountainous area), date, animal species in the locality, soil type, temperature.

The time from sampling to dispatch of the samples to the laboratory is 3 hours from collection from the trap, or 24 hours if they are moved; ambient temperature conditions are maintained immediately after collection and during transport.

Mosquitoes. Mosquito samples are collected using special traps and stored in screw-cap vials of 7–10 ml capacity, provided with a thin layer of silica gel granules; adult mosquitoes are placed on top of the silica gel granules, between two layers of hydrophilic cotton wool.

In terms of quality, the sample (catch) must be fresh or preserved with silica gel granules.

Method of submission: plastic cups with screw caps, with labels indicating: locality, site (river, forest, mountainous area), date, animal species in the locality, soil type, temperature.

The time from collection of the samples to their submission to the laboratory is 48 hours, and ambient temperature conditions are maintained during transport.

Ticks. Tick samples:

a) ticks of the genus Ornithodoros – vectors of African swine fever – are collected by hand or with traps equipped with dry ice, which releases CO2;

b) ticks of other genera are collected from vegetation during the period of peak activity from April to October and/or from animals all year round;

c) samples of oribatid and pasture mites are collected from April to November in jars with a lid, provided with a cotton swab, leaving them in contact with the swab for at least 24 hours.

Method of presentation: ticks must be transferred to a container with a tight stopper, in which several blades of grass, porous paper or slightly moistened cotton wool are placed, or they are submitted alive.

Geographical coordinates – latitude, longitude and altitude above sea level – must be recorded using GPS devices at all tick collection points.

The time from sampling to dispatch to the laboratory is 48 hours, and chilled conditions are maintained during transport.

Soil – minimum quantity/sample 10–20 g. Method of packing the samples taken – samples collected in polyethylene bags, boxes or bottles with lids, packed in polyethylene bags/sacks. Time from sample collection to dispatch to the laboratory: 24 hours. Transport conditions – cooling to 4 °C.

Water – minimum quantity/sample 100–200 ml from open water bodies at a depth of 25–30 cm below the surface. Method of packing the samples taken – bottles with lids, packed in polyethylene bags/sacks. Time from sample collection to dispatch to the laboratory: 24 hours. Transport conditions – cooling to 4 °C.

Specific approaches to sampling biological material for the emerging diseases studied:

Sampling for ASF analysis. Whole blood (with anticoagulant, usually EDTA) is collected from live domestic pigs for detection of viral DNA by PCR, in a quantity of 2–5 cm^3, in a syringe or vacuum sampling tubes such as Vacutec.

From dead/slaughtered pigs – organs (necessarily showing signs of damage): spleen (the main organ for PCR and virus isolation), lymph nodes (especially mesenteric), tonsils, liver, lungs, kidneys (optional), bone marrow (in case of tissue autolysis), blood from the heart (from fresh carcasses), serous fluid from the pleural/abdominal cavity (if present) – in plastic Falcon-type centrifuge tubes or sterile zip bags. Tissues are collected in quantities of 5–10 g/sample, and fluids in quantities of 2–5 cm^3.

In the field, for active monitoring the following are collected from wild boar:

- oral fluid, taken on a rope impregnated with bait,
- dry filter paper cards soaked with blood (FTA cards),
- bones/bone marrow (for dead wild boar that died a long time ago),
- ear pinnae, skin, other available tissues – within the limits of what is possible.

Tissues are collected in quantities of 5–10 g/sample, and fluids in quantities of 2–5 cm^3, in Falcon-type tubes or sterile zip-lock bags.

Organ/tissue samples are chilled (4 °C) and transported in sterile containers. If transport will take more than 24–48 hours, the material is frozen at -20 °C or -80 °C.

Blood with EDTA is stored at 4 °C to avoid haemolysis. FTA cards are stored at room temperature, with no need for a cold chain.

Biosafety considerations during sampling. The ASF virus is very resistant in the environment, so samples are considered high-risk. All manipulations must be carried out wearing personal protective equipment. All procedures must be carried out in accordance with national biosafety protocols and WHO protocols.

If sampling of wild boar is necessary, baits are used. For preparing baits for wild boar, the optimal ratio of mixture components in the recipe, taking consistency into account, is: cornmeal – one part, dried milk – one part, coconut oil – two parts, paraffin

– one part, almond flavouring – 5 drops per 20 g of oil. The solid components are mixed with the cornmeal and dried milk, after which the mixture is poured into moulds and an undyed cotton rope 0.8 cm in diameter is immersed in it. The finished baits are stored at +4 °C until use. For oral fluid sampling in wild boar, baits are placed by tying them to trees so that the bait hangs 15 cm above the ground. GPS coordinates are recorded for all sampling points. The exposure time in different locations will vary from 7 to 20 days, which is acceptable given the low ambient temperature during the sampling period. After exposure, pieces of the chewed bait are placed in labelled plastic tubes, placed in a sealed polyethylene bag. In the laboratory, all samples are stored at -20 °C until further analysis.

Sampling for the diagnosis of avian influenza.

From live birds (with clinical signs or asymptomatic) the following are taken:

- Oropharyngeal swabs for detection of the virus in the respiratory tract, using sterile swabs with transport medium (VTM or PBS with antibiotics). Swabs are carefully inserted into the oral cavity towards the larynx, rotated several times, and then placed in tubes with transport medium.

- Cloacal swabs for detection of the virus in the digestive tract (shedding with faeces).

The swab is inserted 1–2 cm into the cloaca, rotated, and then placed in transport medium with antibiotics.

Tissues are taken from dead or slaughtered birds for PCR testing using sterile instruments.

For testing, samples are taken from the trachea and lungs – respiratory epithelium (the main site of virus replication) – and from the spleen, liver and kidneys in the case of systemic involvement with multiple haemorrhages. In the case of neurological

symptoms, brain tissue samples are also taken. Bursae of Fabricius may also be taken from dead young birds. The heart and intestine are taken as additional material.

Samples are collected in sterile containers or bags with airtight closure (zip bags or Falcon-type tubes).

For monitoring of avian influenza in wild birds, cloacal swabs, oropharyngeal swabs and faecal samples are collected. Tissue, bone and brain samples are collected from carcasses. Samples are collected in sterile containers or bags with airtight closure (zip bags or Falcon-type tubes).

In mammals, 2–5 cm^3 of blood is taken from peripheral vessels and collected in tubes with an anticoagulant, such as Vacutec.

Table 1. Storage and transport conditions

Sample type	Storage conditions	Comment
Washings (oropharyngeal, cloacal)	4 °C for up to 48 hours, or -80 °C	In VTM, sterile conditions
Tissues (organs)	4 °C for a maximum of 24–48 hours	Freezing
Serum	-20 °C or -80 °C	Long-term storage
Swabs	Wet, in VTM	It is important to avoid drying out.

Biosafety aspects of sampling

Avian influenza (especially H5N1, H7N9) poses a zoonotic threat.

All procedures must be carried out in BSL-3-level PPE (mask, protective goggles, coverall, gloves).

Work with live virus (cDNA amplification) is carried out using PPE in class 2 biosafety cabinets (BSCs).

Table 2. Recommended number of samples

Purpose of sampling	Number of samples
Serological testing	≥ 20 birds from the flock

Purpose of sampling	Number of samples
PCR of washings	≥ 10 birds (pooled samples at 5:1 possible)
Organs	From 3–5 carcasses
Faeces (from the environment)	≥ 10 samples

Sampling for the diagnosis of leptospirosis in animals

For leptospirosis testing, the sample types presented in Tables 3 and 4 are taken.

Table 3. Sample types from live animals (cattle, pigs, dogs, etc.)

Sample type	Sampling methodology	Purpose
Blood serum	Venous collection, storage at -20 °C	Serology (MAT, ELISA)
Urine	Sampling by catheter or after massage of the bladder	PCR, bacteriology
Whole blood	With anticoagulant (EDTA)	PCR in the acute phase
Milk	Sterile collection in dairy animals	PCR, less often bacteriological examination

For serological testing by the microscopic agglutination test (MAT), paired samples are taken: the first in the acute phase and the second 2–3 weeks later.

Table 4. Sampling from dead animals

Organ/fluid	Purpose
Kidneys	Microbiological examination, PCR
Liver	PCR
Bladder/urine	PCR, microbiological examination
Blood from the heart	PCR, serological tests
Lacrimal secretion	PCR

Table 5. Transport and storage conditions

Sample	Storage temperature	Conditions
Serum	-20°C	Clean tube without anticoagulant
Blood	4 °C, maximum 24–48 hours	EDTA or heparin

Sample	Storage temperature	Conditions
Urine	4 °C or with preservative	Immediate delivery to the laboratory
Organs	4 °C (up to 24 hours) or -80 °C	Aseptic, airtight

Biosafety measures

Since leptospirosis is also a zoonotic disease, samples are collected using PPE (gloves, gown, protective goggles, mask). Methods and procedures that minimise splashing of fluids and aerosol formation are used.

The clinical and pathological material samples taken are packed in triple packaging.

Recommended number of samples for testing:

- Group monitoring: ≥10 animals from the herd
- In case of suspected leptospirosis outbreak: all clinically ill animals + 2–3 animals without signs of disease

Sampling for the diagnosis of toxoplasmosis in animals

The main aims of diagnostic testing are confirmation of acute infection, detection of persistent forms (cysts), serological screening of herds, and monitoring of abortions (sheep, goats).

For toxoplasmosis testing, the samples listed in Table 6 are taken.

Table 6. Biological materials from live animals (sheep, goats, pigs, cattle, dogs, cats)

Sample type	Sampling methodology	Testing method
Blood serum	From a vein (vacuum tube without anticoagulant)	Serology: ELISA, MAT
Whole blood (EDTA)	Collection in a tube with anticoagulant	PCR (in the acute phase)

Sample type	Sampling methodology	Testing method
Faeces (in cats)	Fresh samples, chilled	Detection of oocysts (PCR)

Cats are the definitive hosts of Toxoplasma, so faeces are the key sample for diagnosis in this species.

In the case of abortions or death of animals, the samples listed in Table 7 are taken.

Table 7. Samples in the case of abortions or death of animals

Sample type	Sampling methodology	Testing method
Placenta	Aseptic collection of fragments	PCR
Foetus (brain, liver, spleen, lungs)	Sterile collection	PCR
Amniotic fluid	Syringe during necropsy	PCR
Maternal uterus	Biopsy or tissue sampling at the time of death	PCR

Biosafety measures

When handling cat faeces, foetal tissues and placenta, personal protective equipment (mask, gloves, protective goggles) will be used. Disposal of residual biomaterials requires compliance with biosafety standards. Toxoplasmosis is a zoonosis, and pregnant women are at increased risk.

Table 8. Storage and transport

Sample	Temperature	Storage time	Conditions
Serum	–20°C	Long-term	Dry tube
Blood (EDTA)	4°C	Up to 24–48 hours	Avoid haemolysis
Faeces	4°C	Up to 3 days	Without preservatives
Tissues	4 °C or –20 °C / in formalin	24 hours / longer	Depends on the method
Placenta, foetus	4 °C or frozen	24–48 hours / longer	Standard biosafety

Sampling for the diagnosis of West Nile fever

Samples of ticks and insects

1. Sampling of Ixodid ticks

- Ixodid ticks are collected directly from animals throughout the year, especially in spring and autumn.

- Ixodid ticks are removed from animals using a feather or a cotton swab moistened with ether, chloroform, acetone or petrol. The use of these substances helps to remove the ticks completely from the animals, including the mouthparts.

- Engorged ixodid ticks are preserved in 10% formalin in distilled water, ordinary water, or 50% alcohol in distilled water.

- Ixodid ticks are transported to the laboratory in a glass tube 10–11 cm long and 1.5 cm in diameter. Ordinary water, less than half of the tube's volume (5–8 cm^3), is placed in the tube. A hydrophilic cotton swab is then inserted into the tube and pushed with a glass rod until it touches the water, avoiding the formation of air bubbles. When the cotton wool becomes wet by capillary action, a second cotton swab is inserted and pushed until it touches the first, so that it too becomes wet by capillary action. The swabs are secured to the tube to prevent water leakage. A layer of gauze measuring 6×6 cm is placed on top of the last layer of cotton wool, and the ticks are introduced on top of it, and the tube is closed with a cotton swab. The tubes are placed in a cardboard or plastic box and sent to the laboratory. Ticks can survive under these conditions for 5 months.

- The tubes are labelled, and the label indicates the date, host identification, site of collection on the host, locality and district. The following is attached to the samples:

- A note recording clinical and epidemiological data;

2. Collection of biting midges (Culicoides)

- Collection of Culicoides larvae and pupae

- A Berlese funnel is used to collect Culicoides larvae and pupae from semi-aquatic or damp environments – forest areas. To facilitate collection of Culicoides larvae and pupae from aquatic environments, sugar or salt is added to the water; this method is usually used only for Culicoides species whose larvae do not swim or whose pupae do not float on the water surface. For other species, Culicoides larvae or pupae can be collected using forceps.

- Pupae can be sampled from the aquatic environment by following the banks of ponds, lakes or rivers.

- Sampling of adult biting midges

- Light traps or CO2 nets are used to collect adult biting midges;

- For trapping insects, it is necessary to know the sites suitable for placing traps, such as: places with relatively high humidity located a short distance from the forest edge, places between inhabited and uninhabited areas, residential areas or recreation zones, located 2–3 m from a building or suspended at a height of 1.5–2 m above the ground, as well as sites where traps should not be placed, such as: over water, directly on the ground, near places with artificial lighting, near animal stalls, in places exposed to smoke, or in places with industrial smog;

- The operating schedule of light traps is as follows: setting them for 1–7 nights per week, switching on in the evening and off in the morning, and collecting insects over 4 weeks.

- Preservation of larvae, pupae and adult biting midges

Alcohol at a concentration of 70% is used to preserve larvae, pupae and adult biting midges. There are other, more complex methods of preserving larvae, pupae and adults, but they can be used only when taxonomic studies are being carried out. Larvae, pupae and adult biting midges are also preserved very well in 70% alcohol, although

the concentration can be gradually increased, up to 100% ethyl alcohol saturated with phenol. The resulting solution is left overnight or even longer.

- Packing tubes with insects for transport to the laboratory

- To pack tubes with insects, tubes, cryovials with stoppers or larger tubes can be used, into which a preservative solution – 70% alcohol – is first placed to half the tube; then 4–5 larvae, pupae or adult biting midges are placed in, and the tubes are closed to prevent leakage of the preservative solution.

- A label indicating the date, locality and district where the samples were collected is attached to the tube, and in addition to the note accompanying the samples, a table is sent if there are several samples, together with a note stating: the sampling site, the soil type in the locality, the ambient temperature at the time of collection, climatic conditions, humidity level, wind speed, atmospheric pressure, whether the locality is inhabited or not, which animal species live in the locality, and the geographical position of the locality within the district and country.

3. Samples of gastropod molluscs – intermediate hosts of geohelminths

Gastropod molluscs can be caught in all seasons. However, the biotopes of the region in which the study will be conducted must be taken into account.

(a) Aquatic gastropod molluscs

- Aquatic gastropod molluscs are collected either with an insect net or with a sieve. The net is moved several times through the water from left to right, at different depths from the surface of aquatic plants to the muddy bottom.

- The material collected in the net is rinsed several times by immersing it in water to remove dirt. This material is then placed in a larger-mesh sieve and rinsed thoroughly to remove all debris so that only the snails remain.

(b) Terrestrial gastropod molluscs

- Terrestrial gastropod molluscs are usually collected after warm summer and even spring rains, when they emerge from their hiding places. Other gastropod molluscs living under leaves or logs are collected all year round by hand or with forceps, placing them directly into glass tubes, cardboard or plastic boxes.

- Terrestrial gastropod molluscs living in meadows are collected with insect nets, especially in the morning.

- Biological material collected from the field is transported in tubes, cardboard, plastic or tin boxes with ventilation, as well as in cloth or polyethylene bags.

- Slugs and snails are usually transported in crates with ventilation mesh. Leaves, grass or moss, which maintain humidity, are placed inside them.

- In the laboratory, gastropod molluscs can be kept alive in aquaria or terraria.

- A sample of gastropod molluscs may consist of a single specimen or several specimens collected at the same place.

