JOINT SCIENTIFIC REPORT

ON THE RESEARCH OF EMERGING DISEASES IN THE ROMANIA–UKRAINE CROSS-BORDER AREA

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REPORT IDENTIFICATION

The report is elaborated within the project: “Development of Screening and Monitoring Systems for Emerging Diseases and Prevention of Cross-Border Epidemics in the Current Context” (SMS RO-UA – ROUA00136) funded by European Union through Interreg VI-A NEXT Romania–Ukraine 2021–2027 Programme.

Project partners:

- Project lead partner: Youth Public Organization “New European Generation”, Ukraine
- Project partner 2: Sanitary Veterinary and Food Safety Directorate of Tulcea, Romania
- Project partner P3: Odesa State Agrarian University, Ukraine

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ABBREVIATIONS AND DEFINITIONS

ASF – African Swine Fever
AI – Avian Influenza
WNV – West Nile Virus
PCR – Polymerase Chain Reaction
RT-PCR – Real-Time Polymerase Chain Reaction
ELISA – Enzyme-Linked Immunosorbent Assay
MAT – Microscopic Agglutination Test
TOR – Terms of Reference
One Health – integrated approach linking animal health, human health, and environmental health
PP2 – Project Partner 2
PP3 – Project Partner 3
SOP – Standard Operating Procedure
Ct – Cycle threshold
IAC – Internal Amplification Control

EXECUTIVE SUMMARY

This joint report summarizes the activities carried out and the results obtained within the cross-border research conducted through project ROUA00136, whose main objective was to develop a common framework for the surveillance, sampling, analysis, and interpretation of data related to emerging and re-emerging diseases in the Lower Danube region, at the border between Romania and Ukraine. The initiative was designed as a practical and scientific contribution to strengthening the capacity for prevention, early detection, and coordinated response to epidemiological risks with cross-border impact.

The study was implemented in the Romania–Ukraine cross-border area, through activities carried out by the partners from Tulcea County and Odesa Region, in a complex ecological and epidemiological context characterized by the mobility of wildlife, seasonal bird migration, the presence of extensive wetland ecosystems, the existence of biological vectors, and the continuous interaction between domestic animals, wild animals, the environment, and human communities. Within this framework, five diseases of major importance for animal health and public health were investigated: African swine fever, avian influenza, leptospirosis, toxoplasmosis, and West Nile virus infection.

INTRODUCTION

Context and relevance

The Lower Danube region, and in particular the Romania–Ukraine cross-border area, represents a space of major epidemiological importance at regional and European level, due to the convergence of geographic, ecological, climatic, and anthropogenic factors that favor the maintenance, circulation, and, under certain conditions, the amplification of emerging and re-emerging pathogens. This area includes extensive wetland ecosystems, highly diverse natural and semi-natural habitats, significant inland water surfaces, flood-prone areas, agricultural land, and dispersed rural settlements, where contact between humans, domestic animals, wildlife, and the environment is frequent and complex.

The Danube Delta and adjacent areas constitute one of the most important ecological zones in Europe for migratory birds, being located on major migration routes that connect different continents and ecosystems. From an epidemiological perspective, this characteristic gives the region a significant role in the introduction, maintenance, and redistribution of pathogens, especially those associated with wild avifauna and biological vectors. At the same time, the presence of wild mammals, including species with high mobility and strong ecological adaptability, as well as the existence of domestic herds raised under extensive or semi-extensive conditions, increases the epidemiological interface potential between the sylvatic, domestic, and human compartments.

The cross-border character of this region enhances its relevance for the surveillance of emerging diseases. The administrative border between Romania and Ukraine does not represent a barrier to the movement of wild birds, certain mammal species, vectors, or the dispersal of environmental factors involved in pathogen transmission. At the same time, the proximity between human communities, animal holdings, fishing areas, agricultural land, and aquatic ecosystems creates favorable conditions for repeated exposure and for the emergence of complex epidemiological chains. In this context, health risks cannot be effectively assessed solely at local or national scale, but require a coordinated, integrated, and comparable approach at the level of the entire cross-border area.

In particular, diseases such as African swine fever, avian influenza, leptospirosis, toxoplasmosis, and West Nile virus infection are highly relevant for this region, both because of their impact on animal health and public health and because of the economic, social, and institutional.

In particular, diseases such as African swine fever, avian influenza, leptospirosis, toxoplasmosis, and West Nile virus infection are highly relevant for this region, both because of their impact on animal health and public health and because of the economic, social, and institutional consequences they may generate. Some of these diseases are closely linked to the movement of wildlife and vectors, while others are influenced by hydrological conditions, seasonality, animal population density, or local farming and husbandry practices. Taken together, they

reflect the region's specific vulnerability to multiple and interconnected epidemiological threats.

From this perspective, the development of integrated systems for monitoring, sampling, analysis, and interpretation of data represents not only a technical necessity, but also an essential condition for substantiating effective prevention and early response measures. Epidemiological surveillance in this area must take into account environmental dynamics, the mobility of animal species, biological seasonality, and the relationships between natural and anthropogenic risk factors. At the same time, its effectiveness depends on the existence of harmonized procedures, an operational exchange of information, and sustained cooperation between the competent institutions on both sides of the border.

In this context, the application of the One Health approach is fully justified. The epidemiological complexity of the Lower Danube region demonstrates that animal health, human health, and environmental health cannot be analyzed separately, but must be understood as interdependent dimensions of the same system. The diseases investigated within the project reflect precisely this interdependence: they involve animal reservoirs, favorable environmental factors, possibilities of human exposure, and the need for genuine collaboration between veterinary medicine, public health, research, and environmental protection. Therefore, the relevance of the study lies not only in generating specific results for each disease, but also in its contribution to building an integrated perspective capable of supporting modern epidemiological surveillance adapted to the cross-border specificity of the region.

Objective of the joint study

The overall objective of the joint study was to develop and apply a harmonized framework for the collection, analysis, and interpretation of data on emerging and re-emerging diseases in the Romania–Ukraine cross-border area, with a view to strengthening epidemiological surveillance, information exchange, and institutional cooperation among the partners involved.

This objective was formulated on the basis of the need for a unified and comparable approach in a region characterized by common epidemiological risks, similar ecological conditions, and a clear interdependence between animal health, human health, and environmental factors. In the specific context of the Lower Danube, the mere parallel implementation of surveillance activities on the two sides of the border would not have been sufficient to provide a coherent epidemiological picture. For this reason, the joint study sought to build a common methodological and operational basis capable of enabling the generation of comparable data and their interpretation within a unified regional logic.

Specifically, the overall objective aimed at harmonizing the main components of the epidemiological research process, namely:

- planning of sample collection;
- selection of relevant species and sample types;

- application of compatible and comparable laboratory methods;
- use of common criteria for the interpretation of results;
- data exchange and correlation of findings obtained by the partners.

Through this approach, the study aimed not only to collect data on the presence or absence of pathogens, but also to generate a clearer picture of the distribution of epidemiological risks in the cross-border area, of the contributing factors, and of any similarities or differences between the two investigated territories. Thus, the joint objective included both a technical component, related to the standardization of field and laboratory activities, and an analytical component oriented toward the integrated interpretation of results in relation to the ecological, epidemiological, and institutional context of the region.

At the same time, the study aimed to strengthen the partners' institutional capacity to cooperate effectively in the field of surveillance of emerging and re-emerging diseases. In this respect, the objective was not limited to obtaining specific results during the implementation period, but also pursued the development of a practical cooperation mechanism based on exchange of experience, methodological harmonization, and professional dialogue. Such cooperation is essential in an area where pathogen circulation is influenced by cross-border factors and where an effective response depends on the speed of communication and on the comparability of the information generated by each side.

Another important element of the overall objective was the creation of a scientific basis capable of supporting future surveillance, prevention, and intervention measures. The data generated within the joint study were conceived not only as project results, but also as useful instruments for risk assessment, for guiding future sampling campaigns, and for substantiating potential recommendations regarding the control of the investigated diseases. In this sense, the study contributes to the transition from a fragmented approach to monitoring to an integrated approach oriented toward anticipation and prevention.

Overall, the general objective of the joint study reflected the need for a coordinated approach based on One Health principles, in which epidemiological research, field surveillance, laboratory diagnosis, and institutional cooperation function as complementary elements of a regional system for monitoring emerging and re-emerging diseases. Through this vision, the study sought to provide not only analytical results, but also a functional model of cross-border cooperation between Romania and Ukraine in the fields of animal health and public health.

Study tasks / objectives

1. Development of a common methodological framework for sampling and analysis activities.
2. Implementation of sample collection campaigns in the cross-border area.
3. Testing of samples through harmonized molecular and serological methods.
4. Comparative evaluation of the results obtained by the partners.
5. Identification of the main epidemiological risks and contributing factors.

6. Formulation of conclusions and recommendations for future monitoring and cooperation.

The list of target diseases for surveillance and justification for their selection

The target diseases for our testing involved:

- African swine fever;
- avian influenza;
- animal leptospirosis;
- animal toxoplasmosis;
- West Nile virus.

The selection of these diseases was based on their regional epidemiological relevance, their impact on animal health and public health, and the recommendations regarding the integrated approach to zoonotic and emerging diseases highlighted in European One Health reports, including the EFSA One Health Report 2023.

Legal and programmatic framework

The activities presented in this report were implemented within project ROUA00136, funded through the Interreg VI-A NEXT Romania–Ukraine 2021–2027 Programme, under Activity 1.4 – Joint research of emerging diseases in cross-border area: sampling and analysis. From a programmatic perspective, this framework provided the formal and operational basis for carrying out joint research activities, data exchange, institutional cooperation, and methodological harmonization among the partners involved in the project.

The Interreg VI-A NEXT Romania–Ukraine 2021–2027 Programme has as its general objective the strengthening of cross-border cooperation between the two states by supporting joint interventions oriented toward sustainable development, institutional resilience, and the addressing of common challenges in areas of strategic interest. In this context, project ROUA00136 fits within the logic of the programme because it addresses an issue with a pronounced cross-border character, namely the monitoring and prevention of emerging and re-emerging diseases, in a region where epidemiological risks are not limited by administrative borders and require a coordinated response.

From a legal and administrative perspective, the implementation of the activities was carried out in accordance with:

- the project approval documents;
- the financing contract and the funding conditions applicable within the programme;
- the specific provisions of Activity 1.4;
- the methodological and administrative documents developed within the partnership;
- the eligibility, reporting, visibility, and implementation rules established by the programme.

A key role in the technical substantiation of the activities was played by the common TOR (Terms of Reference), drafted and agreed by the partners in order to ensure a common understanding of the purpose of the research, the working stages, the methodologies applied, and the expected results. The common TOR represented the reference document for the organization of sampling, testing, data exchange, and comparative interpretation of results, contributing to the harmonization of approaches and to avoiding methodological discrepancies between the two sides.

At the same time, the legal and programmatic framework of the study was also shaped by the need to comply with the rules and procedures applicable to the veterinary and laboratory fields at national and institutional level. Thus, the activities of sample collection, handling, transport, and analysis were carried out in accordance with the regulations and internal procedures applicable to each partner, without departing from the approved objectives and conditions of the project. From this perspective, the programmatic framework of the project functioned as a mechanism for integrating the administrative requirements of the funding with the technical and operational demands of epidemiological surveillance.

The importance of this legal and programmatic framework also lies in the fact that it enabled the conduct of joint research in a sensitive field with implications for animal health, public health, and institutional cooperation. By explicitly including joint research, sampling, and analysis activities in the architecture of the project, the programme provided the necessary basis for the development of a cross-border cooperation model oriented toward concrete results, methodological comparability, and practical utility.