- All samples taken are labelled immediately. The label indicates the date and place of sampling.

- Samples are transported to the laboratory with an accompanying note at chilled temperatures.

- Samples of oribatid mites and pasture mites – intermediate hosts of geohelminths

4. Oribatid mites are collected from April to November.

- Mite samples are collected in jars with lids, provided with a cotton swab moistened with chloroform or acetone, and left in contact with this swab for at least 24 hours. A sample contains at least 4–5 oribatid specimens.

- After the mites are collected, the jar is hermetically closed, the date and place of sample collection are written on the label stuck to the jar, and it is then transported to the laboratory.

- Samples are transported to the laboratory with an accompanying note at chilled temperature.

5. Samples of fruit flies and dragonflies – intermediate hosts of geohelminths

- Adult dragonflies are collected with specially designed nets and placed in screw-cap jars into which a cotton swab moistened with chloroform or acetone has been inserted beforehand, and left in contact with the insects for at least 24 hours.

- It is desirable to collect at least 4–5 dragonfly specimens.

- The date and place of collection of the dragonfly samples are written on the label attached to the jar, and the jar is then transported to the laboratory.

- Samples are transported to the laboratory with an accompanying note at chilled temperature.

6. Samples of flies – intermediate hosts of geohelminths

- At least 5 specimens of flies are collected from grazing sites using nets. They are placed in screw-cap glass jars with a cotton swab moistened with chloroform or acetone, and left in contact with it for at least 24 hours.

- A sample of flies may consist of a single insect or several insects taken from the same place.

- The date and place of sampling of the flies, with an indication of the animal species in that locality, are written on the label attached to the glass.

- Labelled and well-packed samples are transported to the laboratory.

- Samples are transported to the laboratory with an accompanying note at chilled temperature.

7. Samples of formicids (ants) – intermediate hosts of geohelminths

- Ants are collected either directly from the anthill, or blades of grass on which ants are caught are taken.

- It is desirable to take at least 10 ants.

- Ant samples are placed in jars of suitable size, into which a cotton swab moistened with chloroform or acetone has been placed beforehand, and it is left in contact with the insects for at least 24 hours.

- The label attached to the glass is completed as provided in the accompanying note, indicating the date and place of ant collection, after which the samples are transported to the laboratory.

- Samples are transported to the laboratory with an accompanying note at chilled temperature.

8. Samples of beetles (Coleoptera) – intermediate hosts of geohelminths

- Beetles are collected directly by hand or with a specially designed small net from places they frequent: near fresh sheep, cattle and horse faeces.

- It is recommended to collect these insects in damp, dark weather or, in any case, in the morning.

- After sampling, the samples are placed in screw-cap jars into which a cotton swab moistened with chloroform or acetone has been inserted beforehand. The jar is hermetically closed and left in contact with the insects for 24 hours.

- A sample may consist of one or several beetle specimens collected at the same place.

- The date and place of collection of the beetle samples are written on the label attached to the glass, after which they are transported to the laboratory.

- Samples are transported to the laboratory with an accompanying note at chilled temperature.

9. Samples of earthworms – intermediate hosts of geohelminths

- Earthworms, soil worms or ground worms are collected by dredging. They are collected mainly from places with rich soil, from manure, from vegetable gardens and from plains.

- The optimal time for sampling is at night and especially after rain, when they are very abundant.

- Earthworms can be collected for most of the year, from all regions of the country, but not in winter.

- After sampling, the earthworms are placed in screw-cap jars into which 5–10% alcohol or 5% formalin has been placed beforehand for anaesthesia, and they can be preserved in 70% alcohol or 5% formalin.

- The worms are placed for one month in a vessel filled with water with chloroform, which is covered with a glass plate – one part chloroform to 100 parts water, which is mixed well.

- The date and place of collection of the biological material are written on the label attached to the jar, after which it is transported to the laboratory.

10. All samples sent to the laboratory are accompanied by the following identification documents:

- an official accompanying note in accordance with the provisions of the sanitary-veterinary legislation in force;

- a table of the samples taken, completed by the official veterinarian authorised to collect, perform primary processing of, pack and dispatch the samples.

Samples for virological, serological, immunological and haematological testing

Sampling in live animals

1. Blood collection

- For virological testing whole blood is used, and for serological testing – blood on clot or blood serum, obtained by puncture of a deep or superficial blood vessel of suitable size.

- When it is necessary to detect specific antibodies and/or to quantify them over time, after sampling the samples are left at room temperature for serum expression. The serum is then separated from the clot, decanted and even centrifuged, after which it is packed appropriately.

- Serum can be frozen, except for avian serum.

- All these operations will be performed as sterilely as possible.

- Blood samples with clot are not frozen, to avoid haemolysis.

2. Blood collection for virological testing

- Blood is collected with an anticoagulant (sodium citrate, EDTA) from viraemic animals. Sampling is carried out into sterile vacuum tubes.

- After sampling, the collection tubes are gently shaken to homogenise the contents and chilled (2–8 °C). Freezing is prohibited.

- The minimum quantity of blood taken for virological testing is 20–50 ml.

- It is recommended to transport samples to the laboratory on the day of sampling at refrigerator temperature.

3. For blood samples without anticoagulant

- In quantitative terms, the volume of blood samples must be at least 7.5 ml for large animals and at least 1 ml for small animals and birds.

- In terms of quality, samples must not contain anticoagulant substances or foreign bodies, and must not be haemolysed or contaminated.

- Samples are submitted in disposable plastic or glass tubes without additives, with stoppers, clearly marked with appropriate numbering similar to the numbering in the accompanying tables.

- In situations where serum is not expressed or samples are slightly haemolysed, they are centrifuged for 15 minutes at 2,000 revolutions per minute.

4. Blood sampling for haematological testing

- Blood sampling as prescribed is carried out by puncture of large veins, after prior preparation of the chosen site, clipping and disinfection with alcohol or tincture of iodine, preferably before the animal consumes its feed ration.

- The time interval from applying the tourniquet to sampling must not exceed two minutes, to avoid the effect of progressive haemoconcentration.

- Blood is collected into tubes with an anticoagulant such as K3EDTA, in vacuum-container-type tubes, after which it is gently shaken to homogenise by inverting the tube 3–4 times. The tubes are closed tightly to avoid evaporation of plasma, which can cause haemoconcentration, which may be a source of error.

- The tube has a label on which the following is written (sample number; animal serial number; date of collection).

- The sample accompanying note is completed correctly and contains the following: species, age category of the animal, physiological state, clinical data.

e. Storage of blood samples

Blood samples collected in tubes with anticoagulants are stored in a refrigerator until examination at a temperature of 4 °C to preserve the morphological integrity of the blood elements.


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3. ACCURATE AND UNIFIED LABORATORY ANALYSIS METHODS

The laboratory methods described in this section comply with the requirements of the Joint ToR and were applied by both partners to ensure comparability of results. The methods set out in this section were used in the laboratory studies of the Project and are recommended for use by laboratories carrying out surveillance of emerging diseases in the region. Accurate laboratory testing means carrying out and interpreting diagnostic tests with such accuracy that health professionals can rely fully on their results. When we speak about the accuracy of laboratory testing, we mean how accurately test results reflect the true epidemic status of geographical areas. This is important because accurate results directly affect every stage of decision-making on prevention and eradication measures. From diagnosing a disease to monitoring its progression and adjusting treatment, the accuracy of test results is the foundation of effective healthcare.

Laboratory analysis methods

- molecular methods: PCR / RT-PCR (reverse transcription PCR);
- serological methods: ELISA (enzyme-linked immunosorbent assay);
- where necessary – additional methods for interpretation and confirmation of results.

A single methodological approach provides a consistent scientific basis for the activities of partners from different countries, and also makes it possible to compare, correlate and jointly interpret epidemiologically the results obtained by each partner independently.

African swine fever – ELISA

Antibodies to the ASF virus are usually detected 7–10 days after infection or vaccination and may persist in the body for months or even years. Pigs and wild boar

infected with highly pathogenic strains usually die before they have time to develop a detectable level of antibodies. However, in regions where the infection is endemic and attenuated or low-virulence isolates also circulate, serological testing plays a key role in identifying animals that have recovered or are infected but show no clinical signs of disease. To date, no antibodies capable of completely neutralising the virus have been identified.

Antibodies are rarely detected in animals infected with highly virulent ASF virus, because death usually occurs before an antibody response develops, whereas pigs infected with strains of low or moderate virulence produce detectable antibodies.

ELISA directly detects antibodies to ASF in pigs infected with virus strains of low or moderate virulence. A number of validated commercial kits using a competitive or indirect format are currently available for this purpose, and they are suitable for different epidemiological contexts.

The indirect ELISA kit is designed to identify antibodies to ASF in serum, plasma, meat juice and dried blood on filter paper from pigs.

The microplate wells are pre-coated with recombinant ASF proteins p32, p62 and p72. Test samples and controls are dispensed into the coated wells; any antibodies to the ASF virus that are present bind to the coated antigens, forming an antigen–antibody complex. After a washing step, a multispecies antibody conjugate labelled with horseradish peroxidase (HRP) is added; it binds to any captured antibodies, forming an antigen–antibody–HRP conjugate complex. Excess conjugate is removed by washing, after which TMB substrate solution is added.

The intensity of the colour produced is proportional to the quantity of specific antibodies in the sample:

- if antibodies are present, a blue colour develops, which turns yellow after addition of the stop solution;

- if antibodies are absent, no colour change is observed.

<u>African swine fever – PCR (polymerase chain reaction)</u>

PCR methods using primers targeting a highly conserved region of the genome make it possible to detect and characterise isolates covering all known genotypes of African swine fever (ASF) virus, including non-haemadsorbing strains and low-virulence isolates. These methods are particularly valuable for detecting viral DNA in pig tissue samples unsuitable for virus isolation or antigen testing (for example, in cases of material decomposition), as well as in cases where the virus may have been inactivated before the sample reached the laboratory. Owing to its high sensitivity, specificity and suitability for mass testing, PCR is the method of choice for both screening and confirmation of suspected cases.

The ASF primer set can be combined with a primer set specific for classical swine fever (CSF) virus in a multiplex PCR format. This makes it possible to detect and distinguish the genomes of both viruses in a single reaction.

Each PCR run must include at least one positive and one negative control for the amplification step. The positive control consists of 2 µl of ASF virus DNA; its concentration should preferably be close to the detection limit of the method, which allows the amplification efficiency to be verified (for example, a Ct value within 32 ± 2 when real-time PCR is used). The negative control consists of 2 µl of sterile (nuclease-free) water or DNA isolated from blood samples (with EDTA), serum or tissue homogenate not containing ASF virus. Note: the paragraphs are adapted, in the author's own words, from the section on African swine fever (PCR section) of the WOAH Terrestrial Manual.

Avian influenza – multispecies ELISA

Commercial ELISA kits targeting antibodies against influenza A nucleoprotein are widely available. Both indirect and competitive/blocking formats have been developed, validated and are currently used to detect specific antibodies to influenza A in various animal species. The competitive (AIV C-ELISA) and blocking (AIV B-ELISA) formats, in particular, offer greater sensitivity than the AGID test for detecting group-reactive antibodies to influenza A in the sera of poultry and other birds. In both the competitive and blocking formats, the assay works in such a way that sample antibodies compete with a monoclonal antibody for binding sites on a conserved epitope of the nucleoprotein shared by all influenza A viruses.

Kits must be validated for the target species and intended use, since antigen preparation and test formats differ between manufacturers; the manufacturer's instructions on validated use should be followed carefully. These ELISAs are moderately priced, support high-throughput screening for influenza A infections and perform well compared with ELISA in large-scale serosurveillance, although any positive result still requires confirmation by HI to determine the H5/H7 subtype. Subtype-specific kits exist for H5, H7, H9 and some N subtypes, such as N1, although they are generally less sensitive than the general influenza A ELISA. More recently, multiplex assays have been developed using microsphere-based immunoassay technology and recombinant viral proteins, which allow several HA/NA subtypes or additional targets for notifiable diseases to be tested together at lower cost, although they still require further validation before routine diagnostic use.

Mammalian serum can be screened for exposure to influenza virus using a commercial multispecies competitive ELISA that targets antibodies against influenza type A nucleoprotein (NP). Because the diagnostic performance of these kits has not

been established for mammalian species other than those stated by the manufacturer, their suitability should be carefully assessed for the species being tested before routine use, and positive results require confirmation by an additional serological test, such as HI.

Avian influenza – RT-PCR

Well-developed molecular genetic methods can surpass virus isolation in both sensitivity and specificity. Concerns that PCR inhibitors in faecal samples may reduce assay sensitivity have been largely eliminated thanks to improved RNA extraction and sample preparation kits. To obtain reliable results, proper use of positive and negative controls at both the extraction and amplification steps remains critically important.

Most laboratories diagnose influenza type A using real-time RT-PCR (RT-qPCR). This method has shown high performance compared with virus isolation, while being cheaper and considerably faster: results of clinical sample analysis can be obtained in less than three hours. Such screening tools require continuous in silico monitoring and updating as necessary. Thus, the original protocol was later improved by introducing degenerate bases into the primers for the M gene [83]. This change also made it possible to add an internal process control, which helps prevent false-negative results caused by PCR inhibition or extraction errors. Advances in assay development and biochemistry have produced screening tests suitable for detecting all influenza A viruses regardless of host species (both animals and humans) [84]. This is particularly relevant in situations where the virus may be transmitted between birds and other host species.

Laboratories that do not have the equipment for real-time RT-PCR for M-gene screening can use the same primer sets in a conventional (classical) RT-PCR format. Validation data for such alternative use are currently lacking; because the resulting amplicon is small (less than 100 bp), the results should be analysed by SDS

polyacrylamide gel electrophoresis (SDS-PAGE) with silver staining or in a 2–3% agarose gel.

West Nile virus (WNV) infection – ELISA

Antibodies in horses can be detected using an IgM antibody-capture ELISA, the haemagglutination inhibition test (HI), an IgG ELISA, the plaque reduction neutralisation test (PRN) or the micro virus neutralisation test (VN). The IgM antibody-capture ELISA is particularly effective for detecting antibodies in horses after recent natural WNV infection, since IgM in horses is usually detectable from 7–10 days to 1–2 months after infection; in most horses with encephalitis caused by WNV, the test is already positive at the time clinical signs appear. Neutralising antibodies in horses appear approximately two weeks after infection and may persist for more than a year. In birds, ELISA, HI, VN and PRN are most often used to detect antibodies to WNV, although some tests may show cross-reactions with related flaviviruses, such as St. Louis encephalitis virus or Japanese encephalitis virus. The PRN method is the most specific of the serological tests for WNV; where cross-reactivity must be excluded, antibody titres to related flaviviruses can be determined in parallel. Vaccination history should always be taken into account when interpreting PRN, VN or IgG ELISA results. The IgM antibody-capture ELISA can also be used for birds and other animal species provided that species-specific capture antibodies are available (for example, antibodies to chicken IgM), whereas the PRN method is universal across species, including birds.

Most ELISA methods have limited specificity owing to cross-reactions with antibodies to other flaviviruses, in particular those belonging to the Japanese encephalitis virus serogroup; nevertheless, they remain valuable tools for epidemiological surveillance and screening. Any positive result should be confirmed using a more specific method, for example the virus neutralisation test (VN) or the

plaque reduction neutralisation test (PRNT). Some indirect or competitive assay variants make it possible to detect antibodies of different immunoglobulin classes (IgM, IgG, etc.), and in vaccinated horses the results of such tests are often positive.

West Nile virus infection – real-time PCR

Several PCR-based methods for identifying West Nile virus have been published, some of which are available as commercial kits. These include real-time PCR capable of detecting West Nile virus of both lineage 1 and lineage 2, and conventional gel-based real-time PCR for North American lineage 1 strains; both methods have been used successfully on field samples. Lineage 1 West Nile virus strains from France, Egypt, Israel, Italy, Kenya and Mexico have a highly conserved nucleotide sequence in the target region regardless of host species. The conventional nested procedure offers greater sensitivity for North American West Nile virus RNA, especially in field samples from horses, although it requires strict laboratory practice to prevent contamination and its performance against other West Nile virus lineages has not been established. Because West Nile virus continues to evolve and new strains may emerge, PCR test developments should be continually reviewed and updated. Suitable samples for West Nile virus RT-PCR are pools of brain, kidney, heart, liver, spleen and intestine of mammals and birds, and insects. Each PCR run should include positive and no-template controls, and for nested RT-PCR care should be taken to avoid cross-contamination when transferring the outer-primer product to the nested reaction; a run is valid only when its control reactions are within the expected range.

The method given below allows simultaneous identification of West Nile virus lineage 1 and lineage 2; strains can be further identified by sequencing the amplicon and comparing it with reference West Nile virus strains. For each sample, a reaction mixture of 20 µl (according to the kit instructions) must be prepared, containing primers

at a concentration of 0.9 µM and probe at a concentration of 0.25 µM; 20 µl of the mixture is dispensed into the appropriate tube or well. 5.0 µl of isolated RNA is added, the container is sealed hermetically and placed in the thermocycler; amplification is carried out under the conditions given in the kit instructions (starting with reverse transcription, followed by 45 amplification cycles). A Ct value of 37 or less indicates a positive result for West Nile virus (WNV); values in the range of 37.1 to 42 are considered doubtful (retesting required), and values above 42 are considered negative. Assay results are considered valid only if the Ct values for the positive controls are within the expected range and the no-template controls are negative.

Leptospirosis – MAT

The microscopic agglutination test (MAT) using live antigens is the most widely used serological method for the diagnosis of leptospirosis. It serves as the "gold standard" against which all other serological tests are compared, and is also used in the import and export of animals. To achieve optimal sensitivity, the antigen panel should cover all serogroups prevalent in the given region; ideally, strains representing all known serogroups are used. The presence of a particular serogroup is usually presumed on the basis of a characteristic pattern of reactions during screening, but it can be definitively confirmed only by isolating the serovar from a clinically ill animal. The use of local isolates instead of reference strains may increase sensitivity, although reference strains remain useful for comparing results between laboratories.

The specificity of the MAT is good, since antibodies to unrelated bacteria rarely cross-react significantly with Leptospira. However, there is considerable cross-reactivity between different Leptospira serovars and serogroups, so an animal infected with one serovar will usually also show MAT reactivity – usually weaker – against other serovars. For this reason, serology alone cannot definitively identify the infecting

serovar in a given case or outbreak; this requires isolation of the organism. If local serovars are already well characterised through isolation studies, serology may suggest, although not confirm, the probable infecting serovar. Vaccination history is also important, since vaccinated animals may carry antibodies to the vaccine serovars.

Leptospirosis – ELISA

A number of ELISA formats have been developed for detecting anti-leptospiral antibodies, differing in antigen preparation, protocol and platform, including plate-based and dipstick tests. The antigens used are usually either whole-cell preparations or outer membrane protein (OMP) preparations, with more recent work focusing on recombinant OMPs; the choice of antigen determines the specificity of the assay. ELISAs based on recombinant OMPs react broadly with pathogenic leptospires and therefore have limited value for epidemiological work, whereas lipopolysaccharide-based ELISAs are serogroup-specific and useful in epidemiological studies and control programmes. IgM ELISAs are useful for diagnosing acute infection, and a total Ig ELISA can help identify fully susceptible animals for experimental studies. ELISAs for detecting antibodies to serovar Hardjo have also been developed for testing milk from individual cows or bulk-tank milk and have proved useful for identifying herds infected with Hardjo.

Validation remains the main limitation for most leptospirosis ELISA tests, since almost all of them were validated against the MAT (using a titre threshold of 1/100 or higher) – which is itself an imperfect reference standard, with a sensitivity below 50% in some chronic infections. In human medicine this issue is addressed using Bayesian latent class models and random-effects meta-analyses, although the strongest validation is still carried out on serial sera taken from culture-positive cases. Only a few animal ELISA tests have been validated in this way, and even then only up to 6 months after

infection using experimental animals, whereas one commercial Hardjo ELISA was validated on individual serum samples from culture-positive cattle.

Leptospirosis – real-time PCR

PCR-based assays are increasingly used to detect leptospires in animal tissues and body fluids because of their presumed sensitivity and ability to provide an early diagnosis; real-time PCR is faster and less prone to contamination than conventional PCR. These assays target either genes universally present in bacteria, such as gyrB, rrs (16S rRNA) and secY, or genes restricted to pathogenic leptospires, such as lipL21, lipL32, lipL41, ligA and ligB. None of these assays can directly identify the infecting serovar, although sequencing of amplicons from certain primer sets can sometimes allow identification down to species or strain level; this additional analysis is not part of routine diagnostics. Most PCR primer sets were developed and evaluated for human rather than animal samples, and there is no clear consensus on which primers are best suited to animal samples, although those targeting the lipL32 gene are the most widely described.

Validation is still an open issue for PCR diagnosis of animal leptospirosis, and each laboratory is responsible for validating its own assay for the specific tissue, fluid and species tested. To date, only three real-time PCR methods have been officially validated. The presence of amplification inhibitors in clinical samples (for example, owing to faecal contamination or autolysis in samples from animals) may lead to false-negative results. It is therefore necessary to control carefully the organisation of laboratory space, workflows and the use of appropriate control samples in order to avoid contamination of reagents, and sample preparation methods must always be adapted to the type of tissue, body fluid and animal species being tested.

Toxoplasmosis – ELISA. In its simplest form, in ELISA the antigens from the test sample are attached to a surface. A suitable antibody that can bind the antigen is then applied to the surface. This antibody is linked to an enzyme, and any unbound antibodies are then removed. In the last step, a substance containing the enzyme's substrate is added. If binding has occurred, the subsequent reaction produces a signal, most often detected as a colour change. The assay is based on the interaction of at least one antibody with its cognate antigen. For this purpose, the sample with the antigen is immobilised on a solid support (polystyrene microplates are mainly used) either non-specifically (adsorption on the surface) or specifically (capture by another specific antibody – a "sandwich"). After immobilisation, an antibody is added to form an antigen–antibody complex. The antibody may be covalently linked to an enzyme or bound to a secondary antibody. After each stage of the assay, the plates are washed with special buffer solutions to remove any proteins or antibodies bound non-specifically. After the last washing step, an enzyme substrate is added to the microplate to visualise the result, with the intensity of the colour proportional to the amount of antigen in the sample. Quantitative "reading" of the result is based on detecting the intensity of transmitted light by spectrophotometry, which involves quantifying the transmission of light of a certain wavelength through the liquid.

Method of post-mortem (post-slaughter) diagnosis of toxoplasmosis in animals.

Since the meat of cattle and small ruminants is mostly used as food for domestic animals, it should also be regarded as one of the sources of toxoplasmosis infection for various animal species and humans.

The method of post-mortem (post-slaughter) diagnosis of toxoplasmosis in animals involves determining antibodies to the toxoplasmosis agent in the supernatant of a diaphragm homogenate. The total analysis time is 120 minutes.

The use of diaphragm tissue as research material is justified by the presence in it of a larger quantity of antibodies to the toxoplasmosis agent, as confirmed by laboratory studies. For the laboratory studies, diaphragm, back and thigh muscles and lung, kidney and liver tissues were taken from animals with a confirmed diagnosis of toxoplasmosis. According to the results, the highest antibody titre was found in the diaphragm muscles.

The proposed method makes it possible to: diagnose toxoplasmosis post-mortem quickly and with high accuracy; reduce the cost of materials and reagents for diagnostics; and prevent human infection with Toxoplasma through meat and meat products.

The method of post-mortem (post-slaughter) diagnosis of toxoplasmosis in animals includes sample preparation and enzyme-linked immunosorbent assay.

Sample preparation: a 50 g portion of diaphragm is ground through a meat grinder, after which the juice is squeezed out through four layers of gauze, and the liquid obtained is centrifuged for 5 min at 3,000 rpm. Total antibodies to Toxoplasma are detected in the resulting supernatant.

Performing the assay (according to the instructions for the ID Screen Toxoplasmosis Indirect Multi-species reagent kit, manufactured by IDvet): buffer solution (90 µl) is added to the plate with immobilised antigen, followed by the control samples (positive and negative) and the test supernatant samples (50 µl each). It is incubated for 45 min at 21 °C and then washed 3 times in an instrument (adding 300 µl of washing solution to each well). Next, the prepared conjugate is added in a quantity of 100 µl to each well and incubated for 30 min at the same temperature. It is washed

in the same way as before, and 100 µl of substrate solution is added to each well, followed by stop solution to stop the reaction. The result is read on a spectrophotometer at a wavelength of 450 nm.

Assessment procedure

Assessment and interpretation of laboratory results are carried out using a stepwise approach that integrates analytical validity, assay-specific interpretation criteria, biological plausibility and epidemiological context. Molecular and serological results are assessed separately because they provide different types of information: PCR and RT-PCR methods are intended to demonstrate pathogen nucleic acid in the tested material, whereas serological methods detect the immune response and may therefore reflect previous exposure rather than active infection at the time of sampling.

Before individual samples were interpreted, each analytical run was assessed for validity in accordance with the requirements of the method, the manufacturer's instructions, laboratory standard operating procedures and the relevant WOAH recommendations. A run was considered interpretable only when the required positive, negative and, where possible, internal amplification or process controls gave the expected results. Failure of one or more mandatory controls led to invalidation or repetition of the corresponding analytical run.

Sample suitability was also taken into account. Samples showing insufficient preservation, insufficient quantity, signs of degradation, excessive contamination, inhibition or other conditions capable of compromising analytical performance were not interpreted as equivalent to valid negative samples.

Molecular methods – PCR and real-time PCR

Molecular results are assessed on the basis of the amplification profile, the Ct value where applicable, the performance of positive and negative controls, the internal amplification control and the validated interpretation criteria of the specific assay.

A molecular result is classified as positive when specific amplification of the target sequence occurred within the validated analytical criteria of the method and all run controls met the acceptance requirements. A result is classified as negative when no target-specific amplification was detected and the control system demonstrated that the extraction and amplification conditions were valid. Results close to the detection limit of the assay, atypical amplification curves, inconsistent replicates or failure of the internal control were classified, as appropriate, as suspect, inconclusive or invalid, and were re-analysed.

Interpretation of the laboratory testing methods for the diseases defined by the project activity

PCR method

For ASF testing, the PCR framework used included positive and negative amplification controls in line with methodological principles based on WOAH. The positive control was intended to verify amplification efficiency, while the negative control was used to detect contamination. WOAH defines PCR and real-time PCR as highly sensitive, specific and rapid methods for genomic detection of ASF virus and considers them particularly useful in a wide range of diagnostic situations.

For avian influenza, molecular assessment is carried out following a sequential diagnostic logic. Screening iis based on detection of the conserved matrix gene of influenza A. Samples that tested positive on the influenza A screening were to be further examined with subtype-specific molecular methods for H5 and H7, in line with the diagnostic approach described by WOAH. WOAH recognises direct genome

detection by real-time PCR or other validated molecular methods as the main method for the diagnosis and characterisation of influenza A viruses. A negative result for the matrix gene is interpreted as the absence of detected influenza A RNA in the analysed sample at the time of testing, and not as evidence of absence of infection in the population.

For West Nile virus, the real-time PCR method applied the following interpretation criteria: Ct ≤ 37 is considered positive; Ct values from 37.1 to 42 are considered suspect and required repetition; Ct values > 42 are considered negative. A run is considered valid only when the positive control gave amplification within the expected range and the no-template control remained negative. WOAH recognises RT-PCR for detection of West Nile virus nucleic acid, but also stresses that the diagnosis must be interpreted taking into account the biological material and the dynamics of infection.

For leptospirosis, PCR results are interpreted with particular attention to the sample matrix and the stage of infection. WOAH notes that PCR can detect leptospiral genetic material in tissues or body fluids, but its performance depends on sample handling, the type of tissue or fluid and the host species. PCR usually does not identify the infecting serovar. Accordingly, failure to detect leptospiral DNA in blood is interpreted as the absence of detected leptospiral DNA in that specific sample at the time of sampling, and not as definitive exclusion of chronic renal or genital carriage. WOAH separately recognises that leptospires can persist in the kidneys, urine or genital tract of chronic carriers.

For toxoplasmosis, PCR results. For the detection of Toxoplasma DNA, the following protocol for amplification of the 16S gene is used: Lass A, Kontogeorgos I, Ma L, Zhang X, Li X, Karanis P. Investigation of Toxoplasma gondii in wastewater and

surface water in the Qinghai-Tibet Plateau, China, using real-time PCR and multilocus genotyping. Sci Rep. 2022 Mar 31;12(1):5428. doi: 10.1038/s41598-022-09166-0. PMID: 35361820; PMCID: PMC8971506.

Serological methods. Serological results are assessed according to the calculation method and cut-off values defined for each commercial test kit used in the project. Positive ELISA results are regarded as evidence of the presence of antibodies to the target antigen system, and not automatically as proof of active infection.

African swine fever – indirect ELISA

For the indirect ELISA for African swine fever (ASF) used by Romanian partner (Veterinary Sanitary and Food Safety Directorate Tulcea), interpretation was based on the calculated S/P percentage.

Samples were classified as:

- Negative: S/P ≤ 30%;
- Doubtful: 30% < S/P < 40%;
- Positive: S/P ≥ 40%.

These cut-off values correspond to the criteria specific to this method.

Serological results were assessed separately from the results of direct detection of ASF virus. This distinction is important because the World Organisation for Animal Health (WOAH) notes that in pigs that have undergone natural infection, antibodies usually appear approximately 7–10 days after infection and may persist for a long time. Therefore, a result of "ELISA-positive / PCR-negative" was interpreted as the presence of serological reactivity in the absence of direct molecular evidence of viraemia in the sample taken at that time. Such results require consideration of the epidemiological history and, where necessary, additional confirmatory testing.

Avian influenza – multispecies competitive ELISA and haemagglutination inhibition (HI) test

The results of the multispecies competitive ELISA for detection of influenza A virus (manufacturer – Innovative Diagnostics) were interpreted according to the S/N percentage:

- Positive: S/N ≤ 45%;
- Doubtful: 45% < S/N < 50%;
- Negative: S/N ≥ 50%.

These are the cut-off values specific to this method that were applied in this project.

WOAH notes that influenza A viruses have antigenically related nucleoproteins and matrix proteins, and that ELISA methods aimed at detecting antibodies to these antigens are widely used for group-level serological detection of the agents. Accordingly, an ELISA-reactive result is interpreted as group-level reactivity of antibodies to influenza A, and not as evidence of a specific H or N subtype.

A positive general serological result for influenza A with negative H5/H7-specific follow-up was thus is interpreted as the absence of serological confirmation for the tested H5/H7 antigens, and not as a contradiction of the initial screening result.

West Nile virus (WNV) infection – blocking enzyme-linked immunosorbent assay (ELISA)

When the blocking ELISA for WNV (manufactured by Gold Standard Diagnostics Madrid S.A.) was used, interpretation of the results is based on the percentage inhibition (IP).

Samples are classified as follows:

- Positive: IP ≥ 40%;

- Negative: IP ≤ 30%;

- Doubtful: IP in the range from 30% to 40%.

According to the procedure, samples that fall within the doubtful range are subject to additional testing by seroneutralisation.

Positive ELISA results are treated with particular caution because, according to the World Organisation for Animal Health (WOAH), serological cross-reactions with related flaviviruses are possible, in particular Usutu virus, St. Louis encephalitis virus, Japanese encephalitis virus and tick-borne encephalitis virus.

Accordingly, a positive blocking ELISA result is interpreted as serological evidence of reactivity to WNV. In cases where it is epidemiologically appropriate and technically feasible, confirmation of the result by a more specific neutralisation method (for example, the virus neutralisation test or the plaque reduction method) provides higher specificity. A positive serological result combined with a negative reverse transcription–polymerase chain reaction (RT-PCR) result is not considered contradictory, since serological markers may persist longer than the period during which viral RNA can be detected in peripheral blood.

Leptospirosis – ELISA and additional interpretation of results

When the ELISA (manufactured by BIOVET Inc.) was used to detect antibodies to Leptospira interrogans serovars Hardjo and Pomona, the sample ratio is calculated by dividing the optical density of the sample (for the corresponding antigen) by the mean optical density of the positive control for the same antigen.