The activities described in this report were implemented within a clearly defined legal and programmatic framework, which ensured the legitimacy, coherence, and eligibility of the joint research undertaking. Compliance with the approved project documents, the common TOR, and the programme rules constituted an essential condition for carrying out the activities in a coordinated, transparent manner and in line with the objectives assumed by the partners from Romania and Ukraine.

Within this legal and programmatic framework, the specific contribution of Partner 3 — Odesa State Agrarian University — was grounded in the institution's established scientific and educational profile in veterinary medicine. OSAU is a leading higher education institution in the south of Ukraine, combining innovative educational activities at all degrees of higher education, including doctoral studies, with fundamental and applied scientific research. Its Faculty of Veterinary Medicine has, for 85 years, remained the only faculty of its kind in southern Ukraine with the scientific and pedagogical potential required to train specialists in the fields directly relevant to the project. Departments of the faculty provide a strong scientific basis for the study and monitoring of emerging diseases, while systematic monitoring research on the spread of endemic diseases is carried out on the basis of the university's multidisciplinary Laboratory of Veterinary Medicine.

This existing institutional capacity was directly mobilised for the implementation and overall coordination of Activity 1.4 on the Ukrainian side of the border area. Testing of biological and environmental samples was carried out in accordance with the joint Terms of Reference agreed by the partnership, applying PCR/real-time PCR and ELISA methods in line with WOAH recommendations, and complying with BSL-2 biosafety requirements throughout. This ensured that the results generated on the Ukrainian side were methodologically comparable with those obtained by the Romanian partner and could be integrated into a single cross-border analytical framework.

OSAU's long-standing cooperation with the State Service of Ukraine on Food Safety and Consumer Protection and other state veterinary structures, including those operating at the state border, as well as with private veterinary clinics and agricultural production complexes in the region, further reinforced the practical relevance of the university's contribution. These institutional relationships provided both a network of stakeholders for the research and a channel through which the project's findings can continue to inform veterinary practice and animal-health monitoring in the region after the project's completion.

GENERAL CHARACTERISTICS OF THE STUDY REGION

Geographical description

The study area covers the cross-border sector located between south-eastern Romania and south-western Ukraine, including Tulcea County and Izmail District, two territories in direct contact with the Lower Danube basin and functionally integrated into the ecological and hydrographic system of the Danube Delta. From a geographical perspective, this area represents one of the most complex and dynamic border regions in Eastern Europe, both through its physical-geographical structure and through the interdependence between natural components and human activities.

The region belongs to the terminal sector of the Danube, where the river branches out and feeds an extensive system of channels, canals, lakes, marshes, levees, and flood-prone areas, generating a territorial mosaic with very high ecological diversity. On the Romanian side, Tulcea County includes both continental areas and extensive deltaic and lagoon surfaces, while on the Ukrainian side, Izmail District comprises Danube-side territories, agricultural areas, and settlements located near associated aquatic and wetland systems. Overall, the study region is characterized by strong hydrographic fragmentation and by an alternation between natural habitats and anthropized areas.

A defining element of this area is the presence of the Danube Delta, an ecosystem of international importance recognized for its biodiversity and its role as a major biological corridor. From a geomorphological point of view, the analysed space includes fluvial and maritime levees, low-lying areas with slow drainage, periodically flooded land, as well as sectors where agricultural and livestock activities coexist with natural or semi-natural zones. This structure generates strong spatial heterogeneity, with direct implications for the distribution of epidemiological risk factors, the mobility of animal species, and the accessibility of sampling points.

The hydrography of the region is of central importance for defining the study area. The presence of permanent and temporary water bodies, natural and artificial canals, lakes, and areas with excess moisture creates favourable conditions both for the maintenance of valuable habitats and for the persistence of certain pathogens or vectors involved in their transmission. At the same time, the hydrographic network influences the organization of field activities, the duration of travel, the conditions for sample transport, and access to certain localities or areas of epidemiological interest.

Within Tulcea County, the study area includes localities and areas representative of the deltaic and border-specific setting, such as Ceatalchioi, Pardina, Periprava, and Cardon, characterized by proximity to the Danube branches, variable accessibility, and direct contact with natural habitats of epidemiological relevance. In Izmail District, points of interest such as Prymorske, Dunaiske, Stara Nekrasivka, Izmail, and Vylkove reflect a combination of rural, peri-urban, and

riverside territories relevant for the surveillance of emerging diseases in the cross-border context.

The selection of these five points, rather than a single location, reflects both the administrative infrastructure available to Partner 3 and the epidemiological logic of the study.

Administratively, all five settlements fall within the catchment area served by the Izmail District State Hospital of Veterinary Medicine and its subordinate Kiliia District Hospital, which acted as the primary referral network for biological material collected from domestic and wild animals. This existing infrastructure made it possible to organise systematic, repeated sampling across the district within the project's timeframe.

Geographically, the five settlements were chosen to mirror, on the Ukrainian bank, the diversity of sites selected by the Romanian partner in Tulcea County (Ceatalchioi, Pardina, Periprava, Cardon). Prymorske and Dunaiske lie close to the Danube branches and freshwater lakes such as Lake Kryve, used as stopover sites by migratory waterfowl and therefore prioritised for avian influenza surveillance. Stara Nekrasivka and Izmail represent areas with higher concentrations of domestic pig and poultry holdings, relevant to African swine fever and avian influenza surveillance in the domestic compartment. Vylkove, at the mouth of the Danube delta, provided access to the Kar'yer reservoir and the Stentsivsko-Zhebriianivski floodplains, included in the environmental sampling for leptospirosis. Water samples were additionally collected from a wider network of water bodies across these settlements, including Lake Lebyazhe, Buzunchuk Bay, and Lake Pereboinoe, so that the environmental component covered the full range of aquatic habitats characteristic of the district, not a single water body.

From a human-geographical perspective, the region is characterized by the presence of isolated rural settlements, communities dependent on local natural resources, fishing, agriculture, and animal husbandry activities, as well as economic and social ties that extend beyond the administrative border. These particularities increase the relevance of the region for epidemiological studies because they generate multiple forms of interaction between humans, domestic animals, wildlife, and the environment.

Another essential aspect is its character as an ecologically open border area, in which the movement of migratory birds, the mobility of certain wild mammals, and the continuity of natural habitats mean that the administrative boundaries between the two states do not coincide with real epidemiological boundaries. For this reason, the study area must be understood as a territorially unified system from an ecological and functional perspective, even if monitoring activities are institutionally organized within the national territories of each partner.

Therefore, the geographical description of the study region highlights a space characterized by physical-geographical complexity, high ecological connectivity, habitat variability, and intense interaction between the natural environment and human activities. These elements fully justify

the need for a common and harmonized approach to epidemiological surveillance in the Romania–Ukraine cross-border area and explain the scientific and operational relevance of the study carried out within the project.

Environmental and ecological characteristics

The region is characterized by:

- extensive areas of permanent and temporary water bodies;
- wetlands favourable to migratory birds and wildlife;
- variable density of animal holdings;
- the presence of habitats favourable to vectors and natural reservoirs of infection;
- strong interaction between the environment, animals, and humans.

Epizootic context

In recent years, the Romania–Ukraine cross-border area has been exposed to significant epizootic and epidemiological risks generated by the regional, continental, and, in certain situations, seasonal circulation of pathogens of major importance for animal health and public health. The geographical and ecological characteristics of the region, together with wildlife mobility, bird migration, the presence of biological vectors, the movement of domestic animals, and the permanent interaction between natural ecosystems and human activities, have created favourable conditions for the introduction, maintenance, and spread of emerging and re-emerging diseases.

From an epizootic perspective, the region has remained particularly vulnerable to African swine fever and avian influenza, two diseases with high dissemination potential, major economic impact, and important consequences for surveillance, control, and trade activities. In the case of African swine fever, its relevance for the region is determined both by the persistence of the disease at European and regional level and by the risk associated with virus circulation in domestic pig and wild boar populations. In an area where the administrative border does not constitute a real barrier for wildlife and where there is a complex network of agricultural land, wetlands, and natural habitats, the risk of introduction and maintenance of infection has continued to be considered significant. For this reason, African swine fever has remained one of the priority diseases for epidemiological surveillance in the study area.

Avian influenza has also represented a constant threat, especially due to the strategic position of the Danube Delta on the migration routes of wild birds. The region functions as a stopover, feeding, and transit area for a large number of avian species, which gives it an important epidemiological role in the circulation of avian influenza viruses. The interaction between wild avifauna and domestic birds, especially in traditional households or areas with low biosecurity, increases the exposure potential and justifies maintaining active and epidemiologically oriented surveillance. In this context, the studied region must be considered a sensitive area for the early detection of signals of viral circulation.

With regard to leptospirosis, its importance in the regional context is closely linked to the hydrological and ecological specificity of the area. The extensive presence of water bodies, wetlands, flood-prone lands, and environments favourable to the survival of leptospiras, combined with the existence of animal reservoirs and the frequent contact between animals and the environment, makes this disease constantly relevant for the region. From an epidemiological perspective, leptospirosis is particularly important because it reflects the interface between the contaminated environment, wildlife, domestic animals, and the human population. The presence of human cases recorded in various localities of Tulcea County further supports the need to investigate the role of animal species and the environment in the circulation of the pathogen and justifies the inclusion of this disease in the joint research.

West Nile virus infection has also continued to be relevant in the region, especially through its association with aquatic ecosystems, the presence of hematophagous vectors, and the role of birds as natural reservoirs. The climatic and hydrological conditions specific to the Danube Delta and adjacent areas favor the emergence and maintenance of microhabitats suitable for the development of mosquito populations, which creates the conditions for virus transmission. In this context, monitoring West Nile virus is doubly important: on the one hand for animal health, and on the other hand for public health, given its zoonotic nature and its potential impact on the human population.

Although toxoplasmosis does not have the same explosive epizootic profile as African swine fever or avian influenza, it remains relevant within the study because of its zoonotic character and the wide circulation of the pathogen in ecosystems where domestic animals, wild animals, and favourable environmental factors coexist. The importance of including it in the research stems from the need for an integrated perspective on emerging and re-emerging diseases affecting animal health and public health in the region.

Overall, the epizootic context of the study area can be characterized by a high level of epidemiological complexity determined by the overlap of several risk factors: the cross-border mobility of wildlife, seasonal bird migration, variable density of domestic animals, climatic and hydrological particularities, as well as existing biosecurity vulnerabilities at local level. This reality fully justifies the need for joint research and coordinated surveillance between the Romanian and Ukrainian partners, with the aim of obtaining as complete an epidemiological picture as possible and of developing appropriate preventive responses for the cross-border region.

Characteristics of animal husbandry and wildlife

Within the study area, the following coexist:

- non-professional holdings and individual households;
- domestic poultry and pig rearing;
- cattle, dogs, cats, and other domestic species;

- migratory and resident wild birds;
- wild mammals, including the golden jackal and rodents, of epidemiological relevance.