Results were interpreted as follows:

- Negative: ratio < 0.20;

- Suspect/doubtful: ratio ≥ 0.20 and < 0.30;

- Positive: ratio ≥ 0.30.

In the case of suspect results, the assay procedure provides for taking a second serum sample after approximately two weeks and retesting. Epidemiological interpretation of the ELISA results is carried out taking into account WOAH recommendations. WOAH defines the microscopic agglutination test (MAT) as the standard serological method, and also recognises ELISA as a useful tool for detecting antibodies to leptospires, in particular in cases associated with serovar Hardjo in cattle. Serological reactivity alone is not considered sufficient for definitive identification of the infecting serovar. Vaccination history is also taken into account, if available, since vaccinated animals may produce antibodies that can be detected by some serological assays. According to WOAH, for acute infection a four-fold increase in MAT titre between paired acute and convalescent sera is diagnostic evidence.

For the toxoplasmosis agent by ELISA, interpretation in the samples is carried out according to the data given in Table 9.

Table 9. Antibody titre (IgM, IgG) in different tissues and organs of animals with toxoplasmosis

Animal species	Object of study					
	Muscles			Tissues		
	diaphragm	back	thigh	lungs	kidneys	liver
Cattle	51,0	45,3	51,8	10,8	19,7	17,6
Sheep	44,7	39,1	43,6	17,4	15,0	10,3

Note: Interpretation of results: ≤ 40 – negative result; 40–50 – doubtful result; ≥ 50 – positive result

4. RESULTS OF THE RESEARCH OBTAINED WITHIN THE PROJECT AND THEIR INTERPRETATION

This section presents the results of the joint studies carried out in two sampling periods. The figures relate to the samples taken and serve as the basis for the practical conclusions of the Guide. The results were obtained under the single sampling and analysis scheme defined by the Joint ToR.

African swine fever

In Romania, the study on African swine fever (ASF) combined serological screening by ELISA with direct detection of ASF virus genomic material by real-time PCR. In total, 70 serum samples were tested serologically: 33 samples from domestic pigs and 37 samples from golden jackals. Antibodies to ASF virus were detected in three pig serum samples; the remaining 30 pig samples and all 37 golden jackal samples tested negative. The proportion of samples showing a positive ELISA result was thus 9.1% (3/33) of the pig serum samples tested and 4.3% (3/70) of all serum samples included in the dataset for the serological study on ASF. These figures characterise only the sample examined and should not be interpreted as disease prevalence in the population, since sampling within the project was purposive rather than random (as would be required to estimate prevalence).

The three pig samples that gave a positive ELISA result were taken on 29 July 2026 in private backyard holdings in the localities of Slava Rusă (Slava Rusa) and Slava Cercheză (Slava Cercheza). Two positive samples came from 10-month-old gilts (females) from the village of Slava Rusă, and the third from a 12-month-old gilt from the village of Slava Cercheză. These three animals were subsequently tested additionally by real-time PCR (using EDTA blood); ASF virus genomic material was not detected in any of them. In the other pig serum samples – in particular those taken

during the first phase of the study in the localities of Ceatalchioi, Pardina, Cardon and Periprava, as well as in other samples taken in two holdings in July 2026 – antibodies to ASF virus were not detected.

The molecular genetic stage of the study included 65 real-time PCR tests: 24 pig blood samples (with EDTA), 37 golden jackal blood samples (with EDTA) and four tick samples. ASF virus DNA was not detected in any of these materials. The molecular dataset for pigs included samples taken during the campaign in November 2025 in the localities of Ceatalchioi, Pardina, Cardon and Periprava, as well as three pig samples taken in July 2026 that showed serological reactivity. The molecular dataset for wild animals covered golden jackals sampled in the Sulina area and in the hunting grounds of Pandur, Bestepe, Carcaliu, Crapina-Isaccea and Smârdan; all showed negative real-time PCR results. The four tick samples collected during the first campaign also proved negative for African swine fever (ASF) virus genomic material. The "ELISA-positive / real-time PCR-negative" profile found in three pigs indicates serological reactivity in the absence of direct molecular evidence of viraemia at the time of sampling. Such a result should therefore be interpreted taking into account the epidemiological context in which the animals were kept and, where necessary, supplemented by additional confirmatory testing; in itself, this result cannot be regarded as evidence of active circulation of ASF virus. The project results for jackals and ticks (species not determined) should be regarded as additional information for monitoring at the interface between different species; the main epidemiological interpretation of the ASF situation is based on data obtained from members of the family Suidae. Overall, the Romanian dataset did not provide direct molecular evidence of active circulation of ASF virus in the samples tested during the respective periods, but the three pig samples

showing serological reactivity are an important signal that justifies the need for continued risk-based monitoring and further epidemiological investigation.

In Ukraine, examination of animal blood samples, feed, and collected dead blood-sucking arthropods, carried out in autumn and spring, showed the absence of ASF virus DNA.

To determine the immune profile of the pig population in the geographical area studied, blood samples from pigs and piglets were additionally tested by PCR for influenza A virus RNA, porcine reproductive and respiratory syndrome (PRRS) virus RNA, and porcine parvovirus and porcine circovirus DNA. These viral agents may act as potential co-infection agents in ASF, since they have an immunosuppressive effect on the pig organism.

In the examination of 25 pig blood samples from the village of Prymorske, influenza A virus RNA was detected in one sample; this virus did not cause respiratory disorders and did not belong to the H1 or H3 subtypes, which pose a danger to humans.

In the examination of 13 pig blood samples from the village of Stara Nekrasivka, porcine circovirus type 2 DNA was detected in two samples. PRRS virus RNA and parvovirus DNA were not detected. In the examination of 50 pig blood samples taken in spring, porcine circovirus type 2 DNA was detected in one sample. PRRS virus RNA and porcine parvovirus DNA were not detected.

Avian influenza

Testing carried out in Romania by Veterinary Sanitary and Food Safety Directorate Tulcea combined broad serological screening with direct molecular detection and, for ELISA-reactive poultry sera, subtype-oriented haemagglutination inhibition (HI) testing. The serological dataset comprised 408 individual serum samples tested with the multispecies influenza A ELISA: 146 poultry sera (104 chickens, 38

domestic ducks and 4 domestic geese) and 262 mammalian sera (210 cattle, 15 sheep and 37 golden jackals). Five samples (1.2% of the serological set tested) were ELISA-reactive: two chickens and two domestic ducks, taken in non-commercial backyard holdings in Ceatalchioi during the first campaign, and one golden jackal from hunting ground No. 31 Crapina during the second campaign. The remaining 403 ELISA samples were negative. These proportions describe the project sample set and should not be interpreted as population prevalence, since sampling was project- and risk-oriented rather than probability-based.

Four ELISA-reactive poultry sera were further tested by haemagglutination inhibition against the reference antigens H5N3, H5N8 and H7N1, resulting in 12 HI determinations. All titres were below 1/16 and were recorded as negative for antibodies against the tested H5/H7 antigens. In the serological dataset there was no HI record for the ELISA-reactive golden jackal serum. In line with WOAH recommendations, influenza A ELISA tests targeting conserved nucleoprotein/matrix antigens are suitable for group-level serological screening and large-scale surveillance, but positive ELISA results require subtype-oriented follow-up, especially for H5 and H7, and the assay must be validated for the species and intended purpose. Accordingly, the four poultry ELISA reactions were not confirmed as H5/H7-specific, whereas the golden jackal ELISA result should be regarded as a screening signal requiring cautious, species-appropriate interpretation. Molecular testing included 444 real-time PCR determinations targeting the conserved matrix (M) gene of influenza A, corresponding to 379 individual animal records. The material tested included 156 determinations on EDTA blood, 152 on combined cloacal/tracheal swabs, 65 on cloacal swabs, 65 on tracheal swabs and 6 on organ samples. The population tested covered domestic and wild birds (chickens, domestic ducks/ducks, domestic geese, coots and gulls) and

mammals at the wildlife–domestic animal interface (cattle, sheep and golden jackals). Influenza A RNA was not detected in any of the 444 determinations. Notably, the ELISA-reactive golden jackal was also tested on EDTA blood by real-time PCR, and influenza A matrix gene RNA was not detected. WOAH defines direct detection of viral RNA by real-time PCR as the preferred method for rapid laboratory diagnosis and surveillance of influenza A, usually using generic detection of the highly conserved matrix gene as the initial screening step, followed by specific H5 and H7 testing when influenza A RNA is detected. Since all Romanian real-time PCR determinations were negative, the dataset did not provide direct molecular evidence of active influenza A infection in the materials tested at the time of sampling. The combined profile – limited ELISA reactivity, negative H5/H7 HI results in the four reactive poultry sera and uniformly negative real-time PCR – does not support active circulation of H5/H7 virus in the Romanian project sample set during the periods studied. Nevertheless, group-reactive ELISA results may reflect previous exposure to influenza A subtypes other than those tested by HI, antibody waning or non-specific serological reactivity, and therefore remain relevant for further risk-based surveillance at the interface between domestic birds and wildlife.

Results of detection of avian influenza virus in Ukraine (influenza A, H5/H7) (Tables 10, 11). Influenza A virus RNA was detected in three samples of wild bird droppings: in two collected at the sites where migrating dunlins (Calidris alpina) were staying, and in one obtained from a mallard (Anas platyrhynchos). Migratory concentrations of these birds were recorded during sampling near the village of Dunaiske and Lake Kryve; the virus RNA was also detected in coastal-zone water (in the bottom water layer; the samples were prepared by sedimentation and filtration) (see Table 10). Further in-depth testing of the detected influenza A virus RNA for the highly

pathogenic subtypes H5 and H7 gave a negative result, which indicates circulation of low-pathogenic subtypes of influenza A virus.

Table 10. Positive samples for avian influenza in Ukraine

№	"Isolate" name	A	H5	H7	Identification
1	A/Calidris alpina/Kryve/01/H?	+	–	–	Influenza A, low pathogenic
2	A/Calidris alpina/Kryve/02/H?	+	–	–	Influenza A, low pathogenic
3	A/Anas platyrhynchos/Kryve/02/H?	+	–	–	Influenza A, low pathogenic
4	A/water/Kryve/01/H?	+	–	–	Influenza A, low pathogenic

Spring studies detected influenza type A virus RNA in six samples of wild bird droppings: four of them were taken at the sites where migrating mallards (Anas platyrhynchos) were staying, and one each from a ruff (Philomachus pugnax) and a dunlin (Calidris alpina). Migrating flocks of these birds were recorded during sampling in the Danube floodplain area, near the village of Dunaiske and Lake Kryve (in particular, in the coastal waters of Lake Kryve – in the bottom water layer, concentrated by sedimentation filtration). Influenza type A virus RNA was not detected in soil samples taken in areas adjacent to the water bodies.

Table 11. Positive samples for avian influenza in the second period in Ukraine

№	"Isolate" name	A	H5	H7	Identification
1	A/Anas platyrhynchos/Danube/01/H?	+	–	–	Influenza A, low pathogenic
2	A/Anas platyrhynchos/Danube/02/H?	+	–	–	Influenza A, low pathogenic
3	A/Anas platyrhynchos/Danube/03/H?	+	–	–	Influenza A, low pathogenic
4	A/Anas platyrhynchos/Danube/04/H?	+	–	–	Influenza A, low pathogenic

№	"Isolate" name	A	H5	H7	Identification
5	A/Calidris alpina/Kryve/03/H?	+	–	–	Influenza A, low pathogenic
6	A/Philomachus pugnax/Kryve/01/H?	+	–	–	Influenza A, low pathogenic
7	A/water/Kryve/01/H?	+	–	–	Influenza A, low pathogenic

Further in-depth testing of the detected influenza type A virus RNA for the highly pathogenic subtypes H5 and H7 gave a negative result, which indicates circulation of low-pathogenic subtypes of influenza type A virus. This is confirmed by data from parallel ornithological observations: no cases of clinical signs of influenza or mortality of wild or domestic birds were recorded in the areas studied. In the analysis of pig and cattle serum samples, no antibodies to influenza type A viruses were detected.

Leptospirosis

The studies in Romania combined serological screening by enzyme-linked immunosorbent assay (ELISA) with direct molecular detection of leptospiral genetic material by real-time polymerase chain reaction (real-time PCR). The serological dataset comprised 170 serum samples: 132 from cattle, 37 from golden jackals and one from a cat. The ELISA assessed antibody reactivity to Leptospira interrogans serovar Hardjo (type Hardjoprajitno) and L. interrogans serovar Pomona. For serovar Hardjo, 37 samples were positive, 11 doubtful and 122 negative. All positive and doubtful results related to cattle; thus, among the 132 cattle serum samples, 28.0% (37/132) were positive and 8.3% (11/132) doubtful. All 37 samples from golden jackals and the one sample from the cat were negative. For serovar Pomona, all 170 serum samples were negative. In the overall serological dataset, the proportion of samples positive for serovar Hardjo was 21.8% (37/170) and the proportion of doubtful samples was 6.5%

(11/170). These figures relate to the specific sample formed within the project and should not be interpreted as an estimate of the prevalence of infection in the population.

Analysis of the distribution of serological results over time showed reactivity in both sampling periods. During the first phase in November 2025, 41 serum samples were tested (40 from cattle and one from a cat); 23 samples from cattle were positive and 8 were doubtful for serovar Hardjo, while the remaining samples were negative. In the 2026 sampling period, a further 14 positive and 3 doubtful cattle serum samples (for serovar Hardjo) were identified. Positive results in cattle were recorded in samples taken in the localities of Sulina, Letea and Chilia Veche, while all samples from golden jackals collected in 2026 as part of the wildlife study were negative. The detection of cattle with reactivity to serovar Hardjo in different sampling periods and in different localities indicates repeated serological evidence of exposure to infection in the cattle population studied. However, differences in sampling intensity, herd selection and geographical coverage do not allow a direct conclusion about temporal trends or changes in prevalence.

The molecular component consisted of 171 completed real-time PCR determinations, including samples from 112 cattle, 15 sheep, six dogs, 37 golden jackals and one rat. The matrices tested consisted of 124 EDTA blood samples, 46 blood samples and one organ sample. Leptospiral genetic material was not detected in any of the 171 determinations. The molecular dataset therefore does not provide direct evidence of detection of leptospiral DNA in the biological materials examined at the time of sampling, including the wildlife samples included in the project.

The interpretation of the Romanian results is consistent with the WOAH diagnostic framework, which distinguishes between detection of the immune response and direct detection of the agent. WOAH recognises serology as the most widely used

approach to the diagnosis of leptospirosis and defines the microscopic agglutination test (MAT) as the standard serological test, while ELISA may be useful for serological screening and for assessing serovar Hardjo infection in cattle. PCR provides sensitive direct detection of leptospiral nucleic acid, but its diagnostic performance depends strongly on the biological matrix, the stage of infection, sample handling and quality control procedures, and PCR does not identify the infecting serovar. In addition, negative direct detection in blood does not exclude chronic renal or genital carriage, since leptospires can persist in the kidneys, urine or genital tract after the period when leptospiraemia can be detected. Consequently, the picture observed for leptospirosis – namely repeated Hardjo ELISA reactivity in cattle together with uniformly negative real-time PCR results – is consistent with serological evidence of previous exposure or immune response without molecular evidence of active leptospiraemia in the matrices tested at the time of collection. Serological reactivity alone cannot definitively identify the infecting serovar, and vaccination history must also be taken into account, where possible. No serological evidence of exposure to Pomona was found in this dataset. The results of the microscopic agglutination test (MAT) will be taken into account once they are received; they will provide additional information on serogroup affiliation and deepen the epidemiological interpretation, whereas definitive determination of the serovar requires isolation and identification of the microorganism itself.

In the leptospirosis studies carried out in Ukraine in autumn and in the second period (spring) – using whole blood, urine, milk and blood serum samples by PCR and ELISA – neither the DNA of the agent nor antibodies to it were detected.

In the examination of water samples from selected water bodies, DNA of pathogenic leptospires (Leptospira) was not detected. However, in two water samples – from the "Quarry" water body (Vylkove) and the Stentsivsko-Zhebriianivski

floodplains (village of Prymorske) – DNA of microorganisms of the genus Leptospira was detected which most likely belong to non-pathogenic species.

Toxoplasmosis (Toxoplasma gondii)

In the "Serology" or "Molecular Biology and Genetic Analysis" datasets used for the study of the Ukrainian project territory, no tests for Toxoplasma gondii were recorded. As a consequence, it is not possible to present laboratory results specific to Romania in this subsection on the basis of these two datasets.

Molecular genetic studies of whole blood and faeces carried out in the Ukrainian part of the project during the first (autumn) period, as well as serological studies of blood sera (by PCR and ELISA), showed the absence of the DNA of the agent and of antibodies to it in the samples examined. Toxoplasma DNA was also not detected in water samples.

West Nile virus infection

The studies carried out in Romania combined serological screening by competitive enzyme-linked immunosorbent assay (ELISA) with direct molecular detection of West Nile virus (WNV) RNA by reverse transcription and real-time polymerase chain reaction (RT-qPCR). The serological dataset comprised 432 serum samples. In total, 168 samples tested positive, 8 were classified as doubtful and 256 as negative, corresponding to 38.9%, 1.9% and 59.3% respectively of all samples taken within the project. The distribution by animal species was uneven: among 316 cattle serum samples, 122 were positive, 7 doubtful and 187 negative; among 40 samples from chickens, 11 were positive, 1 doubtful and 28 negative; all 15 samples from sheep and all 12 samples from pigs were positive; among 37 samples from golden jackals, 7 were positive and 30 negative; among 5 samples from ducks, 1 was positive and 4 negative. All 6 samples from dogs and the single sample from a cat were negative.

These figures characterise only the animals sampled within the project and should not be interpreted as an estimate of the prevalence of infection in the population, since sampling intensity, species composition and geographical coverage were not statistically uniform. A clear difference was found between the two sampling periods within the project, although the composition of the populations studied also differed substantially. During the first phase in November 2025, 65 serum samples were tested (from 58 cattle, six dogs and one cat); all results were negative. In the 2026 sampling period, 367 serum samples obtained from cattle, chickens, jackals, sheep, pigs and ducks were tested; 168 samples tested positive, eight doubtful and 191 negative. Thus, in 2026 serological evidence of infection was recorded in representatives of several domestic and wild animal species. However, because the second period covered different animal species, locations and sampling intensity, the differences between the periods cannot be regarded as evidence of an increase in incidence or prevalence of infection over time.

The molecular studies included 140 real-time PCR (RT-PCR) tests: 81 samples from cattle, 37 from jackals, 15 from sheep, six from dogs and one from a cat. The material tested consisted of 125 EDTA blood samples and 15 blood samples (anticoagulant not specified). Sixteen molecular tests were carried out in the 2025 phase and 124 in the 2026 sampling period. West Nile virus (WNV) RNA was not detected in any of the 140 samples. The molecular data obtained therefore do not provide direct evidence of viraemia in the animals examined at the time of sampling. This result does not contradict the serological data, since the possibility of detecting the virus by molecular methods depends largely on the time of sampling and the type of biological material, whereas antibodies may persist in the body longer than the period during which viral RNA can be detected in peripheral blood.

Interpretation of these results is based on the diagnostic recommendations of the World Organisation for Animal Health (WOAH) on West Nile fever. WOAH defines West Nile virus as a mosquito-borne flavivirus maintained mainly in a "bird–mosquito–bird" transmission cycle, and also recognises real-time PCR (RT-PCR) as appropriate for direct detection of viral nucleic acid in suitable clinical material.

For serological surveillance, indirect and competitive ELISAs are useful screening tools, but WOAH stresses that these assays may cross-react with antibodies to related flaviviruses, including Usutu virus and other members of the Japanese encephalitis serogroup. Accordingly, positive ELISA reactions, where epidemiologically appropriate and technically feasible, should be confirmed by a more specific neutralisation method, such as plaque reduction neutralisation (PRN/PRNT) or virus neutralisation (VN). Thus, the Romanian results demonstrate a substantial WNV-reactive serological signal in the 2026 target sample set, especially among cattle and several other species sampled, but in themselves they do not establish active WNV infection or WNV-specific prevalence in the population.

The uniformly negative real-time PCR results indicate that WNV RNA was not detected in the biological materials examined during the two sampling periods of the project.

Results of analysis of environmental samples

In order to identify the possible source of pathogens and trace the routes of their spread, the project also examined samples taken from the environment in the project implementation area in Ukraine. These include water, soil, insect and feed samples; they are presented separately, in line with the specifics of the One Health approach.

According to the results of examination of biological material from the environment (Table 12), namely water samples from the water bodies studied, DNA of

pathogenic leptospires was not detected in the water. At the same time, in two water samples – from the "Quarry" water body (Vylkove) and the Stentsivsko-Zhebriianivski floodplains (village of Prymorske) – DNA of microorganisms of the genus Leptospira was detected which most likely belong to non-pathogenic species.

The joint GIS platform (https://danube-emerging.org/en) is a web tool for visualising and comparing the epidemiological situation regarding emerging infectious diseases in the border territories of Odesa Oblast (Ukraine) and Tulcea County (Romania). The platform was developed by the lead partner of the project – the Youth Public Organization "New European Generation" (YPO NEG) – within Activity 1.4 of the SMS-RO-UA project (ROUA00136). It is intended for coordinated presentation of data from Romania and Ukraine, which makes it possible to assess risks across the entire cross-border territory rather than separately for each country.

Table 12. Total number of environmental samples for laboratory testing in Ukraine

№	Infection	Species/samples	Method to be used for testing the material	Number of samples		
				Period I	Period II	Total
1	African swine fever	Feed samples from risk zones	PCR/real-time PCR (WOAH recommendation)	8	8	16
		Insects that are potential mechanical vectors (ticks, blackflies, etc.)	PCR/real-time PCR (in accordance with WOAH recommendations)	10	30	40
2	Avian influenza	Water	PCR/real-time PCR – influenza A, H5/H7	17	17	34
		Soil	PCR/real-time PCR – influenza A, H5/H7		15	15
3	Leptospirosis	From environmental objects – stagnant water body	PCR	25	29	54

№	Infection	Species/samples	Method to be used for testing the material	Number of samples		
				Period I	Period II	Total
4	Toxoplasmosis	Water in an open water body	PCR	25	29	54
Total samples tested				85	128	213

The joint GIS platform (danube-emerging.org) (Fig. 10) is an interactive web tool for visualizing, analyzing and comparing the epidemiological situation of priority infectious diseases in the border areas of Odesa Oblast, Ukraine, and Tulcea County, Romania.

The platform was developed by the Youth Public Organization “"New European Generation” (YPO NEG), the lead partner of the SMS-RO-UA project (ROUA00136), under Activity 1.4. It aims to present Ukrainian and Romanian data in a consistent way, strengthen cross-border monitoring and create a shared information environment for assessing epidemiological risks.

Platform features. The platform covers five priority diseases identified by the project: African swine fever, avian influenza, leptospirosis, West Nile fever and toxoplasmosis.

The main tool is an interactive map that lets users explore the geographic distribution of reported cases and analyze the epidemiological situation across the Ukrainian–Romanian border area.

A filter system allows users to select the disease, year or time period, region and country. Additional criteria include population at risk, host type, detection method, epizootic unit and data source type. This lets users tailor the display to specific research and analytical tasks.

Users can choose which indicators to show on the map: risk level, number of reported cases or population density. A legend explains the symbols and color scale. Outbreaks can be shown as points sized by the number of cases.

Additional settings let users choose a base map and manage thematic layers, such as urban areas, rivers and lakes, forests and water bodies. This makes it possible to analyze the spatial distribution of diseases in relation to environmental features and to explore potential links between animals, water, soil and humans in line with the One Health approach.

Data analytics and visualization. The map is complemented by an analytics section with charts and key indicators. It allows users to examine the distribution of reported cases by region, track trends over the years, analyze disease structure by region and host type, compare risk levels by region and disease, and assess the number of cases per 100,000 population.

Combining the interactive map with analytical tools gives a more complete picture of the data, helps identify territorial differences and makes it possible to track changes in the epidemiological situation over time.

Cross-border cooperation and practical value. The platform's key advantage is that it presents and compares Ukrainian and Romanian data consistently within a single information environment. This makes it possible to assess risks across the entire border area rather than within each country separately, and supports information exchange between stakeholders.

The platform can be used by project partners, government authorities, specialists and researchers to analyze the epidemiological situation, identify areas of concern and support the planning of prevention and monitoring measures.

To make the information accessible, the interface is available in three languages: English, Ukrainian and Romanian.

Sustainability. The joint GIS platform is a practical outcome of the SMS-RO-UA project, aimed at developing cross-border epidemiological monitoring and strengthening cooperation between Ukraine and Romania. After the project ends, YPO NEG will maintain the platform and support its continued use as a tool for working with geospatial data and analyzing epidemiological risks.

The platform thus provides a shared information base for cross-border surveillance of priority infectious diseases, supports consistent data analysis, and strengthens cooperation between Ukraine and Romania in assessing and managing risks to human, animal and environmental health.

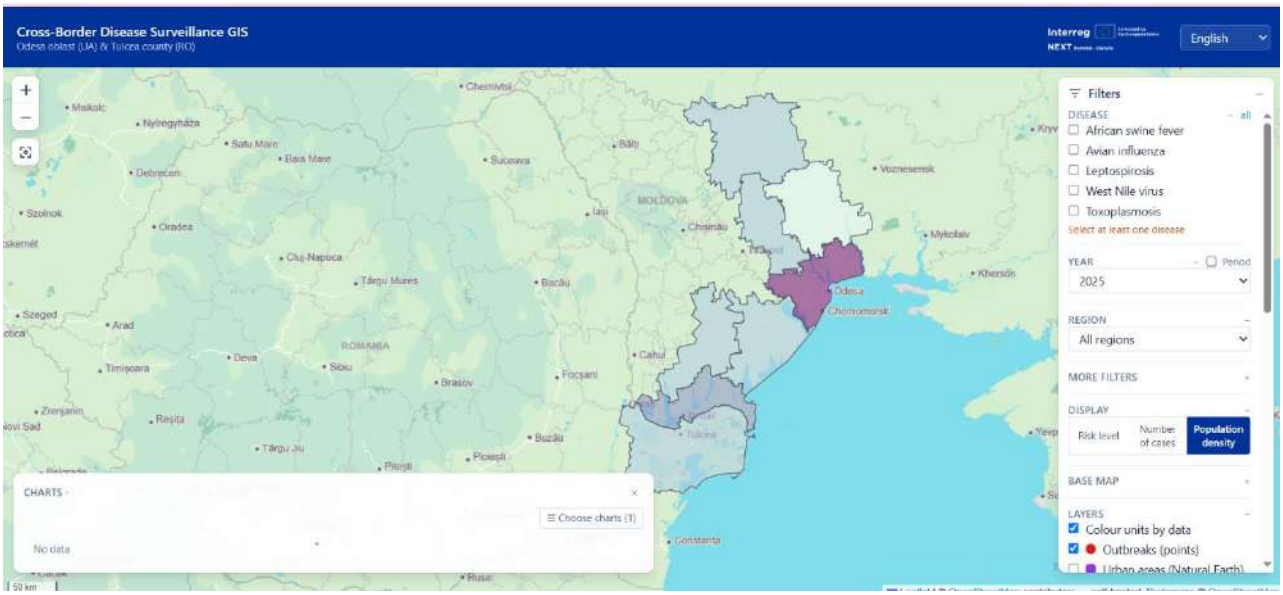


Figure 10. Interface of the joint GIS platform Cross-Border Disease Surveillance GIS (date viewed: 27.10.2026).

5. CROSS-BORDER EPIDEMIOLOGICAL INTERPRETATION, METHODOLOGICAL VALIDITY AND HARMONISATION

This section is intended for bodies coordinating surveillance in the cross-border territory and shows how the results of both sides can be interpreted together. The results were obtained under the single sampling and analysis scheme defined by the Joint ToR, which ensures harmonisation of the relevant information.

Method of assessment and interpretation of results

The assessment of emerging infectious diseases in this Guide is based on joint interpretation of laboratory results, epidemiological context and environmental data. The method applies to all five diseases of the project and equally to the Ukrainian and Romanian parts of the cross-border territory. It consists of six sequential steps.

Step 1. Description of the sample. For each result, the following are recorded: the animal species or the type of environmental sample and biological material from animals, the place and period of sampling, and the sampling method. Sampling in the project was purposive and risk-oriented, so the figures obtained relate to the samples examined and are not an estimate of prevalence in the population.

Step 2. Assessment of the suitability of the method. It is taken into account what exactly the test detects: pathogen nucleic acid (PCR, real-time PCR) or antibodies (ELISA, MAT, HI, PRN/VN), and whether it has been validated for the animal species studied and the purpose of the study.

Step 3. Interpretation of the result in accordance with Table 13.

Table 13.

Interpretation of results

Result	Interpretation	Further actions

PCR positive	Direct detection of the agent in the material tested at the time of sampling	Confirmation by a specific method (for influenza A: testing for H5/H7), notification of the competent authority, additional sampling, epizootiological investigation
Serology positive, PCR negative	Exposure to the agent or immune response; active infection (viraemia) not proven	Confirmation by a specific method (MAT, HI, PRN/VN), paired sera after about two weeks, consideration of vaccination history, enhanced surveillance
Serology doubtful	Insufficient for a conclusion	Repeat testing, paired sera
Serology and PCR negative	No signs of the agent in the samples examined; carriage or infection outside the detection time window is not excluded	Continuation of risk-based monitoring, expansion of the list of matrices (urine, kidneys, genital tract)
DNA of a microorganism genus detected without confirmation of pathogenicity (for example, Leptospira in water)	Presence of the microorganism in the environment; pathogenicity not established	Species identification, repeat sampling at the same site
No data (dataset absent)	Conclusion not possible	Planning of comparable sampling in future

Step 4. Taking context into account. The following are added to the result: vaccination history, season and migration periods of wild birds, mosquito and tick activity, contact between domestic and wild animals, presence of water bodies and wetlands, and characteristics of holdings (in particular backyard holdings).

Step 5. Determination of risk level. On the basis of the combined results (Step 3) and context (Step 4), the territory or epizootic unit is assigned a risk level: low, medium or high. The assessment is an expert one, combines animal and environmental results and is displayed on the joint GIS platform.

Step 6. Formulation of conclusions and measures. The conclusion is presented within what has been proven: for example, "serological reactivity without molecular

evidence of active circulation" is not treated as confirmation of an outbreak, and a negative result is not treated as proof of the absence of disease. Measures (Section 6) and the need for confirmatory testing are determined.

Limitations. The method does not replace official diagnostics and epizootiological investigation, does not provide an estimate of prevalence, and its reliability depends on the completeness of data on both sides of the border.

The joint results demonstrate that the circulation of pathogens in the cross-border territory is heterogeneous and specific to particular diseases, which reflects the complex interaction between domestic animals, wildlife, vectors, aquatic ecosystems and seasonal environmental conditions.

Regarding African swine fever, neither the Romanian nor the Ukrainian molecular studies detected ASFV DNA in the biological or environmental materials examined during the project periods. However, in Romania three domestic pigs showed ASFV serological reactivity without concurrent PCR detection. In Ukraine, ASF virus DNA was not detected in animal blood, feed or collected blood-sucking arthropods. Taken together, the joint results do not provide direct molecular evidence of active circulation of ASF virus in the project samples, while the Romanian serological results justify continued targeted surveillance of susceptible pig populations.

Regarding avian influenza, the cross-border comparison revealed an important ecological difference. In the Romanian molecular dataset, influenza A RNA was not detected, whereas the Ukrainian studies demonstrated influenza A RNA in samples associated with migrating wild birds and the aquatic environment. Further testing of the Ukrainian positive samples did not detect H5 or H7, and the results were interpreted as circulation of low-pathogenic influenza A viruses. During the spring period, influenza A RNA was again detected in samples associated with wild birds, including ruff

(Philomachus pugnax) and dunlin (Calidris alpina), whereas influenza A RNA was not detected in the corresponding soil samples.

This result is particularly relevant for the ecology of the Danube Delta, since WOAH identifies waterfowl as an important natural reservoir of influenza A viruses and notes that many viruses circulating in these populations have low pathogenicity. The detections in Ukraine, together with the absence of H5/H7 identification, do not indicate circulation of H5/H7 viruses in the respective samples, but they demonstrate that the wild bird and wetland interface functions as an important surveillance target. The results thus confirm the need for continued synchronised surveillance on both sides of the border during the main migration periods.