MATERIALS AND METHODS

Timeframe and study stages

Stage	Period	Description	Responsible partner
Development of the joint TOR	Period 1	Establishment of the common methodological framework	All partners
Sample collection	Periods 1 and 3	Field campaigns in Romania and Ukraine	PP2, PP3
Laboratory analysis	Periods 2 and 4	Molecular and serological testing	PP2, PP3
Preparation of reporting documentation	Period 4	Consolidation of results and drafting of the report	All partners

Geographical coverage and sampling sites

- PP3 (Ukraine): Izmail District — Prymorske, Dunaiske, Stara Nekrasivka, Izmail, Vylkove
- PP2 (Romania): Tulcea — Ceatalchioi, Pardina, Periprava, Cardon, various areas of Tulcea County

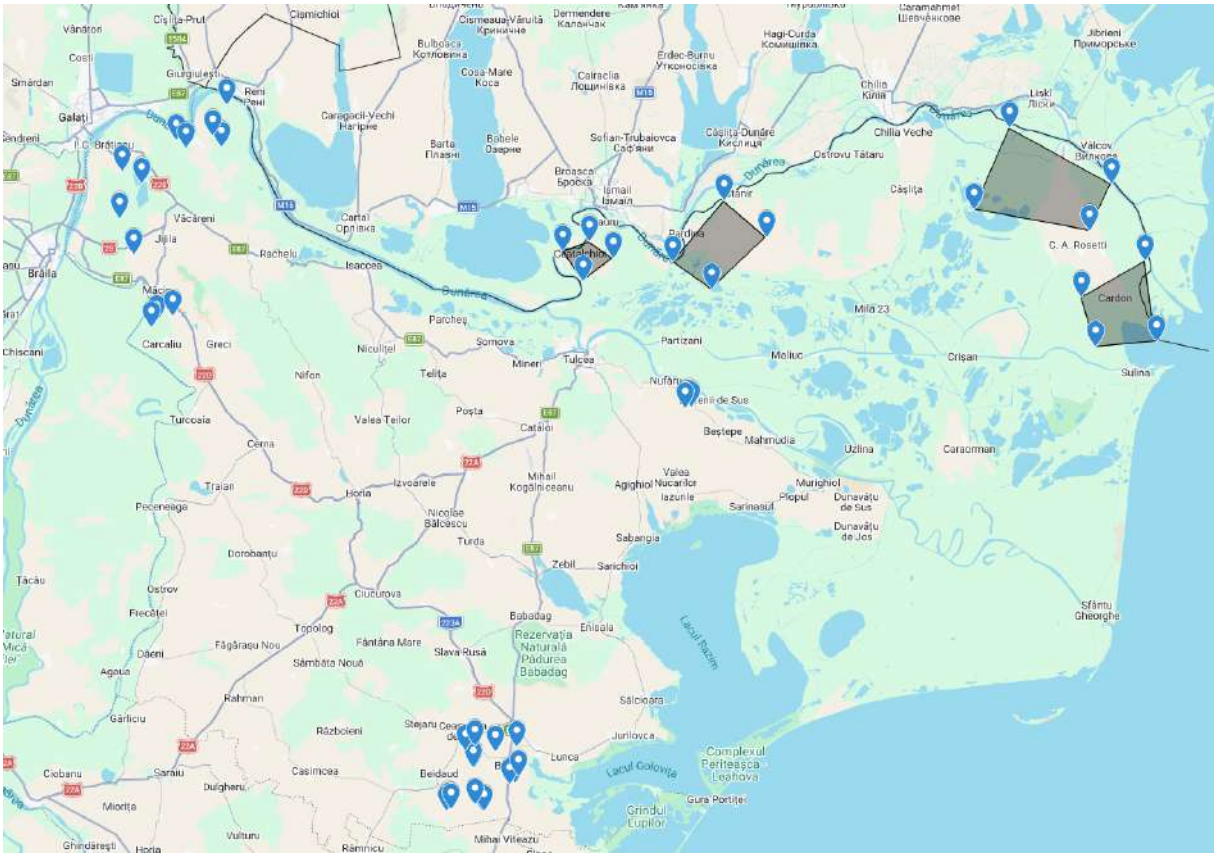


Figure 1. Study area in Romania



Figure 2. Study area in Ukraine

Sampling requirements and conditions

For each disease, specific sample types, target species, and handling conditions were established. Biological samples were collected from domestic and wild animals, as well as, where applicable, environmental samples.

Examples of sample types:

- blood / serum;
- cloacal and tracheal swabs;
- organs and tissues;
- ticks / vectors;
- water and soil.

Storage and transport conditions complied with the cold chain, packaging rules, and biosecurity requirements. Seasonality and bird migration were taken into account in the planning.

Laboratory analysis methods

The following were used:

- molecular methods: PCR / real-time PCR;
- serological methods: ELISA;
- where necessary, complementary interpretation and confirmation methods.

Sample testing was carried out in accordance with the joint Terms of Reference agreed by the partnership, while observing the applicable biosafety and biosecurity requirements for the protection of personnel, the environment, and sample integrity, as well as the internal procedures and legal requirements applicable to each partner institution.

Molecular testing, including PCR and real-time PCR, was performed in line with the general principles and methodological recommendations provided by WOAH, while serological testing was based on ELISA methods applied in accordance with the relevant WOAH guidance and the validated laboratory procedures of each partner. Specific operational details related to individual commercial diagnostic kits, such as incubation times, reagent volumes, cut-off values, or plate validation criteria, were applied according to the corresponding SOPs and manufacturers' instructions and are therefore not reproduced in detail in this report.

This common methodological framework ensured a consistent scientific basis for the activities performed by PP2 and PP3 and supported the comparability, correlation, and joint epidemiological interpretation of the results generated independently by each partner.

African swine fever – ELISA

Serological methods are widely applied for ASFV diagnosis because they are simple to run, inexpensive and do not require sophisticated laboratory infrastructure. Anti-ASFV antibodies typically become detectable within 7–10 days after infection or vaccination and can remain present for months to years. Pigs and wild boar infected with highly virulent strains generally succumb before mounting a measurable antibody response. However, in regions where the infection is endemic and attenuated or low-virulence isolates also circulate, serology plays a key role in identifying animals that have recovered or are infected without showing clinical signs. To date, no antibodies capable of fully neutralising the virus have been identified.

ELISA (Gallardo et al., 2015b; Sánchez-Vizcaíno, 1987) is the technique most frequently applied, and it works well on serum and plasma samples. Any sample giving a positive ELISA result should be re-checked using a different method, such as IFAT, IPT or immunoblotting (Gallardo et al., 2015b; Pastor et al., 1989). Antibodies are rarely found in animals infected with highly virulent ASFV, since death usually occurs before an antibody response develops, whereas pigs infected with low- or moderate-virulence strains do produce detectable antibodies. A validated, commercially available lateral-flow (pen-side) test can also be used for rapid antibody detection.

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

The indirect ELISA kit is designed to identify anti-ASFV antibodies in pig serum, plasma, meat juice and dried blood on filter paper.

The wells of the microplate are pre-coated with the recombinant ASFV proteins p32, p62 and p72.

Test samples and controls are dispensed into the coated wells; any anti-ASFV antibodies present will bind to the coated antigens, forming an antigen–antibody complex.

Following a wash step, a multi-species anti-antibody conjugate labelled with horseradish peroxidase (HRP) is added; this binds to any captured antibodies, forming an antigen–antibody–HRP-conjugate complex.

Excess conjugate is removed by washing, after which the TMB substrate solution is added.

The intensity of the colour produced is proportional to the amount of specific antibody in the sample:

- if antibodies are present, a blue colour develops, turning yellow once the stop solution is added.
- if antibodies are absent, no colour change is observed.

Note: paragraphs adapted, in the author's own words, from the WOAH Terrestrial Manual, chapter on African Swine Fever (ELISA section).

African swine fever – real-time PCR

PCR assays targeting primers from a highly conserved genomic region have been designed to detect and characterise isolates spanning all recognised ASFV genotypes, including non-haemadsorbing strains and low-virulence isolates. These assays are especially valuable for detecting viral DNA in pig tissue samples that are unfit for virus isolation or antigen testing — for example, decomposed material — or in cases where the virus may have been inactivated before the sample reached the laboratory. Because of its high sensitivity, specificity and suitability for high-throughput testing, PCR is the method of choice for both screening and confirmation of suspected cases.

Several conventional and real-time PCR protocols have been published for ASFV detection (Agüero et al., 2003; Basto et al., 2006; Fernández-Pinero et al., 2013; King et al., 2003; Tignon et al., 2011), and multiple commercial kits are available, one of which is formally registered with OIE. Duplex real-time PCR assays enabling simultaneous, differential detection of ASFV and CSFV have also been reported (Agüero et al., 2004; Haines et al., 2013).

The ASFV primer set used here can be paired with a CSFV-specific primer set in a multiplex RT-PCR, allowing both viral genomes to be detected and distinguished within a single reaction (Agüero et al., 2004).

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 ASFV-positive DNA and should ideally sit near the assay's detection limit, so that the amplification yield can be checked (for example, a Ct value of 32±2 in real-time PCR). The negative control consists of 2 µl of nuclease-free sterile water, or of DNA extracted from ASFV-negative EDTA-blood, serum or tissue homogenate.

Note: paragraphs adapted, in the author's own words, from the WOAH Terrestrial Manual, chapter on African Swine Fever (PCR section).

Avian Influenza – multi-species ELISA

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

Kits need to be validated for the target species and the intended application, since antigen preparation and test formats vary between manufacturers; the manufacturer's instructions for validated use should be followed closely. These ELISAs are moderately priced, support high-throughput screening for influenza A infections, and perform well compared with HI in large-scale serosurveillance, although any positive result still needs HI confirmation for H5/H7 subtyping. Subtype-specific kits exist for H5, H7, H9 and certain N subtypes such as N1, though these tend to be less sensitive than the general influenza A ELISA. More recently, multiplex assays using bead-based immunoassay technology and recombinant viral proteins have been developed, allowing several HA/NA subtypes or additional notifiable-disease targets to be tested together at lower cost — though these still require further validation before routine diagnostic use.

Mammalian serum can be screened for AIV exposure using a commercial multispecies competitive ELISA that targets antibodies against the type A influenza nucleoprotein (NP). Because these kits' diagnostic performance is not established for mammalian species beyond those specified by the manufacturer, their suitability should be carefully assessed for the species being tested before routine use, and positive results require confirmation with an additional serological test such as HI.

Note: paragraphs adapted, in the author's own words, from the WOAH Terrestrial Manual, chapter on Avian Influenza (ELISA section).

Avian Influenza – real-time RT-PCR

Well-designed molecular assays can outperform virus isolation in both sensitivity and specificity. Concerns that PCR inhibitors in faecal samples might reduce assay sensitivity have largely been resolved through improved RNA extraction and treatment kits. Proper use of positive and negative controls at both the extraction and amplification stages remains essential for generating valid results.

Most laboratories diagnose influenza A using real-time RT-PCR, beginning with a screening step that targets the highly conserved matrix (M) gene common to all influenza A viruses (Table 2); samples positive for the M gene are then followed up with subtype-specific real-time RT-PCR for H5 and H7. Numerous highly sensitive M-gene assays meeting the requirements of a suitable

screening test have been reported. Spackman et al. (2002), for instance, developed a single-step real-time RT-PCR using a primer/fluorogenic hydrolysis-probe system that performed well against virus isolation while being cheaper and considerably faster, delivering results from clinical samples in under three hours. Screening tools such as this should be monitored in silico and updated as needed — the Spackman et al. (2002) protocol itself has since been revised through the introduction of degenerate bases in the M-gene primers (Hassan et al., 2022; Laconi et al., 2020; Spackman et al., 2020), a change that also enables inclusion of an internal process control to guard against false negatives caused by PCR inhibition or extraction failure. Advances in assay design and biochemistry have further produced screening assays applicable across all influenza A viruses and hosts, animal and human alike (Nagy et al., 2021), which is particularly relevant at the avian/other-host interface.

Laboratories lacking a real-time RT-PCR platform for M-gene screening can instead use the same primer set from the Spackman et al. (2022), Heine et al. (2015) or Hassan et al. (2022) protocols in a conventional RT-PCR. Validation data for this alternative use are not yet available, and because the resulting amplicon is small (under 100 bp), results should be read on silver-stained SDS-PAGE gels or on 2–3% agarose gel.