Regarding leptospirosis, the two national datasets reveal additional epidemiological information. Romania showed repeated Hardjo-related serological reactivity in cattle without PCR detection of leptospiral DNA. In Ukraine, pathogenic leptospiral DNA and specific antibodies were not detected in the animals tested, whereas two environmental water samples contained DNA belonging to the genus Leptospira. These results illustrate the value of combining animal and environmental surveillance, and demonstrate why serological, molecular and environmental data should be interpreted together rather than separately.

Regarding toxoplasmosis, Ukrainian testing did not detect Toxoplasma gondii DNA or specific antibodies in the animal samples examined, and environmental water samples were also negative. There were no comparable results in the Romanian datasets. Consequently, the available data do not permit a balanced two-sided assessment of T. gondii circulation, and future project-related surveillance would benefit from comparable sampling and testing on both sides of the border.

Regarding West Nile virus (WNV), the results obtained in Romania provide strong serological evidence of exposure to the virus in several animal species, although WNV RNA was not detected in the samples examined. This picture is epidemiologically consistent with the description of WNV infection given by the World Organisation for Animal Health (WOAH): virus circulation is maintained mainly through the "mosquito–bird" cycle, viraemia in vertebrates may be short-lived, and serological evidence of infection may persist longer than the period during which viral RNA can be detected. Because serological cross-reactivity with other flaviviruses is possible, these results should be regarded only as evidence of reactivity to WNV until confirmed by a more specific neutralisation method.

Overall, the results obtained demonstrate that no single animal species, sample type or diagnostic method is sufficient to fully characterise the epidemiological risks in the Lower Danube ecosystem. The project thus confirms the appropriateness of the One Health approach, which entails integrating data on farm and wild animals, vectors and environmental objects. This makes it possible to identify epidemiological signals that would remain unnoticed if these components were studied separately.

Another important result was the creation of a unified technical basis for future epidemiological surveillance. In the Ukrainian component alone, about 1,160 biological and environmental samples were collected over the two periods, while in Romania systematic sampling made it possible to build large molecular and serological datasets on domestic and wild animals. More importantly, this activity proved that institutions of both countries are capable of carrying out coordinated sampling, exchanging technical information and interpreting laboratory results within a common epidemiological framework.

At the same time, the results point to certain methodological limitations that should be taken into account. Sampling was purposive (risk-oriented) rather than random (probability-based); the host animal species, the number of samples and the types of materials examined differed depending on the country and the sampling period; in Ukraine environmental sampling was carried out on a larger scale; in addition, data on toxoplasmosis in Romania were not available for this analysis, nor were data on West Nile virus in Ukraine. Therefore, the prevalence figures given within the project relate only to the samples examined and should not be interpreted as an estimate of disease prevalence in the entire animal population or in the entire border region.

Despite these limitations, an important goal was achieved: a joint epidemiological basis for future cross-border monitoring was created. The results of the study identify specific signals that require continuous attention: ASF serological reactivity in Romanian pigs, influenza A circulation associated with wild birds and aquatic habitats in Ukraine, Hardjo-related serological exposure in Romanian cattle, environmental detection of leptospires in Ukraine, and widespread West Nile virus-reactive serology in the Romanian multispecies dataset. The Lower Danube and the Danube Delta should be approached as a single, epidemiologically interconnected ecological system, even if patterns of pathogen detection may differ locally and over time between the Romanian and Ukrainian territories. Movements of wild animals, migratory bird routes, wetlands, vector populations and hydrological connections function independently of administrative borders and require coordinated surveillance.

The activity has delivered not only disease-specific laboratory results but also a functional model for cross-border early detection and epidemiological cooperation. Maintaining harmonised protocols, synchronising future sampling campaigns, strengthening confirmatory testing, integrating animal and environmental data, and

ensuring rapid exchange of epidemiological information between partners will turn the infrastructure and cooperation established under Activity A1.4 into a sustainable mechanism for surveillance of emerging and re-emerging diseases in the Lower Danube region.

In this respect, one of the most important long-term results of the activity is the transition from isolated national surveillance to a joint Romanian–Ukrainian surveillance system based on coordinated field studies, comparable laboratory methods, joint interpretation and the One Health concept. This system is an appropriate foundation for prevention, early detection and coordinated management of future cross-border epidemiological threats.

6. PROTOCOLS FOR JOINT PREVENTIVE AND ERADICATION MEASURES

The measures in this section are intended for veterinarians, owners of animals and holdings, state supervisory authorities, local self-government bodies, healthcare institutions and all stakeholders. They do not replace the regulatory acts in force in Ukraine and Romania, but supplement them with practical recommendations based on the One Health approach.

Each infectious disease has specific biological and ecological aspects, affecting geographical regions differently. Therefore, each disease requires specific control and prevention measures. However, there are effective "universal strategies" for the prevention and mitigation of infectious diseases in general that give very convincing results, especially when such strategies take into account aspects relating to humans, the environment, animals and pathogens in an integrated approach from the point of view of the One Health concept.

Preventive and eradication measures for African swine fever.

Farm biosecurity. Biosecurity is the most effective means of preventing the introduction of ASF virus into holdings. The main measures are:

- keeping pigs indoors, without contact with wild boar;
- controlling access of unauthorised persons to the holding's territory;
- equipping sanitary checkpoints and disinfection barriers;
- regular disinfection of premises, vehicles and equipment;
- use of special clothing and footwear for staff;
- prohibiting the use of untreated food waste for feeding pigs;
- controlling the origin of feed and water;
- limiting contact between different production groups of animals.

Special attention should be paid to backyard-type holdings, where the risk of virus introduction is often higher owing to a lower level of biosecurity.

Epizootic monitoring and early detection. Timely detection of the disease is a key condition for preventing large-scale spread of infection. The monitoring system must include:

- continuous clinical surveillance of the pig population;
- laboratory testing of suspected cases of disease;
- examination of pathological material from dead pigs;
- passive and active monitoring of wild boar populations;
- prompt notification of the competent authorities of cases of increased mortality or suspected ASF.

Measures in case of suspected ASF. If disease is suspected, it is necessary to:

- immediately notify the territorial body of the State Service of Ukraine on Food Safety and Consumer Protection (Derzhprodspozhyvsluzhba) or another responsible specialised body;
- stop the movement of pigs, feed and pig products;
- isolate suspected animals;
- arrange the collection of pathological material for laboratory testing;
- ensure that all contact animals and possible factors of infection transmission are recorded;
- carry out a preliminary epizootiological investigation.

Measures in case of confirmed ASF. After laboratory confirmation of the disease, quarantine restrictions are introduced in accordance with the legislation of Ukraine in force and WOAH recommendations. The main measures include:

- declaring quarantine;

- delimiting the epizootic focus, protection and surveillance zones;
- prohibiting the movement of pigs and pig products;
- destruction (depopulation) of sick, suspected and contact animals;
- safe disposal of carcasses, feed and waste;
- cleaning and disinfection of premises, vehicles and equipment;
- carrying out an epizootiological investigation to establish the source of infection and possible routes of its spread.

Control of the wild boar population. Wild boar play an important role in maintaining virus circulation under natural conditions. To reduce the risk of ASF spread, the following are carried out:

- monitoring of population numbers;
- examination of dead animals found;
- controlled regulation of wild boar numbers;
- searching for and safe disposal of carcasses of dead animals;
- prohibiting supplementary feeding of wild boar in affected regions.

Disinfection and decontamination. The ASF agent is relatively resistant in the external environment, so effective disinfection is important for eradicating foci of disease. Preparations based on sodium hydroxide, sodium hypochlorite, formaldehyde, glutaraldehyde and other agents recommended by veterinary authorities are used for decontamination.

Before disinfection, thorough mechanical cleaning of surfaces from organic contamination is mandatory.

Informing the public and training staff. The effectiveness of prevention depends largely on the level of awareness of animal owners, hunters, pig farm workers and veterinary specialists. Information and awareness-raising work should be aimed at:

- raising awareness of the clinical signs of ASF;
- promoting the principles of biosecurity;
- explaining the rules for handling carcasses of dead animals;
- preventing illegal movement of pigs and pig products;
- timely reporting of suspected cases of disease.

The importance of epizootiological investigation. One of the most important elements of ASF control is establishing the source of introduction of the virus and the factors of its spread. A detailed epizootiological investigation makes it possible to identify the mechanisms of transmission of infection, assess the risks for neighbouring holdings and determine the additional control measures needed to eradicate the outbreak and prevent recurrence of the disease.

The integrated application of biosecurity, monitoring, quarantine, disinfection and epizootiological investigation measures is the basis of effective prevention and control of African swine fever both in Ukraine and in other countries of the world.

Consequences of failure to observe African swine fever control measures.

Failure to observe the requirements for prevention, biosecurity and control of African swine fever (ASF) (Fig. 9) creates the preconditions for rapid spread of the virus among domestic pigs and wild boar, the emergence of new epizootic foci and significant economic losses.

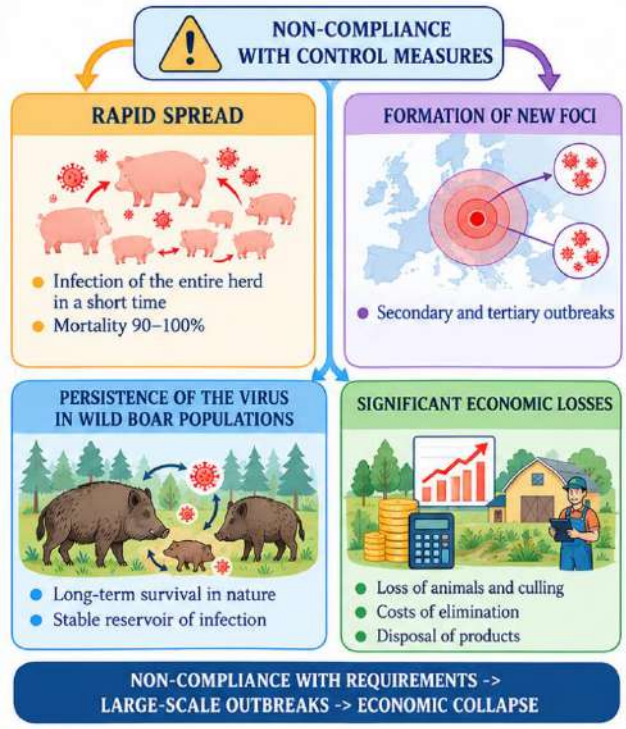


Figure 9. Consequences of failure to observe ASF control measures

Given the high contagiousness of the disease, the absence of effective treatment and the considerable persistence of the agent in the environment, even minor violations of veterinary and sanitary requirements can lead to large-scale outbreaks of infection.

Spread of the disease among the pig population. The most obvious consequence of failure to observe control measures is the introduction and rapid spread of the virus in a holding. Under conditions of intensive pig farming, the disease can affect the entire population within a short time. For highly virulent ASF strains, the case fatality rate can reach 90–100%, which leads to mass death of animals or the need to destroy them as part of anti-epizootic measures.

Formation of new epizootic foci. Violation of the rules for moving animals, transporting pig products, failure to observe quarantine restrictions and inadequate disinfection can contribute to the spread of the virus beyond the primary focus. In such

cases secondary and tertiary outbreaks occur, which considerably complicates eradication of the disease and increases the duration of quarantine measures.

Maintaining virus circulation among wild boar. Late detection and disposal of carcasses of dead animals, absence of monitoring of wild boar populations and inadequate control of hunting activity contribute to long-term persistence of the virus in the natural environment. As a result, a persistent natural reservoir of infection forms, which can remain for years a source of new cases of disease among domestic pigs.

Significant economic losses. ASF outbreaks are accompanied by substantial economic losses for animal owners and the state. The main losses include:

- death and forced destruction of the pig population;
- costs of eradicating foci of disease;
- disinfection measures;
- disposal of carcasses and contaminated products;
- compensation payments to animal owners;
- reduced productivity of the pig sector;
- job losses in the livestock sector.

Trade restrictions. Detection of ASF outbreaks leads to the introduction of domestic and international restrictions on the movement of live pigs, meat and pig products. Such restrictions negatively affect a country's export potential, the competitiveness of producers and the development of the agricultural sector as a whole.

Threat to food security. In regions with developed pig farming, substantial losses of livestock can lead to reduced pork production, shortages of products on the market and higher prices. This may negatively affect the food security of the population and the availability of protein foods.

Complication of epizootiological investigation. Late reporting of disease, concealment of animal deaths or violation of the rules for recording pig movements significantly complicate establishing the source of infection and its routes of spread. As a result, the risk of further virus circulation and recurrent outbreaks of the disease increases.

Social consequences. ASF outbreaks may have substantial social consequences for the population, especially in rural areas, where pig farming is an important source of income. Mass destruction of animals, restrictions on economic activity and economic losses negatively affect the well-being of farmers and owners of private smallholdings.

Thus, failure to observe African swine fever control measures poses a serious threat to animal health, the economic stability of holdings, food security and the epizootic well-being of the country. Strict compliance with biosecurity requirements, timely detection of the disease and prompt implementation of anti-epizootic measures are necessary conditions for effective control of ASF and for preventing its further spread (Fig. 10).

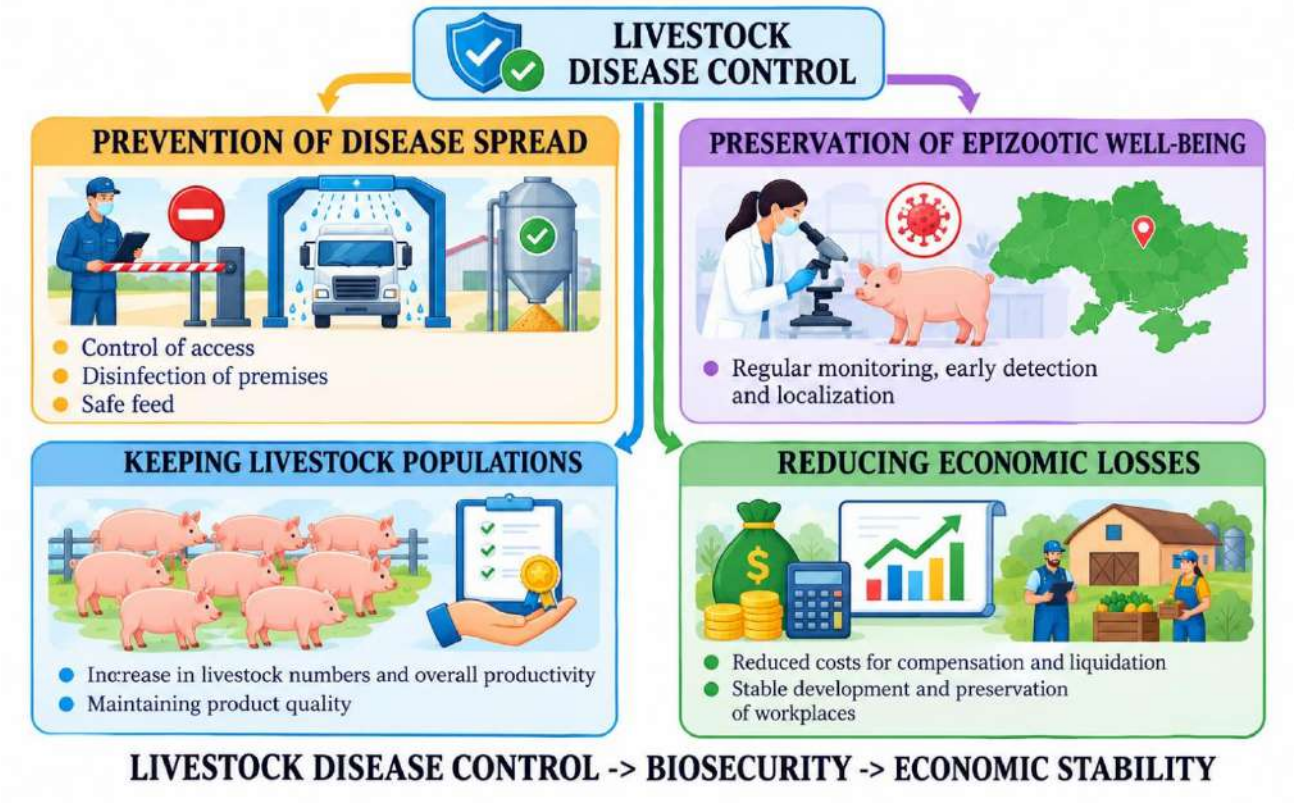


Figure 10. Benefits of observing ASF control measures

Preventive and eradication measures for avian influenza

Effective control of avian influenza is based on the integrated implementation of biosecurity measures, epizootic monitoring and veterinary and sanitary control that comply with the recommendations of the Food and Agriculture Organization of the United Nations (FAO), the World Organisation for Animal Health (WOAH) and the regulatory legal acts of Ukraine.