Note: paragraphs adapted, in the author's own words, from the WOAH Terrestrial Manual, chapter on Avian Influenza (PCR section).

West Nile virus infection – ELISA

Equine antibodies can be detected using IgM-capture ELISA, haemagglutination inhibition (HI), IgG ELISA, plaque-reduction neutralisation (PRN) or microtitre virus neutralisation (VN) (Beaty et al., 1989; Hayes, 1989). IgM-capture ELISA is particularly suited to identifying equine antibodies from a recent natural WNV exposure, since equine IgM is generally detectable from 7–10 days to 1–2 months post-infection, and most horses with WN encephalitis already test positive by the time clinical signs appear. Neutralising antibodies in horses become detectable around two weeks post-infection and can persist beyond a year. In birds, ELISA, HI, VN and PRN are the methods most often used to detect WNV antibodies, although cross-reactions with related flaviviruses such as St Louis or Japanese encephalitis virus can occur in some assays. PRN is the most specific of the WNV serological tests, and antibody titres against related flaviviruses can be run in parallel where cross-reactivity needs to be ruled out. Vaccination history should always be taken into account when interpreting PRN, VN or IgG ELISA results. IgM-capture ELISA can also be applied to birds or other species provided a species-specific capture antibody is available (e.g. anti-chicken IgM), while PRN can be used across species, including birds.

Both commercial and in-house indirect and competitive ELISAs are used for WNV antibody detection. Competitive formats work across all species, whereas indirect formats need species-specific enzyme-labelled secondary antibodies. Most ELISAs have limited specificity because they cross-react with antibodies to other flaviviruses, particularly those in the Japanese

encephalitis serogroup, but they remain valuable for surveillance and screening purposes; any positive should be confirmed with a more specific test such as VN or PRNT. Some indirect or competitive formats can detect antibodies across immunoglobulin classes (IgM, IgG, etc.), and vaccinated horses frequently test positive on these assays.

Note: paragraphs adapted, in the author's own words, from the WOAH Terrestrial Manual, chapter on West Nile Virus infection (ELISA section).

West Nile virus infection – real-time RT-PCR

Multiple PCR-based methods for WNV identification have been published, and some are available as commercial kits. This includes a real-time RT-PCR capable of detecting both lineage 1 and lineage 2 WNV (Eiden et al., 2010), as well as a conventional gel-based RT-PCR for lineage 1 North American strains (Johnson et al., 2001); both have been used successfully on field samples. Lineage 1 WNV strains from France, Egypt, Israel, Italy, Kenya, Mexico and Russia share a highly conserved nucleotide sequence in the target region regardless of host species (Lanciotti et al., 2000). The conventional nested procedure offers greater sensitivity for North American WNV RNA, particularly in equine field samples, although it demands strict laboratory practices to prevent contamination, and its performance against other WNV lineages has not been established. Because WNV continues to evolve and new strains may emerge, PCR test designs should be reviewed and updated on an ongoing basis. Suitable specimens for WNV real-time RT-PCR include mammalian and avian brain, kidney, heart, liver, spleen, intestine, and insect pools. Every PCR run must include positive and no-template controls, and for nested RT-PCR, care must be taken to avoid cross-contamination when transferring the outer-primer product to the nested reaction; a run is only valid if its control reactions fall within the expected range.

The method below, developed by Eiden et al. (2010), allows simultaneous identification of lineage 1 and lineage 2 WNV; strains can be further identified by sequencing the amplicon and comparing it against WNV reference strains. The version presented here is slightly modified from the original publication and uses primers and a probe targeting the NS2A region of the WNV genome. Any commercial kit that gives the expected control amplification can be used, with cycling parameters adjusted to match the kit's requirements and the primers'/probe's melting temperatures; primer and probe concentrations can likewise be optimised. An internal control with its own primers and probe may be added to verify valid test conditions.

Primers/probe (NS2A region of genome):

- Forward primer: GGG-CCT-TCT-GGT-CGT-GTT-C
- Reverse primer: GAT-CTT-GGC-YGT-CCA-CCT-C
- Probe: FAM-CCA-CCC-AGG-AGG-TCC-TTC-GCA-A-BHQ

For each sample, prepare a 20 µl reaction mix (as per kit instructions) containing each primer at 0.9 µM and the probe at 0.25 µM, and dispense 20 µl into the corresponding tube or well. Add 5.0 µl of extracted RNA, seal, and load into the thermocycler; run under the kit-specified

conditions, starting with reverse transcription and followed by 45 amplification cycles. A Ct value of 37 or below is read as positive for WNV, values between 37.1 and 42 are considered suspect and should be repeated, and values above 42 are negative. The run is valid only if the positive controls' Ct values fall within the expected range and the no-template controls are negative.

Note: paragraphs adapted, in the author's own words, from the WOAH Terrestrial Manual, chapter on West Nile Virus infection (PCR section).

Leptospirosis – MAT

The Microscopic Agglutination Test (MAT) using live antigens is the most widely applied serological test for leptospirosis, serving as the benchmark against which all other serological tests are measured, and it is also the test used for import/export purposes. To achieve optimum sensitivity it should include antigens covering every serogroup known to occur in the region, ideally using strains representative of all recognised serogroups. A serogroup's presence is usually suggested by a frequent reaction pattern in screening, but can only be confirmed by isolating a serovar from a clinically affected animal. Using local isolates rather than reference strains can improve sensitivity, though reference strains remain useful for comparing results between laboratories.

MAT specificity is good, since antibodies to unrelated bacteria rarely cross-react significantly with Leptospira. However, extensive cross-reactivity exists between different Leptospira serovars and serogroups, so an animal infected with one serovar will typically 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; that requires isolating the organism. Where the local serovars have already been well characterised through isolation studies, serology can suggest, though not confirm, the likely infecting serovar. Vaccination history is also important, as vaccinated animals may carry antibodies to the vaccine serovars. Detailed testing procedures are described elsewhere (Faine et al., 2000; Goris & Hartskeerl, 2014; USDA, 1987).

Note: paragraphs adapted, in the author's own words, from the WOAH Terrestrial Manual, chapter on Leptospirosis (MAT section).

Leptospirosis – ELISA

A range of ELISA formats for detecting anti-leptospiral antibodies have been developed, differing in antigen preparation, protocol and platform, including plate- and dipstick-based tests. Antigens used are generally either whole-cell preparations or outer membrane protein (OMP) preparations, with more recent work focusing on recombinant OMPs; the antigen choice determines the assay's specificity. Recombinant OMP-based ELISAs react broadly across pathogenic Leptospira and therefore have limited value for epidemiological work, whereas lipopolysaccharide-based ELISAs are serogroup-specific and useful in epidemiological investigations and control programmes. IgM ELISAs are useful for diagnosing acute infection,

and a total-Ig ELISA can help identify fully susceptible animals for experimental challenge studies. ELISAs for serovar Hardjo antibodies have also been developed for individual-cow or bulk-tank milk testing, and have proved useful for identifying Hardjo-infected herds and supporting Hardjo control/eradication programmes (Pritchard, 2001); however, herds vaccinated against Hardjo will also test positive, limiting their usefulness where vaccination is routine. OMP-based tests are not yet widely available and, while potentially useful for diagnosing incidental infections, are unlikely to be useful in control programmes for host-adapted infections such as Hardjo, where naturally infected cattle mount a weak or absent response to outer membrane proteins but a strong response to outer lipopolysaccharide antigens instead (Ellis et al., 2000).

Validation remains a major limitation for most leptospirosis ELISAs, since nearly all have been validated against the MAT (using a titre threshold of 1/100 or higher) — itself an imperfect reference, with sensitivity below 50% in some chronic infections. In human medicine, this has been addressed using Bayesian latent-class models and random-effects meta-analyses (Limmathurotsakul et al., 2012; Signorini et al., 2013), though the strongest validation still comes from sequential sera taken from culture-positive cases (Goris et al., 2012). Only a handful of animal ELISAs have been validated this way, and even then only up to 6 months post-challenge using experimental animals, while a single commercial Hardjo ELISA has been validated against single serum samples from culture-positive cattle.

Note: paragraphs adapted, in the author's own words, from the WOAH Terrestrial Manual, chapter on Leptospirosis (ELISA section).

Leptospirosis – real-time PCR

PCR-based assays are increasingly applied to detect leptospires in animal tissues and body fluids because of their perceived sensitivity and ability to give an early diagnosis; real-time PCR is faster and less prone to contamination than conventional PCR (Picardeau, 2013). These assays target either genes universally present in bacteria — such as gryB, rrs (16S rRNA) and secY — or genes restricted to pathogenic Leptospira, such as lipL21, lipL32, lipL41, ligA and ligB (Thaipadunpanit et al., 2011). None of these assays can identify the infecting serovar directly, though sequencing the 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 designed and evaluated for human rather than animal specimens, 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 reported.

Validation is still an open issue for PCR-based diagnosis of animal leptospirosis, and each laboratory is responsible for validating its own assay for the specific tissue, fluid and species being tested. So far, only three real-time PCR assays have been formally validated (Ahmed et al., 2009; 2012b; Slack et al., 2007; Thaipadungpanit et al., 2011). Amplification inhibitors in clinical samples — for example, faecal contamination or autolysis in animal specimens — can cause

false negatives, so laboratory design, workflow and appropriate control samples must be carefully managed to avoid reagent contamination (McCreedy & Callawayth, 1993), and sample processing must always be adapted to the tissue, fluid and species under investigation.

Note: paragraphs adapted, in the author's own words, from the WOAH Terrestrial Manual, chapter on Leptospirosis (PCR section).

Assessment procedure

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

Before interpretation of individual samples, each analytical run was assessed for validity according to the requirements of the method, the manufacturer's instructions, the laboratory SOPs and the applicable WOAH recommendations. A run was considered interpretable only when the required positive, negative and, where applicable, internal amplification or process controls produced the expected results. Failure of one or more mandatory controls resulted in invalidation or repetition of the affected analytical run.

Sample suitability was also taken into consideration. Samples showing inadequate preservation, insufficient quantity, evidence of degradation, excessive contamination, inhibition or other conditions capable of compromising analytical performance were not interpreted as equivalent to valid negative samples. Where technically possible, such samples were retested following re-extraction, dilution, additional processing or collection of a new specimen.

Molecular methods – PCR and real-time PCR

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

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

For ASFV testing, the PCR framework used in the report included positive and negative amplification controls, in accordance with the methodological principles described in the WOAH-based section of the report. The positive control was intended to verify amplification performance, while the negative control was used to detect contamination. WOAH identifies PCR and real-time PCR as highly sensitive, specific and rapid techniques for ASFV genomic detection and considers them particularly useful across a wide range of diagnostic situations.

For avian influenza, molecular assessment followed a sequential diagnostic logic. Screening was based on detection of the conserved influenza A matrix gene. Samples producing a positive influenza A screening result were to be further investigated by subtype-specific molecular methods for H5 and H7, in accordance with the diagnostic approach described by WOAH. WOAH recognises direct genome detection by real-time RT-PCR or other validated molecular techniques as a major method for diagnosis and characterisation of influenza A viruses. A negative matrix-gene result was interpreted as absence of detectable influenza A RNA in the analysed specimen at the time of testing and not as evidence of population freedom from infection.

For WNV, the real-time RT-PCR method described in the report applied the following interpretative criteria: Ct ≤37 was considered positive; Ct values between 37.1 and 42 were considered suspect and required repetition; and Ct values >42 were considered negative. The run was regarded as valid only if the positive control produced amplification within the expected range and the no-template control remained negative. WOAH recognises RT-PCR for detection of WNV nucleic acid but also emphasises that diagnosis must be interpreted in relation to the biological material and infection dynamics.

For leptospirosis, PCR results were interpreted with particular attention to the sample matrix and stage of infection. WOAH notes that PCR can detect leptospiral genetic material in tissues or body fluids, but its performance depends on sample processing, tissue or fluid type and host species. PCR does not generally identify the infecting serovar. Consequently, failure to detect Leptospira DNA in blood was interpreted as absence of detectable leptospiral DNA in that particular specimen at the time of sampling and not as definitive exclusion of chronic renal or genital carriage. WOAH specifically recognises that leptospires may persist in kidney, urine or genital tract in chronic carriers.

For Toxoplasma gondii, molecular results obtained within the Ukrainian component were assessed according to the validated laboratory procedures and joint Terms of Reference used by the partner laboratory. No dedicated WOAH toxoplasmosis document was included among the source documents used for the present update; consequently, no additional WOAH-specific diagnostic thresholds have been introduced into this section.

Serological methods – general interpretation

Serological results were evaluated according to the calculation method and cut-off values specified for each commercial assay used in the project. ELISA-positive results were interpreted as evidence of antibody reactivity to the target antigen system and not automatically as evidence of active infection.

Where a screening assay was known to detect group-level antibodies rather than pathogen subtype-specific antibodies, the interpretation was limited accordingly. Doubtful or suspicious results were repeated or subjected to supplementary testing when required by the assay protocol or epidemiological context.

African swine fever – indirect ELISA

For the indirect ASF ELISA used by PP2, manufactured by Innovative Diagnostics, 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 thresholds correspond to the assay-specific criteria already incorporated into the common report.

Serological results were evaluated separately from direct ASFV detection. This distinction is essential because WOAH notes that pigs surviving natural infection generally develop antibodies approximately 7–10 days after infection and that antibodies may persist for long periods. Therefore, an ELISA-positive/PCR-negative profile was interpreted as serological reactivity without direct molecular evidence of viraemia in the specimen collected at that time. Such findings require consideration of the epidemiological history and, where appropriate, supplementary confirmatory investigation.

Avian influenza – multispecies competitive ELISA and HI

The multispecies competitive influenza A ELISA manufactured by Innovative Diagnostics was interpreted according to the S/N percentage:

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

These are the assay-specific thresholds applied in the project.

WOAH notes that influenza A viruses share antigenically related nucleoprotein and matrix proteins and that ELISA assays directed against these antigens are widely used for group-level

serological detection. Consequently, an ELISA-reactive result was interpreted as influenza A group-level antibody reactivity and not as evidence of a particular H or N subtype.

Where indicated, ELISA-reactive poultry sera were subjected to haemagglutination inhibition testing against the H5N3, H5N8 and H7N1 antigens used in the project. HI findings were interpreted using the laboratory criteria applied for the assay. The presence or absence of antibodies against the tested H5/H7 antigens was reported independently from the initial influenza A ELISA result.

A positive generic influenza A serological result with negative H5/H7-specific follow-up was therefore interpreted as lack of serological confirmation for the tested H5/H7 antigens rather than as contradiction of the original screening result.

West Nile virus infection – blocking ELISA

For the WNV blocking ELISA manufactured by Gold Standard Diagnostics Madrid S.A., interpretation was based on the inhibition percentage (IP).

Samples were classified as:

- Positive: IP ≥40%;
- Negative: IP ≤30%;
- Doubtful: IP between 30% and 40%.

According to the procedure incorporated in the report, samples in the doubtful interval should be further investigated by seroneutralisation.

Additional caution was applied to positive ELISA findings because WOAH notes that serological cross-reactions can occur with related flaviviruses, including Usutu virus, St Louis encephalitis virus, Japanese encephalitis virus and tick-borne encephalitis virus.

Accordingly, a positive blocking ELISA result was interpreted as WNV-reactive serological evidence. Where epidemiologically relevant and technically feasible, confirmation by a more specific neutralisation method, such as virus neutralisation or plaque-reduction neutralisation, provides greater specificity. A positive serological result combined with negative RT-PCR was not considered contradictory, because serological evidence may persist after the period during which viral RNA is detectable in peripheral blood.

Leptospirosis – ELISA and complementary interpretation

For the BIOVET Inc. ELISA used for Leptospira interrogans serovars Hardjo and Pomona, the sample ratio was calculated by dividing the optical density of the sample for the respective antigen by the mean optical density of the positive control for that antigen.

Results were interpreted as follows:

- Negative: ratio <0.20;

- Suspicious/doubtful: ratio ≥0.20 and <0.30;
- Positive: ratio ≥0.30.

For suspicious results, the assay procedure recommends collection of a second serum sample approximately two weeks later and repeat testing.

The epidemiological interpretation of ELISA findings took into account WOAH guidance. WOAH identifies the Microscopic Agglutination Test (MAT) as the standard serological method and recognises ELISA as useful for detection of leptospiral antibodies, including applications related to serovar Hardjo in cattle.

Serological reactivity alone was not considered sufficient to definitively identify the infecting serovar. Vaccination history, when available, was also taken into consideration because vaccinated animals may produce antibodies detectable by some serological assays. For acute infection, a four-fold rise in MAT titre between paired acute and convalescent sera represents diagnostic evidence according to WOAH.

Integrated interpretation of discordant results

Whenever serological and molecular results differed, the findings were not automatically regarded as analytically contradictory. Instead, interpretation considered the biological window detected by each method.

A serology-positive/PCR-negative combination could represent previous exposure, an immune response occurring after the period of detectable viraemia or bacteraemia, vaccination where relevant, or non-specific/cross-reactive serological activity. Conversely, a molecular-positive/serology-negative profile could be compatible with early infection before development of detectable antibodies.

For this reason, final interpretation was based on the complete diagnostic profile rather than on a single test result and took into account the species, sample type, collection date, clinical information, epidemiological context and, where available, vaccination history.

Management of doubtful, inconclusive and non-specific results

Doubtful, suspect, inconclusive or non-specific findings were managed according to the relevant assay instructions and laboratory SOPs. Depending on the method, this included repeat testing of the original sample, repeat extraction, analysis in duplicate, use of a supplementary method, collection of a second serum sample or referral for confirmatory testing.

Results from samples considered analytically unsuitable or producing unresolved non-specific reactions were excluded from the corresponding quantitative totals when they could not be reliably classified. This is consistent with the reporting approach already used in the common report, where certain samples were excluded because sample preparation or non-specific results did not allow inclusion in the final measurement totals.

Statistical and epidemiological assessment

Descriptive statistics were calculated from the valid analytical results, including the number and proportion of positive, negative, doubtful or suspect samples by disease, species, sampling period, geographical origin and diagnostic method, where appropriate.

Because sampling under Activity A1.4 was predominantly targeted and risk-based rather than probabilistic, proportions of reactive or positive samples were interpreted as characteristics of the investigated project dataset and not as estimates of population prevalence.

Direct comparisons between the Romanian and Ukrainian datasets or between the two sampling periods were made cautiously, taking into account differences in sample numbers, host species, biological matrices, geographical coverage and seasonality.

Where differences, unexpected results or discordant findings were identified between partners or between diagnostic methods, they were reviewed within the framework of joint technical coordination. Final interpretation was based on the agreed Terms of Reference, laboratory documentation, applicable WOAH principles and the epidemiological context of the Romania–Ukraine cross-border area.

RESULTS

General characteristics of the collected biological material

Collection of biological material was carried out in accordance with international and national standards, the Terms of Reference (approved within the Project), and veterinary medicine requirements, in compliance with all biosafety rules. Samples from animals were collected at their natural habitats and holding locations. Particular attention was paid to areas with high animal density, the wetlands of the Danube Delta, which serve as a migration site for wild birds, and to zones of possible contact between wild and domestic animals.

Table 1. Total number of biological material samples from animals for laboratory tests in Romania

№	Infection	Species/samples	Method to be used for testing the material	Quantity of samples		
				Period I	Period II	Total for both periods
1	African Swine Fever	Swine (domestic/wild where possible) – sodium citrate- or EDTA-stabilised blood	real-time PCR (per WOAH recommendations)	21	3	24
		Samples from mammals (wild carnivorous mammals), that come into direct contact with wildlife		-	37	37
		Insects acting as vectors in disease transmission		3	-	3
		Samples from mammals (wild carnivorous mammals), that come into direct contact with wildlife	ELISA (per WOAH recommendations)	-	37	37

№	Infection	Species/samples	Method to be used for testing the material	Quantity of samples		
				Period I	Period II	Total for both periods
		Swine (domestic/wild where possible) –blood sera	ELISA (per WOAH recommendations)	21	12	33
2	Avian Influenza	Poultry (domestic waterfowl, chickens in case of death, wild waterfowl) – cloacal swabs, blood from suspected cases, poultry carcasses where available	PCR/real-time – influenza A, H5/H7	131	152	283
			ELISA (per WOAH recommendations)	66	92	158
			Haemagglutination and haemagglutination inhibition tests – H7N1, H5N3, H5N8	4	-	4
		Wild bird	RT-PCR – influenza A, H5/H7	2	3	5
		Samples from mammals (swine and cattle), that come into direct contact with wildlife	RT-PCR – influenza A	-	87	87
			ELISA (per WOAH recommendations)	13	225	238
		Samples from mammals (wild carnivorous mammals), which consumes wild bird carcasses	RT-PCR – influenza A	-	37	37
			ELISA (per WOAH recommendations)	-	37	37
3	Leptospirosis		ELISA	41	129	170

№	Infection	Species/samples	Method to be used for testing the material	Quantity of samples		
				Period I	Period II	Total for both periods
		Cats, dogs, rodents, swine and cattle – blood sera, pathological material where available, cattle and swine semen	RT-PCR	47	124	171
4	WestNile	Sheep, cattle, cats – blood and vaginal swabs	RT-PCR	16	124	140
			ELISA	65	367	432
Total tested samples				430	1466	1896

Table 2. Total number of biological material samples from animals for laboratory tests in Ukraine

№	Infection	Species/samples	Method to be used for testing the material	Quantity of samples		
				Period I	Period II	Total for both periods
1	African Swine Fever	Swine (domestic/wild where possible) – sodium citrate- or EDTA-stabilised blood	PCR/real-time PCR (per WOAH recommendations)	50	50	100
	Avian Influenza	Poultry (domestic waterfowl, chickens in case of death, wild waterfowl) – cloacal swabs,	PCR/real-time – influenza A, H5/H7	100	100	200

№	Infection	Species/samples	Method to be used for testing the material	Quantity of samples		
				Period I	Period II	Total for both periods
		blood from suspected cases, poultry carcasses where available				
		Wild bird faeces	PCR/real-time – influenza A, H5/H7		50	50
		Samples from mammals (swine and cattle), in case of positive results among domestic poultry	PCR/real-time – influenza A	11	8	19
3	Leptospirosis	Cats, dogs, rodents, swine and cattle – blood sera, pathological material where available, cattle and swine semen	ELISA	100	101	201
			PCR	100	89	189
4	Toxoplasmosis	Sheep, cattle, cats – blood and vaginal swabs	PCR	29+5	45+5	84
			ELISA	32	70	102
Total tested samples				427	518	945

Disease-specific results

African Swine Fever

In Romania, the African swine fever (ASF) component of the study combined serological screening by ELISA with direct detection of ASFV genomic material by real-time PCR. A total of 70 serum samples were examined serologically, comprising 33 samples from domestic swine

and 37 samples from golden jackals. Anti-ASFV antibodies were detected in three swine serum samples; the remaining 30 swine sera and all 37 golden-jackal sera were negative. Consequently, ELISA-reactive samples represented 9.1% (3/33) of the tested swine sera and 4.3% (3/70) of all sera included in the ASF serological dataset. These proportions describe the investigated sample set and should not be interpreted as population prevalence because sampling was targeted within the project framework rather than designed as a random prevalence survey.