One of the key areas of prevention is ensuring bio-isolation of the flock by keeping domestic poultry in enclosed premises or specially equipped enclosures that prevent contact with wild migratory and synanthropic birds. This measure is of

particular importance in regions located near water bodies and on seasonal bird migration routes, which are a natural reservoir of influenza type A viruses.

An important component of the prevention system is the introduction of strict veterinary biosecurity measures at poultry enterprises. For this purpose, sanitary checkpoints are organised for staff, mandatory changes of work clothing and footwear are ensured, hands and vehicles are disinfected, and access of unauthorised persons to production premises is restricted. In addition, a ban on keeping domestic poultry by workers of industrial holdings is recommended, which reduces the risk of introducing the agent into production.

Particular attention is paid to control of feed and water. Feed must be stored in sealed storage facilities inaccessible to wild birds and rodents, and only good-quality drinking water or water that has been disinfected beforehand should be used for watering poultry. When grain feed is used, heat treatment is advisable, which helps to inactivate possible pathogenic agents.

Epizootiological monitoring occupies an important place in the system of anti-epizootic measures. It involves regular serological, virological and molecular genetic testing using the polymerase chain reaction method for timely detection of virus circulation in poultry populations. Particular attention is paid to holdings located in high-risk zones, in particular along wild bird migration routes.

To prevent the spread of infection through poultry products, veterinary and sanitary control is carried out at all stages of production and sale of products. Slaughter of poultry must be carried out exclusively at specialised enterprises under the supervision of veterinary specialists, and sale of meat and eggs without the relevant accompanying documents is not permitted.

An important component of prevention is raising the awareness of the public and industry workers about the signs of the disease, routes of transmission of the agent and methods of personal protection. When in contact with sick poultry or poultry suspected of being ill, personal protective equipment must be used, in particular gloves, protective clothing and respirators.

If an outbreak of highly pathogenic avian influenza is confirmed, quarantine restrictions are introduced to localise and eradicate the focus of infection. Depending on the epizootic situation, protection and surveillance zones are established, the movement of poultry, products and transport is restricted, and depopulation of the susceptible population is carried out, followed by safe disposal of biological waste. After eradication of the focus, thorough cleaning and repeated disinfection of premises, equipment and the territory of the holding are carried out.

Consequences of failure to observe control measures. Ignoring the established biosecurity and veterinary and sanitary control requirements may lead to rapid spread of infection among the susceptible population and to large-scale epizootic outbreaks. Highly pathogenic strains of the virus are characterised by extremely high contagiousness and are able to cause mass mortality of poultry within a short period of time.

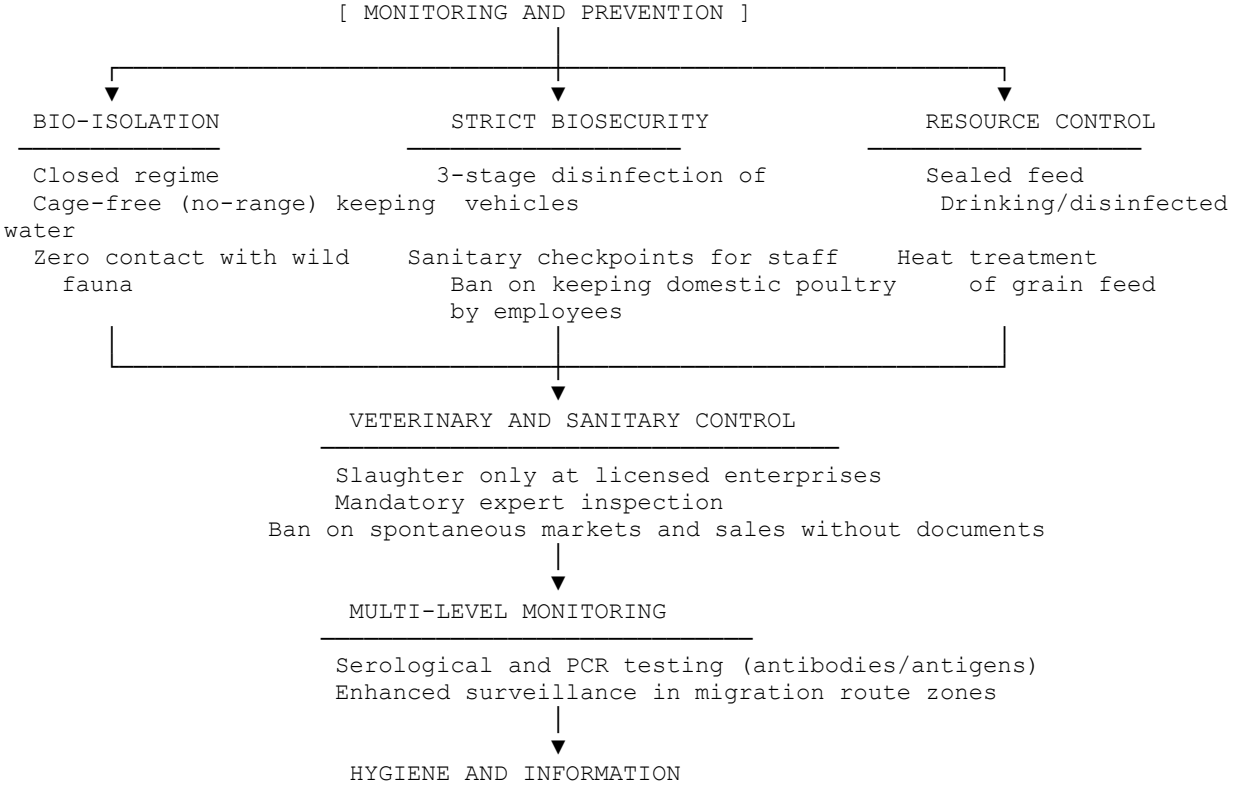
The spread of the disease results in significant economic losses associated with destruction of the flock, cessation of production, the costs of eradicating outbreaks and the introduction of international trade restrictions. In addition, circulation of the virus in poultry populations increases the risk of human infection and creates potential preconditions for the emergence of new pandemic variants of the agent through mutational processes or genetic reassortment.

The spread of avian influenza can also negatively affect the food security of the state through reduced production of poultry meat and eggs, rising prices for products of animal origin and disruption of the stability of the agricultural sector. Additional consequences may be socio-economic problems associated with reduced profitability of holdings, job losses and risks of environmental contamination.

Benefits of observing biosecurity measures. Comprehensive implementation of preventive and anti-epizootic measures ensures effective containment of virus circulation and prevents its penetration into industrial poultry holdings. Implementation of biosecurity principles helps to preserve the economic stability of enterprises, minimise the costs of eradicating outbreaks and maintain the export potential of the sector.

AVIAN INFLUENZA CONTROL SYSTEM

(According to the standards of the State Service of Ukraine on Food Safety and Consumer Protection and FAO)



Briefing of the public and farmers (clinical symptoms)
PPE (masks, gloves) during care and feeding

▼

RADICAL MEASURES (IN CASE OF OUTBREAK)

Immediate quarantine at the epicentre
Buffer zones with a radius of 3–10 km
Bloodless slaughter (depopulation)
Safe incineration / deep burial

In addition to the economic benefits, observing control measures is important for protecting public health. Reducing the level of virus circulation in the environment lowers the risk of interspecies transmission of the agent and the occurrence of cases of disease in humans. Prevention of avian influenza is thus an integral part of the One Health concept, which provides for an integrated approach to protecting the health of animals, humans and the environment.

Preventive and eradication measures for leptospirosis.

Prevention:

- swim only in designated places; leptospirosis is most often contracted in closed stagnant water bodies, especially shallow ones;
- do not swim in water bodies and rivers if there are wounds, scratches or inflamed areas on the skin and mucous membranes;
- do not walk barefoot in marshy areas;
- do not drink unboiled water from open water bodies or unverified sources;
- wash fruit and vegetables thoroughly and scald them with boiling water;
- do not eat vegetables and fruit (especially if they have been stored in pantries and cellars) showing signs of damage by animals;
- protect sources of drinking water and food products from access by rodents;
- do not eat the meat of animals ill with leptospirosis;
- observe the rules of personal hygiene when caring for livestock – wash hands regularly, use protective equipment (work clothing, rubber boots, gloves);
- wash hands with soap before eating or preparing food;
- carry out regular rodent control in residential premises and places where livestock are kept (snap traps, cage traps, mousetraps, poisons);

Eradication measures

1. When a diagnosis of leptospirosis is established, local self-government bodies and local state executive authorities, on the submission of the chief veterinarian of the district, city or city district, take a decision to declare the holding (or an independent part of it) or the settlement unfavourable (affected), introduce quarantine restrictions on animals and approve a plan of measures for eradicating the disease. At the same time, the chief veterinarian of the district, city or city district notifies the relevant authorities of the oblast and district.

2. Veterinary specialists carry out a clinical examination of the animals, measuring body temperature in animals suspected of the disease. Sick and suspected animals are isolated and treated with hyperimmune serum in accordance with the instructions for use and with antibiotics (streptomycin sulphate – intramuscularly at 25,000 units per kg of animal body weight at intervals of 12 hours for 4–5 days; for pigs, in addition, ditetracycline intramuscularly at 30,000 units/kg of body weight twice at an interval of 48–72 hours; kanamycin is administered intramuscularly at a dose of 15,000 units/kg of body weight three times a day at intervals of 8 hours for 4–5 days).

3. Under the quarantine restrictions, the following are prohibited:

- removal (export) of animals from the affected holding and use of sick animals for breeding, and sale of animals to the public;

- regrouping of animals without the permission of the chief veterinarian of the holding;

- removal of animals not vaccinated against leptospirosis;

- grazing and watering of unvaccinated animals where animals ill with leptospirosis have grazed;

- use of meat and slaughter products from sick and suspected animals for food and feed purposes without appropriate decontamination;

- use of milk from sick animals for farm purposes without boiling. Milk from clinically healthy animals whose blood sera give a positive MAT without a rise in titre is used without restrictions.

4. Breeding males infected with leptospires (positive MAT or leptospires in urine) are isolated, semen collection is stopped, and they are treated with leptospirocidal preparations. After 10–12 days, the effectiveness of treatment is monitored by urine microscopy. If leptospires are detected in the urine, the course of treatment and the check of its effectiveness are repeated. Stocks of semen obtained from leptospirosis-carrier breeding males in the 6 months before the diagnosis was established are destroyed.

5. Feed to which animals ill with leptospirosis had access is fed to livestock vaccinated against leptospirosis.

6. Breeding males at artificial insemination enterprises (stations, centres) located in zones of natural foci of leptospirosis are vaccinated against leptospirosis.

7. Repeat testing of blood serum by MAT and urine microscopy of all breeding males at a previously affected enterprise (station) is carried out after three months and, if negative results are obtained, every 6 months thereafter.

8. Slaughter of sick animals is carried out at a sanitary slaughterhouse, and in its absence – in the slaughter shop of a meat-processing plant at the end of the shift after removal of all carcasses and offal from the hall. The premises and equipment of the shop are disinfected after the slaughter of the animals.

9. In working-dog breeding kennels, clinically ill animals and dogs suspected of the disease are isolated and treated with hyperimmune serum and streptomycin sulphate. Streptomycin sulphate is administered to sick dogs once a day for 4–5 days at 25,000 units/kg of body weight.

Clinically healthy dogs of all age groups are vaccinated against leptospirosis. The sale of dogs from a kennel affected by leptospirosis is prohibited.

10. After the isolation of sick animals and animals suspected of leptospirosis, mechanical cleaning and disinfection are carried out in the premises and enclosures with a 1–2 per cent formalin solution, a 2 per cent caustic soda solution, or a clarified bleaching-powder solution containing 3 per cent active chlorine.

A holding (farm, herd) is considered free of leptospirosis after veterinary measures have been carried out and when there are no sick animals or leptospirosis carriers. Before quarantine restrictions are lifted, 1–2 months after the measures, no fewer than 50 blood serum samples from young animals are tested by MAT (there must be no positive reactions), and no fewer than 100 urine samples per 1,000 adult animals, but from no fewer than 100 animals, among which there must be no leptospirosis carriers. The microagglutination reaction in adult animals may remain positive. Repeat examination for leptospirosis in previously affected holdings is carried out 6 months after quarantine restrictions are lifted.

Preventive and eradication measures for West Nile fever.

Preventive measures. The best way to avoid infection with West Nile virus is to prevent mosquito bites.

Mosquitoes are most often most active at dawn (in the first rays of the sun) and at dusk (just before darkness falls).

Exposed areas of skin should be covered at this time.

When outdoors, it is recommended to wear:

- long trousers, loose long-sleeved shirts, socks and a hat;
- a mosquito net over the hat to protect the head;
- light-coloured clothing (mosquitoes are attracted to dark colours).

When outdoors, insect repellents (sprays and lotions) containing repellents or icaridin should be used.

How can the number of mosquito breeding sites near the home be reduced?

Mosquitoes lay eggs in standing water (water that does not move or flow). Therefore, standing water around the house should be eliminated, in particular by:

- draining or drying water from old tyres, rain barrels, children's toys, flower pots and paddling pools;
- cleaning gutters regularly to prevent blockages in which water accumulates;
- turning fishing boats and tackle on their sides to drain the water;
- changing the water in outdoor pet bowls and bird drinkers at least twice a week;
- installing screens on windows and doors so that mosquitoes cannot enter the home.

*How should dead animals and wild birds be handled***?**

A dead animal or bird must never be touched with bare hands: a dead bird or animal should be touched only while wearing rubber gloves. Dead birds suspected of being infected with West Nile virus must be reported. When hunting wild animals or skinning them, it should be remembered that West Nile virus can be transmitted through blood-to-blood contact.

To protect against diseases to which animals may be susceptible:

- always wear gloves when handling dead animals;
- make sure that all open wounds on the hands are covered;
- wash hands while still wearing gloves, and then wash bare hands every time after contact with a dead animal.

There is no evidence that people can become infected with West Nile virus by eating fully cooked birds or animals.

Other preventive measures worth noting include:

- raising the awareness of healthcare workers about West Nile virus so that it is taken into account in the differential diagnosis of travellers returning from areas affected by the epidemic;
- outreach to travellers heading to affected areas on ways to reduce the risk;
- ensuring that laboratory capacity for diagnosis is available in the country.

Eradication measures. When cases of serous meningitis, meningoencephalitis, and milder forms of viral infections with general intoxication syndrome without pronounced catarrhal-exudative signs are detected with suspected WNV, an emergency notification is submitted to the Public Health Department.

From all patients with the above diagnoses, as well as other manifestations of disease suspected to be WNV, paired blood sera, taken during the acute period of the disease and then after 10–14 days, are sent to virological laboratories. Collection and transport of material from sick people and persons suspected of infection for testing must be carried out in accordance with the sampling rules.

The process of epidemiological investigation of a West Nile fever focus includes interviewing the patient, collecting the epidemiological history, laboratory tests, studying documentation, observation of the focus, and completing the epidemiological investigation card of the focus, taking into account additional information obtained during the investigation of the focus:

- whether the person previously had the diseases listed above, and in which year;
- whether the person was under dispensary observation (whether previously tested for WNV), since which year;

- residential sector (private, public), presence of mosquitoes, presence of a water body and tree plantations near the house;
- presence of contact with birds (wild, domestic) and rodents;
- presumed source of infection;
- whether the patient travelled to a dacha or for recreation in natural settings in the last 14 days.