The three ELISA-positive swine samples were collected on 29 July 2026 from non-professional holdings in Slava Rusa and Slava Cercheza. Two positive samples originated from 10-month-old female pigs in Slava Rusa, while the third was obtained from a 12-month-old female pig in Slava Cercheza. The same three animals were subsequently investigated by real-time PCR using EDTA blood, and ASFV genomic material was not detected in any of them. In the remaining swine sera, including samples collected during the first campaign in Ceatalchioi, Pardina, Cardon and Periprava and the other samples collected in the two July 2026 holdings, anti-ASFV antibodies were not detected.

The molecular component comprised 65 completed real-time PCR determinations: 24 EDTA-blood samples from swine, 37 EDTA-blood samples from golden jackals and four tick samples. ASFV DNA was not detected in any of these materials. The swine molecular dataset included the samples collected during the November 2025 campaign in Ceatalchioi, Pardina, Cardon and Periprava, together with the three July 2026 swine samples that had shown serological reactivity. The wildlife-related molecular dataset included golden jackals sampled in the Sulina area and in the hunting grounds of Panduru, Bestepe, Carcaliu, Crapina-Isaccea and Smardan; all yielded negative real-time PCR results. The four tick samples collected during the first campaign were likewise negative for ASFV genomic material.

The interpretation of these findings follows the diagnostic framework described by WOAH, which separates ASF laboratory diagnosis into direct agent detection and serology. WOAH identifies PCR and real-time PCR as highly sensitive, specific and rapid methods for ASFV detection and notes that viral DNA can be detected from an early stage of infection in tissues, EDTA blood and serum. At the same time, pigs surviving natural infection generally develop ASFV-specific antibodies from approximately 7–10 days post-infection, and these antibodies may persist for long periods. Within this framework, the ELISA-positive/real-time-PCR-negative profile observed in the three pigs indicates serological reactivity without direct molecular evidence of viraemia at the time of sampling. The result therefore requires interpretation together with the animals' epidemiological context and, where appropriate, supplementary confirmatory investigations; it should not, on its own, be considered evidence of active ASFV circulation.

WOAH further identifies domestic pigs, wild boar and feral pigs as the principal naturally susceptible hosts and recognises soft ticks of the genus Ornithodoros as established reservoirs

and transmission vectors in epidemiological settings where these ticks occur. Accordingly, the golden-jackal and non-speciated tick results obtained in the project are best regarded as supplementary interface-surveillance information, while the primary ASF epidemiological interpretation remains centred on suid findings. Overall, the Romanian dataset provided no direct molecular evidence of active ASFV circulation in the tested materials during the investigated periods, while the three serologically reactive swine samples constitute a specific signal warranting continued risk-based surveillance and epidemiological follow-up.

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

To determine the immune profile of the swine population in the study geographic zone, blood samples from pigs and piglets were additionally tested by PCR for the presence of influenza A virus RNA, as well as PRRS virus RNA and swine parvovirus and circovirus DNA. These viral agents may be potential co-infection pathogens with ASF, since they have an immunosuppressive effect on the pig organism.

When testing blood samples from pigs in Prymors'ke village – in a quantity of 25 – influenza A virus RNA was detected in one sample; the virus did not cause respiratory disorders and did not belong to subtypes H1 and H3, which are dangerous to humans.

When testing blood samples from pigs in the village of Stara Nekrasivka, in a quantity of 13, porcine circovirus type 2 DNA was detected in two samples. PRRS virus RNA and parvovirus DNA were not detected. When testing spring blood samples from pigs, in a quantity of 50 samples, porcine circovirus type 2 DNA was detected in one sample. PRRS virus RNA and swine parvovirus DNA were not detected.

Avian Influenza

Testing performed by PP2 in Romania combined broad serological screening with direct molecular detection and, for ELISA-reactive poultry sera, subtype-oriented haemagglutination inhibition (HI). The serology dataset contained 408 individual serum samples examined by a multispecies influenza A ELISA: 146 avian 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 tested serological set) were ELISA-reactive: two chickens and two domestic ducks sampled in non-professional 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 because sampling was project- and risk-oriented rather than probabilistic.

The four ELISA-reactive poultry sera were further examined by haemagglutination inhibition against H5N3, H5N8 and H7N1 reference antigens, generating 12 HI determinations. All titres were below 1/16 and were reported as negative for antibodies against the tested H5/H7

antigens. No HI record was present in the serology dataset for the ELISA-reactive golden jackal serum. In line with WOAH guidance, influenza A ELISAs targeting conserved nucleoprotein/matrix antigens are appropriate for group-level serological screening and large-scale surveillance, but positive ELISA findings require subtype-oriented follow-up, particularly 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, while the golden-jackal finding should be regarded as a screening signal requiring cautious, species-appropriate interpretation.

Molecular testing comprised 444 real-time RT-PCR determinations targeting the conserved influenza A matrix (M) gene, corresponding to 379 individual animal records. The tested material included 156 EDTA-blood tested samples, 152 tested samples on combined cloacal/tracheal swabs, 65 on cloacal swabs, 65 on tracheal swabs and 6 on organ samples. The tested population covered domestic and wild birds (chickens, domestic ducks/ducks, domestic geese, coots and gulls) and mammals at the wildlife-domestic interface (cattle, sheep and golden jackals). Influenza A viral RNA was not detected in any of the 444 determinations. Of particular relevance, the golden jackal with ELISA reactivity was also tested on EDTA blood by real-time RT-PCR, and no influenza A matrix-gene RNA was detected.

WOAH identifies direct viral RNA detection by real-time RT-PCR as a preferred method for rapid laboratory diagnosis and surveillance of influenza A, commonly using generic detection of the highly conserved matrix gene as the initial screening step, followed by H5- and H7-specific testing when influenza A RNA is detected. Because all Romanian matrix-gene RT-PCR determinations were negative, the PP2 dataset provided no direct molecular evidence of active influenza A infection in the tested materials 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 matrix-gene RT-PCR - does not support active H5/H7 virus circulation within the Romanian project sample set during the investigated periods. Nevertheless, group-reactive ELISA findings may reflect previous exposure to influenza A subtypes other than those tested by HI, waning antibody responses or non-specific serological reactivity and therefore remain relevant for continued risk-based surveillance at the domestic-bird/wildlife interface.

Results of detection of avian influenza virus in Ukraine (Influenza A, H5/H7). Influenza A virus RNA was detected in three samples of wild bird droppings – 2 collected in the localisation zone of migrating dunlin (Calidris alpina) and one from a mallard (Anas platyrhynchos), whose migratory colonies were recorded during sampling in the area of Dunais'ke village, the Kryve lake water body, and also in the coastal-line water (bottom water layer, prepared by the sedimentation filtration method) (table). The detected influenza A virus RNA proved negative during in-depth testing for the presence of the highly pathogenic subtypes H5 and H7, indicating the circulation of low-pathogenic influenza A virus subtypes. This is confirmed by parallel

ornithological observations, which showed no cases of clinical manifestation of influenza or deaths of wild or domestic birds in the study areas.

Table 3. Positive samples for Avian Influenza in Ukraine

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

Spring testing showed that influenza A virus RNA was detected in six samples of wild bird droppings – 4 collected in the localisation zone of migrating mallard (Anas platyrhynchos), and one each from ruff (Philomachus pugnax) and dunlin (Calidris alpina), whose migratory colonies were recorded during sampling in the area of the Danube floodplains, Dunaiske village, the Kryve lake water body, and in the coastal-line water of Lake Kryve (bottom water layer, concentrated by the sedimentation filtration method). No influenza A virus RNA was detected in soil samples from the surrounding water-adjacent territories.

The detected influenza A virus RNA proved negative during in-depth testing for the presence of the highly pathogenic subtypes H5 and H7, indicating the circulation of low-pathogenic influenza A virus subtypes. This is confirmed by parallel ornithological observations, which showed no cases of clinical manifestation of influenza or deaths of wild or domestic birds in the study areas.

No antibodies to influenza A viruses were detected when testing blood serum samples from pigs and cattle.

Table 4. 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 path
2	A/Anas platyrhynchos/Danube/02/H?	+	–	–	Influenza A, low path
3	A/Anas platyrhynchos/Danube/03/H?	+	–	–	Influenza A, low path
4	A/Anas platyrhynchos/Danube/04/H?	+	–	–	Influenza A, low path

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

Leptospirosis

Testing performed by PP2 in Romania combined serological screening by ELISA with direct molecular detection of Leptospira genetic material by real-time PCR. The serological dataset comprised 170 serum samples: 132 from cattle, 37 from golden jackals and one from a cat. The ELISA records assessed antibody reactivity against Leptospira interrogans serovar Hardjo (type Hardjoprajitno) and L. interrogans serovar Pomona. For Hardjo, 37 samples were positive, 11 were classified as doubtful and 122 were negative. All positive and doubtful reactions occurred in cattle; therefore, among the 132 bovine sera, 28.0% (37/132) were positive and 8.3% (11/132) were doubtful. All 37 golden-jackal sera and the single feline serum were negative. For Pomona, all 170 serum samples were negative. Across the complete serological dataset, Hardjo-positive samples represented 21.8% (37/170) and doubtful samples 6.5% (11/170). These proportions describe the targeted project sample set and should not be interpreted as population prevalence estimates.

The temporal distribution of the serological findings showed reactivity in both project sampling periods. During the first campaign in November 2025, 41 sera were examined (40 bovine and one feline); 23 bovine samples were positive and eight were doubtful for Hardjo, while the remaining samples were negative. In the 2026 sampling period, a further 14 Hardjo-positive and three doubtful bovine sera were identified. Positive bovine results were recorded in samples originating from Sulina, Letea and Chilia Veche, while all golden-jackal sera collected during the 2026 wildlife component remained negative. The presence of Hardjo-reactive cattle in more than one sampling period and locality constitutes a repeated serological signal of exposure within the investigated bovine population; however, differences in sampling intensity, herd selection and geographical coverage do not allow direct inference of temporal trends or prevalence changes.

The molecular component comprised 171 completed real-time PCR determinations, including samples from 112 cattle, 15 sheep, six dogs, 37 golden jackals and one rat. The tested matrices consisted of 124 EDTA-blood samples, 46 blood samples and one organ sample. Leptospira genomic material was not detected in any of the 171 determinations. Thus, the molecular dataset provides no direct evidence of detectable leptospiral DNA in the biological materials examined at the time of sampling, including the wildlife samples incorporated into the project.

Interpretation of the Romanian findings follows 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 for leptospirosis diagnosis and identifies the Microscopic Agglutination Test (MAT) as the standard serological test, while ELISA may be useful for serological screening and for assessment of serovar Hardjo infection in cattle. PCR provides sensitive direct detection of leptospiral nucleic acid, but its diagnostic performance depends strongly on the biological matrix, stage of infection, sample processing and quality-control procedures, and PCR does not identify the infecting serovar. In addition, negative direct detection in blood does not exclude a chronic renal or genital carrier state, because leptospires may persist in the kidney, urine or genital tract after the period of detectable leptospiraemia. Consequently, the pattern observed by PP2- 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 tested matrices at the time of collection. Serological reactivity alone cannot definitively identify the infecting serovar, and vaccination history, where applicable, must also be considered. No serological evidence of exposure to Pomona was identified in the present dataset. MAT results will be incorporated when available to provide additional serogroup-oriented information and to strengthen the epidemiological interpretation, while definitive serovar attribution would require isolation and identification of the organism.

Testing conducted in Ukraine in autumn and in the second period (spring) for leptospirosis on whole blood, urine, milk and blood serum samples using PCR and ELISA methods detected neither pathogen DNA nor antibodies to it.

When testing water samples from target water bodies, non- pathogenic Leptospira DNA was detected in the water; however, in 2 water samples from the Kar'yer water body (Vylkove) and the Stentsivsko-Zhebriianivski floodplains (Prymorske village), DNA of biological agents of the genus Leptospira was detected, most likely belonging to non-pathogenic species.

Toxoplasmosis (Toxoplasma gondii)

No investigations for Toxoplasma gondii were recorded in the PP2 Serology or Molecular Biology and Genetic Analysis datasets used for the present update. Consequently, no Romania-specific laboratory results can be reported in this subsection on the basis of these two datasets.

Molecular-genetic testing in Ukrainian part during the first (autumn) period of whole blood and faeces, and serological testing of blood sera by PCR and ELISA methods, showed the absence of pathogen DNA and antibodies to it in the samples tested. Molecular-genetic testing of whole blood and faeces, as well as serological testing of blood sera by PCR and ELISA methods, showed the absence of pathogen DNA and antibodies to it in the samples tested. Water samples were also negative for the presence of Toxoplasma DNA.

West Nile virus infection

Testing performed by PP2 in Romania combined serological screening by competitive ELISA with direct molecular detection of West Nile virus (WNV) RNA by real-time RT-PCR. The serological dataset comprised 432 serum samples. Overall, 168 samples were positive, eight were classified as doubtful and 256 were negative, corresponding to observed proportions of 38.9%, 1.9% and 59.3%, respectively, within the targeted project sample set. The species distribution was heterogeneous: among 316 cattle sera, 122 were positive, seven doubtful and 187 negative; among 40 chicken sera, 11 were positive, one doubtful and 28 negative; all 15 sheep sera and all 12 swine sera were positive; among 37 golden-jackal sera, seven were positive and 30 negative; and among five duck sera, one was positive and four were negative. All six canine sera and the single feline serum were negative. These proportions describe the animals sampled within the project and should not be interpreted as population prevalence estimates because sampling intensity, species composition and geographical coverage were not statistically uniform.

A clear difference was observed between the two project sampling periods, although the composition of the sampled populations was also markedly different. During the first campaign in November 2025, 65 sera were tested (58 cattle, six dogs and one cat), and all were negative. During the 2026 sampling period, 367 sera were examined, including cattle, chickens, golden jackals, sheep, swine and ducks; 168 were positive, eight doubtful and 191 negative. The 2026 serological signal was therefore distributed across several domestic and wildlife species. However, because the second period included different species, locations and sampling intensity, the contrast between periods cannot by itself be interpreted as evidence of a temporal increase in incidence or prevalence.

The molecular component comprised 140 real-time RT-PCR determinations: 81 from cattle, 37 from golden jackals, 15 from sheep, six from dogs and one from a cat. The tested matrices consisted of 125 EDTA-blood samples and 15 blood samples. Sixteen molecular determinations were performed during the 2025 campaign and 124 during the 2026 sampling period. WNV RNA was not detected in any of the 140 samples. Thus, the molecular dataset provides no direct evidence of detectable viraemia in the tested animals at the time of sampling. This finding is not incompatible with the serological results, because molecular detection is strongly dependent on the timing of sampling and the biological matrix, while antibody responses may persist after the period during which viral RNA is detectable in peripheral blood.

Interpretation of these findings follows the WOAH diagnostic framework for West Nile fever. WOAH describes WNV as a mosquito-borne flavivirus maintained primarily in a bird-mosquito-bird transmission cycle and recognises real-time RT-PCR as an appropriate method for direct detection of viral nucleic acid in suitable clinical material. For serological surveillance, indirect and competitive ELISAs are useful screening tools, but WOAH emphasises 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 should, where epidemiologically relevant and technically feasible, be confirmed by a more specific neutralisation method such as plaque-reduction neutralisation (PRN/PRNT) or virus neutralisation (VN). Consequently, the Romanian results demonstrate a substantial WNV-reactive serological signal in the targeted 2026 sample set, especially among cattle and several other sampled species, but they do not by themselves establish active WNV infection or WNV-specific population prevalence. The uniformly negative real-time RT-PCR results indicate that WNV RNA was not detected in the biological materials examined during the two project sampling periods.

Results for environmental samples

In order to identify the possible source of pathogens and track their possible circulation, environmental samples in Ukrainian project territory were also tested within the framework of the project. This material includes water, soil, insect and feed samples, presented separately in accordance with the specifics of the “One Health” project.

Table 5. Total number of environmental samples for laboratory tests in Ukraine

№	Infection	Species/samples	Method to be used for testing the material	Quantity of samples		
				Period I	Period II	Total for both periods
1	African Swine Fever	Feed samples from at-risk areas	PCR/real-time PCR (per WOAH recommendations)	8	8	16
		Insects that are potential mechanical vectors (ticks, midges, etc.)	PCR/real-time PCR (per WOAH recommendations)	10	30	40
2	Avian Influenza	Water	PCR/real-time – influenza A, H5/H7	17	17	34
		Soil	PCR/real-time – influenza A, H5/H7		15	15
3	Leptospirosis	From environmental objects –	PCR	25	29	54

№	Infection	Species/samples	Method to be used for testing the material	Quantity of samples		
				Period I	Period II	Total for both periods
		standing water body water				
4	Toxoplasmosis	Open water body water	PCR	25	29	54
Total tested samples				85	128	213

Based on the results of testing biological material from the environment, namely water samples from target water bodies, no pathogenic Leptospira DNA was detected in the water; however, in 2 water samples from the Kar'yer water body (Vylkove) and the Stentsivsko-Zhebriianivski floodplains (Prymorske village), DNA of biological agents of the genus Leptospira was detected, most likely belonging to non-pathogenic species.

DISCUSSION OF RESULTS

The results obtained during the joint Romania–Ukraine study demonstrate the epidemiological complexity of the Lower Danube and Danube Delta cross-border area and confirm the need to interpret laboratory findings within a broader ecological, temporal and methodological context. The investigated territory is characterised by extensive wetlands, important migratory bird corridors, permanent interfaces between wildlife and domestic animals, seasonal vector activity and hydrological connectivity between habitats. Consequently, the absence of direct detection of a pathogen in a particular host population or sampling period cannot be interpreted as proof of absence from the wider ecosystem. Conversely, serological reactivity or isolated molecular detections within a targeted sampling framework cannot be extrapolated directly to population-level prevalence.

The two project sampling periods also differed with regard to species composition, number of samples, geographical coverage and, in some cases, biological matrices tested. For this reason, comparisons between periods and between the Romanian and Ukrainian datasets should primarily be interpreted as descriptive epidemiological comparisons rather than as estimates of temporal trends or differences in prevalence. The study was designed as targeted, risk-based cross-border surveillance and not as a statistically representative prevalence survey. This distinction is particularly important for diseases for which markedly different serological patterns were obtained between sampling periods.

African swine fever

The combined Romanian ASF results indicate no direct molecular evidence of active ASFV circulation in the materials tested during the study periods. All 65 completed real-time PCR determinations from swine, golden jackals and ticks were negative. At the same time, three of the 33 tested domestic swine sera were ELISA-reactive. Importantly, the same three pigs were negative by real-time PCR on EDTA blood. Thus, the observed pattern was one of serological reactivity without simultaneous molecular evidence of viraemia.

This interpretation is consistent with the WOAH diagnostic framework. WOAH distinguishes between direct detection of ASFV and detection of the immune response, identifying PCR and real-time PCR as highly sensitive, specific and rapid techniques for the detection of ASFV genomic DNA. It also notes that surviving pigs generally develop detectable antibodies approximately 7–10 days after infection and that these antibodies may persist for long periods. Accordingly, an ELISA-positive/PCR-negative profile cannot, by itself, demonstrate active infection at the time of sampling and should be interpreted together with epidemiological history and, where appropriate, additional confirmatory investigations.

In Ukraine, ASFV DNA was not detected in the tested animal blood, feed samples or collected blood-sucking arthropods during either sampling period. This similarity between the two

national datasets is epidemiologically relevant because neither component of the project provided direct molecular evidence of ASFV circulation in the materials examined. Nevertheless, the Romanian serological signal in domestic pigs warrants continued risk-based surveillance, particularly because WOAH identifies domestic pigs and wild boar as the principal naturally susceptible hosts and emphasises the importance of laboratory confirmation in ASF surveillance.

The inclusion of golden jackals and arthropods in the Romanian sampling scheme provides additional information on the wildlife–domestic animal interface but should not be assigned the same epidemiological weight as investigations in Suidae. Similarly, WOAH specifically recognises Ornithodoros soft ticks as biological reservoirs and vectors in epidemiological systems where the sylvatic tick cycle is present; therefore, negative results obtained from a small number of non-speciated tick samples should not be generalised to the wider arthropod fauna.

Avian influenza

Avian influenza produced one of the clearest contrasts between the Romanian and Ukrainian components of the study. In Romania, 408 serum samples from birds and mammals were examined by multispecies influenza A ELISA, with only five reactive samples: two chickens, two domestic ducks and one golden jackal. The four ELISA-reactive poultry samples were subsequently negative by haemagglutination inhibition against the H5N3, H5N8 and H7N1 antigens tested. Moreover, all 444 Romanian real-time RT-PCR determinations targeting the conserved influenza A matrix gene were negative. Taken together, these findings did not provide direct molecular evidence of active influenza A infection or evidence supporting active H5/H7 circulation within the Romanian project sample set during the investigated periods.

WOAH considers direct detection of influenza A viral RNA by real-time RT-PCR a major diagnostic approach and notes that direct molecular detection has largely replaced virus isolation as an initial diagnostic method. WOAH also recognises ELISA assays directed against conserved nucleoprotein or matrix antigens as useful group-level serological methods, including competitive ELISA formats that can be applied across species. Consequently, the limited Romanian ELISA reactivity should primarily be interpreted as evidence of influenza A group-level antibody reactivity rather than as evidence of H5 or H7 infection, particularly because the four reactive poultry samples were not confirmed by the subtype-oriented HI investigations performed.

In contrast, the Ukrainian component detected influenza A viral RNA in wild-bird faecal samples and in water associated with wetland habitats during both sampling periods. In the first period, positive detections involved material associated with dunlin (Calidris alpina) and mallard (Anas platyrhynchos), together with a water sample. Additional influenza A RNA-positive wild-bird faecal and water samples were identified in spring, including material associated with mallard,

ruff (Philomachus pugnax) and dunlin. However, subsequent testing did not identify H5 or H7 in these samples.

This pattern is biologically plausible in the context described by WOAH, which identifies aquatic birds as a major natural reservoir of influenza A viruses and notes that the overwhelming majority of viruses circulating in these hosts are of low pathogenicity for poultry. The Ukrainian detections therefore demonstrate circulation of influenza A viruses within the wetland/wild-bird component of the investigated ecosystem, while the available project results do not demonstrate H5/H7 involvement. Because WOAH emphasises that H5 and H7 low-pathogenicity viruses require particular attention due to their potential to acquire high-pathogenicity characteristics, continued subtype-oriented surveillance remains justified even when screening samples are H5/H7-negative.

The Romania–Ukraine difference should not be interpreted simply as absence of circulation on one side of the border and presence on the other. The Ukrainian positive results arose mainly from samples directly connected to wild migratory birds and aquatic environments, whereas the Romanian dataset incorporated a substantial domestic-animal and mammalian-interface component. Differences in sampling matrices, locations, timing relative to migration and concentration of birds at particular wetlands therefore represent plausible contributors to the different detection patterns. From a surveillance perspective, these findings support maintaining coordinated sampling on both sides of the border during periods of major bird migration and prioritising wild waterfowl, fresh faecal material, cloacal/oropharyngeal swabs and strategically selected environmental samples.