A brief description of the locality and possible contacts with mosquitoes is provided, including the conditions of infection (circumstances and situations in which the patient was within the infection period, indicating dates and places of stay over the last 2 weeks):

- if the presumed infection is associated with mosquito bites, indicate under what conditions and where the infection occurred (additionally, indicate the address according to the patient);

- indicate the mosquito species and their numbers per premises according to entomological control data at the patient's place of residence (in residential and non-residential premises);

- indicate the presence of water bodies, their characteristics and approximate area in the focus within a radius of 3 km, and whether cases of WNV disease territorially linked to this water body have been registered previously;

Among the measures for eradicating the epidemic focus, when a WNV patient is identified, the focus is immediately examined together with the adjacent territory for the presence of vector mosquitoes. The areas to be treated are determined by specialists.

Mosquito control in premises and breeding sites (cellars, water bodies) is carried out by institutions holding a licence to carry out rodent and pest control work, regardless of form of ownership, immediately when a focus of disease is detected. In addition,

during the period of mass mosquito breeding (April–August), continuous anti-mosquito treatment of potentially dangerous water bodies and cellars of buildings is carried out according to entomological indicators and in accordance with a comprehensive plan.

If the focus is located in a small settlement, every yard with residential and non-residential premises, farm buildings and possible resting places of virus-carrying mosquitoes is treated. If there are small water bodies of no economic importance near the settlement that are populated by mosquito larvae, they are treated at the same time or filled in.

In large settlements, the cellars of multi-storey buildings and water bodies of no economic importance are treated.

When cases of WNV are detected where the disease has been registered previously, the scope of measures depends on the intensity of transmission of the infection and must be decided in each individual case.

When 5 or more cases of WNV are registered in the territory and the situation worsens compared with baseline, the epidemiological situation is assessed as an emergency and the entire set of anti-epidemic measures provided for in such cases is carried out.

Active case finding is carried out, in particular in somatic non-infectious hospitals, among patients who have sought medical care with complaints of high fever, headaches and other manifestations of viral infections, including meningitis and encephalitis, with clinical material sent for testing for antibodies to WNV.

Measures are taken to reduce the populations of synanthropic birds – food sources for vector mosquitoes – in settlements by reducing the availability of food of anthropogenic origin (elimination of spontaneous rubbish dumps), destroying nesting

sites, and preventing birds from using buildings and structures for breeding (blocking access).

The choice and justification of priority directions of the set of sanitary-hygienic and anti-epidemic measures for the prevention of WNV are made depending on the results of epidemiological surveillance in the territory and are accompanied by the development and implementation of a comprehensive plan of preventive and anti-epidemic measures.

The content of the comprehensive plan, approved at national and oblast levels, must include preventive and anti-epidemic measures indicating the scope of work, deadlines and responsible executors. The priority areas of such a plan are:

1) Treatment and preventive:

• identification of WNV patients (passive and active methods);

• collection of material and laboratory testing for specific antigens and antibodies to WNV;

• symptomatic treatment of patients;

• epidemiological investigation of the focus;

• verification that there are no other patients in the focus;

• dispensary observation of patients.

2) Anti-mosquito and anti-tick measures (including entomological surveillance):

• counting the numbers of mosquitoes and ticks;

• determining the infection rate of mosquitoes and ticks, taking into account seasonal fluctuations in infection rate;

• monitoring of mosquito and tick breeding sites and the dynamics of their areas;

• preventing the formation of water bodies – mosquito breeding sites – and reducing the areas of existing ones;

• removal of reed thickets along the banks of water bodies and especially along roadside ditches;

• treatment of water bodies (chemical, biological and engineering methods);

• prevention of mosquito and tick bites;

• treatment of cellar premises with insecticides with evaluation of effectiveness;

• acaricidal treatment of cattle and small ruminants.

3) Reduction of populations of synanthropic birds – food sources for vector mosquitoes – in settlements by destroying their feeding grounds (elimination of unorganised rubbish dumps), destroying nesting sites, etc.

4) Health education work through:

• mass media;

• leaflets;

• information sheets, etc.

The presence of WNV-vector mosquitoes creates a potential danger of cases of West Nile fever and the possible emergence of new natural foci in the territory of the country.

The set of measures for combating mosquitoes includes:

- preventive and ongoing sanitary supervision, which are the main links in preventing the formation of mosquito breeding sites in settlements and are aimed at preventing flooding of the cellars of residential and public buildings, as well as at improving the condition of territories;
- sanitary-engineering and extermination measures.

In the summer-autumn period, anti-mosquito and anti-tick treatment of water bodies and the territory of children's health and recreation facilities, recreation zones

and tourist routes must be carried out before each arrival of visitors, in the necessary volumes.

When treating open water bodies and cellar premises, insecticides authorised for use in the country must be used.

For treating cellar premises and building entrances against adult mosquitoes, various agents authorised in the country are used – pyrethroids and organophosphorus insecticides. All insecticides must be applied in accordance with the instructions supplied with them and only for their intended purpose. Treatment is carried out in strict compliance with the precautions for working with toxic chemicals.

In addition, to destroy mosquito larvae in surface and closed water bodies not used for fisheries, preparations from various classes of chemicals (organophosphorus compounds, carbamic acid derivatives, pyrethroids, neonicotinoids) can be used, as well as agents based on entomopathogenic bacteria and growth regulators; microbiological preparations made on the basis of the δ-endotoxin of the entomopathogenic bacterium Bacillus thuringiensis H-14 var. israelensis, which are selective insecticides with an intestinal mode of action. When they enter the digestive tract of mosquito larvae, the preparations cause toxicosis and subsequent death; insect growth regulators.

The preparations used may be in the form of blocks, tablets, emulsion concentrates, wettable powders, microemulsions, aqueous solutions and water-soluble granules and briquettes.

Only microbiological preparations may be used to treat water bodies of fisheries importance.

For individual and collective protection against mosquitoes, repellents authorised for use in the country must be used. For adults, products of the highest and first category in the form of sprays, lotions, and aerosol and propellant-free packaging can be used.

Counts of winged mosquitoes and larvae are carried out on the day of, or immediately before, treatment, and then 5–7 days after treatment.

Tick control is comprehensive in nature and must cover the entire life cycle of the arthropod, in order to reduce its numbers in territories affected by the virus.

Farm management measures for tick control:

- changing pastures for each herd after the end of the period of mass tick parasitism (June);
- surface treatment of land to destroy engorged females and their egg clutches;
- destruction of crow nesting sites in shelterbelts near pastures, followed by chemical treatment of the area;
- barrier dusting of shelterbelts;
- improvement of the territory of holdings.

Chemical tick control measures are carried out using acaricides and consist of:

- dipping animals;
- wiping with acaricidal liquids;
- spraying.

Physical precautionary measures:

- do not crush ticks with the hands;
- if a tick has attached, apply a fatty substance to it and after 10–15 minutes remove it, carefully turning it in one direction with soft tweezers or with hands in rubber gloves;
- in pastures and shelterbelts, close-fitting clothing must be worn. Trousers must be tucked into socks and boots;

As acaricidal agents, toxic chemicals authorised for use in the territory of the country must be used, in accordance with the instructions for their use and observing precautions.

Authorised personal protective products against ticks must be used in accordance with the instructions for use. Acaricidal and acaricidal-repellent products must be applied only to clothing; repellents may be applied to the skin.

Preventive and eradication measures for toxoplasmosis.

*Preventive measures***.-** Wear gloves when gardening or when in contact with soil or sand that may be contaminated with cat faeces containing Toxoplasma. After gardening or contact with soil or sand, wash hands with soap and water;

- Change the litter in the cat litter tray daily. The Toxoplasma parasite becomes infectious only 1–5 days after a cat has shed it in its faeces. Daily cleaning of the tray removes the faeces before they become infectious. Cover outdoor sandpits;

- Regularly test the pet cat for toxoplasmosis at a veterinary clinic;

- Prevent cats from hunting mice and rats, as they are the main sources of infection;

- Feed cats only canned or dry commercial food and well-cooked table food. Do not feed cats raw or undercooked meat;

- Do not eat raw or undercooked oysters, mussels or clams (they may be infected with Toxoplasma that has entered the sea water);

- Oocysts may be spread by coprophagous insects, which are capable of contaminating vegetables and other food. Flies can also carry oocysts onto food and drinking water. Disinfecting hands and carefully protecting food from flies are important preventive measures against infection;

- Wash fruit and vegetables under running water;

- Do not drink unpasteurised milk and avoid drinking untreated water;

- People with immunodeficiency (HIV, chemotherapy) should avoid any sources of Toxoplasma. Dishes made of raw meat, unpasteurised milk and contact with stray cats are contraindicated for them;

- It is advisable to walk dogs wearing a muzzle to prevent oral contact with soil where cat faeces may be present;

Toxoplasmosis eradication measures. If there are grounds to suspect toxoplasmosis, pending the results of diagnostic tests the following must be done on the holding:

- isolate animals suspected of the disease;

- submit stillborn offspring and aborted foetuses to the laboratory immediately for testing;

- maintain veterinary observation of all animals suspected of toxoplasmosis, and carry out serological testing of blood serum once a month for 6 calendar months;

When a final diagnosis of toxoplasmosis is established on the holding:

- animals with a confirmed diagnosis are slaughtered. Their meat is used after boiling (+66 °C inside the product) or deep freezing (-18 °C inside the product). Hides obtained after slaughter of sick animals or animals suspected of the disease are released without restrictions after preservation;

- premises where sick animals were kept must be treated with disinfecting solutions;

- when working with acutely ill animals, pathological material and carcasses suspected of toxoplasmosis, careful observance of safety rules is necessary (work clothing, gauze masks, rubber gloves and footwear);

- on a holding affected by toxoplasmosis, after slaughter of sick animals and isolation of animals suspected of the disease, removal, movement and sale of other animals and use of all types of livestock products from them are permitted without restrictions;

- quarantine of animals newly arriving at the affected holding is carried out for 30 days; where indicated, serological testing for toxoplasmosis is performed;

- animals suspected of the disease are isolated. All animals suspected of the disease are kept under veterinary observation, and serological testing of blood serum for toxoplasmosis is carried out once a month for 6 consecutive months. If during this period no positive reactions are detected among these animals, or a decrease in antibody titre to 1:5–1:10 is noted, such animals are transferred to the healthy group;

- clinically healthy animals on holdings affected by toxoplasmosis are tested serologically once a month until the holding is declared recovered. If clinical signs appear and positive serological reactions are obtained in 2 repeats and with rising antibody titres, the animals are transferred to the group suspected of the disease, and if they have deaths, stillbirths, abortions or other pregnancy pathologies, the material is sent to the laboratory;

- milk from sick animals may be used only after pasteurisation or boiling;

- in a location affected by toxoplasmosis, after slaughter of sick animals and isolation of animals suspected of the disease, the sale and removal of other animals and the use of all types of livestock products from them are permitted without restrictions;

- a business entity in which toxoplasmosis in animals was established is considered recovered if for one year no sick animals were identified and no pronounced clinical signs of the disease were noted, including cases of pregnancy pathology.

Thus, given the diversity of measures for the control and prevention of infectious diseases, it is necessary to involve specialists from various fields. For example, biologists can take part in developing new tools for controlling disease vectors, as well as in identifying host genetic factors associated with infectious disease phenotypes; veterinarians can contribute to monitoring pathogenic agents, such as protozoa, viruses and bacteria, in wild and domestic animals; social scientists can work on identifying the populations most vulnerable to infection; geneticists and molecular biologists can improve pathogen detection methods.

SUMMARY AND CONCLUSIONS

The Practical Guide “Assessment of Emerging Diseases” was prepared under Activity 1.4 of the SMS-RO-UA project (ROUA00136), which is co-funded by the EU through the Interreg VI-A NEXT Romania–Ukraine Programme 2021–2027. It brings together the results of the joint sampling and laboratory work done by Odesa State Agrarian University (Ukraine) and the Veterinary Sanitary and Food Safety Directorate Tulcea (Romania). It offers one practical way to find and assess new infectious diseases in the chain linking animals, water, soil and people along the Lower Danube.

The Guide’s sampling protocols, laboratory methods, advice on reading results and joint prevention measures are intended for use in a shared surveillance system, supported by the joint GIS platform that YPO NEG maintains. They also form the basis for the joint actions on animal health and welfare that the project is developing.

The joint research was carried out by the Veterinary Sanitary and Food Safety Directorate Tulcea and Odesa State Agrarian University. The Romanian side collected biological samples from domestic and wild animals and from species living where the two groups meet, using both blood tests and molecular tests. Over two sampling periods the Ukrainian side collected and tested about 1,160 samples of biological material from wild and domestic animals, together with environmental samples from wetlands, farmland and settlements, under BSL-2 biosafety conditions.

The studies found the following. For African swine fever in Romania, none of the 65 real-time PCR tests (pigs, golden jackals, ticks) detected the virus’s genetic material, although three pigs had antibodies. In Ukraine, no virus DNA was found in animal blood, feed or blood-sucking arthropods, while influenza A virus RNA was found in pig blood. For bird flu in Romania, all 444 PCR tests for the M gene were negative. Five blood samples reacted in the ELISA test (chickens, domestic ducks and

one golden jackal), and four poultry samples were negative in the specific HI test for H5N3, H5N8 and H7N1. In Ukraine, influenza A RNA was found in wild bird droppings and in water, while the H5 and H7 types were not found, which points to low-pathogenic viruses. For leptospirosis in Romania, antibodies to *Leptospira interrogans* serovar Hardjo were found repeatedly in cattle, in both periods and in several places, including Sulina, Letea and Chilia Veche. The bacterium's DNA was not found in any of the 171 PCR tests. In Ukraine, no DNA of disease-causing leptospires and no antibodies were found in animals, while *Leptospira* DNA, most likely from non-disease-causing species, was found in the water of several water bodies in Odesa Oblast. For toxoplasmosis, the Romanian datasets were not envisaged to be collected. In Ukraine, no *T. gondii* DNA or antibodies were found in animals, and no DNA in water. For West Nile fever in Romania, 168 of 432 blood samples were positive and 8 doubtful, yet the virus's RNA was not found in any of the 140 PCR tests, in Ukraine respective researches were not envisaged. All these figures describe only the samples taken. Because sampling was targeted, they do not show how common each disease is in the whole population.

For practical use, the Guide recommends that sampling follow the unified protocols in Section 2 and the Joint Terms of Reference. Samples from animals, birds, the environment and insect carriers are to be combined, so that results can be compared on both sides of the border. PCR detects the germ itself, whereas blood tests show that an animal has met it, so the Guide advises using the two together. Antibodies without a positive PCR do not prove an active infection, and a negative PCR does not rule out past contact or carriage. According to the Guide, positive and doubtful results should therefore be confirmed with more specific tests (MAT; the HI test for H5/H7; the neutralisation test for West Nile virus), following the scheme in Section 5. The Guide

sees the greatest need for surveillance attention in wetlands and among wild waterbirds during migration, in cattle for leptospirosis (with extra sampling of urine and of urinary and reproductive organs from animals slaughtered out of necessity or from carcasses), on backyard pig farms for African swine fever, and in mosquito-active areas for West Nile virus. For toxoplasmosis, it points out that both countries need to sample in a comparable way.

When a warning sign appears, the Guide directs readers to Section 6. This section calls for observing biosecurity rules, informing the competent authorities quickly, restricting the movement of animals and animal products, cleaning and disinfecting, controlling disease carriers, and training staff and the public. The Guide does not replace the laws in Ukraine and Romania; it adds practical advice to them. The data of both countries are planned to be entered on the joint GIS platform (https://danube-emerging.org/en), which YPO NEG maintains. The platform supports early detection of outbreaks and coordinated action by the partners.

The Guide lays a common foundation for moving from separate national checks to coordinated cross-border surveillance, based on synchronised sampling campaigns, comparable laboratory methods and joint reading of results. The cooperation model built on the joint terms of reference, synchronised sampling campaigns and joint interpretation can serve as a template for monitoring cross-border diseases in the Lower Danube region and can extend to neighbouring countries that share the same ecological corridor. The Guide's recommendations matter for health and welfare in Odesa Oblast, Tulcea County and the whole cross-border region, so monitoring to detect the agents of the relevant cross-border diseases should continue.

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