Leptospirosis

The leptospirosis findings show a different epidemiological pattern. In Romania, serological reactivity against Leptospira interrogans serovar Hardjo was repeatedly detected in cattle: 37 of 132 bovine sera were positive and 11 were classified as doubtful. Reactive cattle originated from several localities and were identified during both project periods. In contrast, all tested sera were negative for Pomona, while the golden-jackal and feline sera included in this component were negative. All 171 Romanian real-time PCR determinations were also negative for Leptospira genomic material.

The combined finding of repeated Hardjo seroreactivity and negative blood-based molecular testing should not be regarded as contradictory. WOAH identifies serological testing as the most widely used approach to leptospirosis diagnosis and considers the microscopic agglutination test (MAT) the standard serological test. ELISA may nevertheless be useful for screening and is specifically used in cattle health programmes to assess infection with serovar Hardjo. In parallel, PCR can detect leptospiral nucleic acid in tissues or body fluids, but WOAH emphasises that performance depends on sample processing, matrix, species and quality-control procedures and that molecular tests generally do not identify the infecting serovar.

This distinction is particularly relevant to chronic leptospirosis. WOAH notes that leptospires may persist in the kidney, urine or genital tract in clinically normal carrier animals and that localisation in these sites may continue after leptospiraemia has ceased. Consequently, uniformly negative blood PCR results do not eliminate the possibility of previous exposure or chronic renal/genital carriage. Indeed, WOAH explicitly states that failure to demonstrate leptospires in urine does not necessarily eliminate chronic renal carriage because shedding may be intermittent.

The Romanian Hardjo pattern therefore represents an epidemiologically relevant signal that merits additional investigation. Confirmation and refinement of this signal would benefit from MAT using a panel of locally relevant serogroups, documentation of vaccination history and, where epidemiologically indicated, examination of urine, kidney or genital material rather than reliance exclusively on peripheral blood. WOAH also cautions that vaccination against the serovar of interest can generate positivity in some ELISA assays and should therefore be taken into account when interpreting individual and herd results.

In the Ukrainian animal dataset, neither antibodies nor pathogenic Leptospira DNA were detected. However, Leptospira-genus DNA was identified in two environmental water samples, from the Kar'yer water body at Vylkove and the Stentsivsko-Zhebriianivski floodplains near Prymorske, and was interpreted in the report as most probably representing non-pathogenic species. These environmental findings should therefore not be treated as evidence of pathogenic leptospiral transmission, but they demonstrate the usefulness of environmental sampling as an additional component of the One Health surveillance framework.

Taken together, the Romanian and Ukrainian findings illustrate different components of the leptospirosis epidemiological system: evidence of bovine immune exposure on the Romanian side, absence of corresponding direct detection in tested animal matrices, and environmental detection of Leptospira-genus DNA without evidence of pathogenic species on the Ukrainian side. This combination reinforces the value of integrating host, environmental and serological information rather than relying on a single diagnostic method.

Toxoplasmosis

Interpretation of toxoplasmosis is more limited because the datasets available for the present Romanian update contained no PP2 serological or molecular investigations for Toxoplasma gondii. Consequently, no Romania-specific laboratory comparison can currently be made for this disease. The Ukrainian investigations of whole blood, faecal material and serum, together with water testing, did not detect T. gondii DNA or antibodies in the materials examined.

These negative findings describe only the investigated samples and should not be interpreted as evidence that T. gondii is absent from the cross-border ecosystem. The absence of comparable Romanian laboratory data is also an important limitation for the joint analysis. A

more balanced cross-border assessment would require comparable host species, diagnostic methods, sampling periods and environmental matrices on both sides of the border.

West Nile virus infection

West Nile virus generated the strongest serological signal in the Romanian component. Among 432 serum samples, 168 were positive and eight doubtful by competitive ELISA. Reactivity occurred in several species, including cattle, chickens, sheep, swine, golden jackals and ducks. All samples from dogs and the single cat were negative. In contrast, WNV RNA was not detected in any of the 140 real-time RT-PCR determinations performed on animal blood samples.

The coexistence of extensive serological reactivity and uniformly negative RT-PCR results is epidemiologically plausible. WOAH describes WNV as a mosquito-borne flavivirus maintained through a bird–mosquito–bird cycle, with humans and horses functioning primarily as dead-end hosts. It also emphasises the importance of migratory birds for geographic dispersal and reintroduction of the virus. WNV infections are frequently inapparent, and diagnostic interpretation therefore requires integration of clinical and laboratory evidence.

RT-PCR is appropriate for detecting viral nucleic acid, but detection depends strongly on sampling during the period in which virus is present in the tested matrix. Serological responses can remain detectable after peripheral viraemia has disappeared. Accordingly, negative blood RT-PCR results do not negate the epidemiological significance of antibody detection and instead indicate that detectable viraemia was not demonstrated in the sampled animals at the time of collection. WOAH recognises RT-PCR for direct agent detection and ELISA, virus neutralisation and plaque-reduction neutralisation methods for antibody detection.

At the same time, interpretation of the high Romanian ELISA reactivity requires caution because WOAH reports possible serological cross-reactions with related flaviviruses, including Usutu virus, Japanese encephalitis virus and tick-borne encephalitis virus. Therefore, the observed ELISA positivity should be described as WNV-reactive serological evidence unless confirmed by a more specific neutralisation technique such as PRNT or VN. This limitation is particularly important when interpreting results from species for which the assay has not been established as a definitive diagnostic method.

The difference between the completely negative first Romanian sampling period and the extensive serological reactivity observed during 2026 should likewise not be interpreted as direct evidence of a rapid increase in infection. The populations sampled during the two periods differed substantially in species composition, number of animals and geographical coverage. Nevertheless, detection of reactive antibodies across several species during the second period represents an epidemiologically important signal and is consistent with the ecological suitability of the Danube Delta for mosquito-borne transmission. WOAH notes that WNV transmission is

seasonal in temperate climates and generally reaches maximum intensity toward late summer and early autumn in the Northern Hemisphere.

For future surveillance, these findings support closer integration of vertebrate serology with mosquito surveillance, wild-bird surveillance and, when possible, confirmatory neutralisation testing. Such a design would improve discrimination between historical exposure, current transmission and possible cross-reactivity with other flaviviruses.

Cross-border epidemiological interpretation

When considered collectively, the project results reveal heterogeneous rather than uniform pathogen circulation across the Romania–Ukraine study area. ASF showed no molecular evidence of active circulation in either project component, although three Romanian pigs displayed ASFV antibody reactivity. Avian influenza showed the opposite geographical pattern: Romania generated only limited group-level serological signals with no molecular detection, whereas Ukraine demonstrated influenza A RNA in wild-bird-associated and aquatic samples, without detection of H5 or H7. Leptospirosis produced a repeated bovine Hardjo serological signal in Romania but no direct molecular detection in animals, while Ukrainian environmental testing detected Leptospira-genus DNA in two water samples interpreted as probably non-pathogenic. Toxoplasmosis testing was negative in Ukraine, but comparable Romanian results were unavailable. Finally, WNV generated substantial multispecies serological reactivity in Romania without concurrent viral RNA detection.

These differences emphasise that the cross-border area should be regarded as a connected ecological system with pathogen-specific transmission pathways, rather than as two epidemiologically isolated national territories. Migratory birds can cross the border independently of administrative boundaries; hydrological connections can transport biological material between wetlands; mosquito abundance and activity vary according to temperature and rainfall; and movements of domestic animals and wildlife create repeated opportunities for pathogen introduction or exposure. Surveillance should therefore remain coordinated at cross-border level while retaining disease-specific sampling strategies.

The results are also consistent with a One Health surveillance concept. Information derived from domestic animals, wildlife and environmental samples was complementary rather than interchangeable. Wild-bird and water sampling contributed particularly to influenza surveillance; domestic cattle were informative for leptospiral and WNV exposure; swine remained the central target for ASF investigation; and wildlife samples provided information on interfaces between natural ecosystems and animal-production systems. Environmental sampling in Ukraine added a further layer of information that could not have been obtained from animal testing alone.

Methodological robustness and harmonisation

An important strength of the study was the combined use of molecular and serological approaches. Direct detection by PCR or real-time RT-PCR addresses the presence of pathogen nucleic acid at or around the time of sampling, while serology provides information on previous exposure or immune response. The two approaches therefore answer different epidemiological questions and should not be expected to produce identical patterns.

The diagnostic framework used for ASF, avian influenza, leptospirosis and WNV was broadly aligned with the methodological principles described in the corresponding WOAH chapters. For ASF, molecular detection and serology were combined; for influenza A, group-level molecular and serological screening was complemented, where relevant, by H5/H7-oriented testing; for leptospirosis, PCR and antibody detection were used in parallel; and for WNV, RT-PCR and serological screening provided complementary information.

Nevertheless, further harmonisation would strengthen subsequent joint investigations. Particular attention should be given to comparable biological matrices, synchronised sampling periods, common case and sampling definitions, assay validation for each host species, harmonised criteria for positive/doubtful results and predefined confirmatory algorithms. For leptospirosis, incorporation of MAT and appropriate renal/urinary/genital samples would strengthen interpretation of serological findings. For WNV, confirmatory PRNT/VN testing of selected ELISA-reactive samples would substantially increase specificity. For avian influenza, synchronised surveillance focused on wild-bird migration periods and comparable bird and environmental matrices would facilitate more direct cross-border comparisons.

Study limitations

Several limitations must be considered when interpreting the findings. First, sampling was targeted and risk-oriented rather than probabilistic; consequently, proportions of positive, doubtful and negative samples describe the investigated project material and must not be interpreted as population prevalence estimates. Second, sample numbers, species composition and geographical coverage differed substantially between diseases, countries and sampling periods.

Third, the diagnostic value of a negative molecular result depends on the pathogen, biological matrix and timing of collection. Transient viraemia or leptospiraemia, intermittent shedding and localisation of pathogens in tissues not sampled during the study may reduce the probability of direct detection. This consideration is particularly relevant for WNV and leptospirosis.

Fourth, serology also presents pathogen-specific limitations. Influenza A antibody detection is primarily group-specific unless additional subtype testing is undertaken; WNV ELISA may cross-react with related flaviviruses; leptospiral antibody results require consideration of vaccination

and ideally confirmation or characterisation using MAT; and ASF serological reactivity must be interpreted together with direct agent detection and the epidemiological situation.

Fifth, environmental detections require particularly cautious interpretation. Detection of nucleic acid in water or other environmental matrices does not necessarily demonstrate the presence of viable infectious organisms or transmission to susceptible hosts. Conversely, negative environmental samples provide information only for the specific locations, matrices and collection times represented.

Finally, direct Romania–Ukraine comparisons are affected by differences in sampling design. The Ukrainian avian influenza component, for example, included intensive examination of material associated with migratory wild birds and aquatic environments, whereas the Romanian dataset contained a larger domestic-animal and wildlife-interface component. Similarly, Romania generated extensive multispecies WNV serological information, whereas equivalent cross-border coverage was not available for all categories. These differences should be preserved explicitly in interpretation rather than masked through aggregation.

Overall interpretation

Overall, the study does not indicate a single uniform epidemiological pattern across the Lower Danube cross-border area. Instead, it identifies a series of disease-specific epidemiological signals: ASF serological reactivity requiring follow-up in Romanian domestic pigs; influenza A viral RNA circulation associated with wild birds and aquatic environments in Ukraine but without H5/H7 detection; repeated Hardjo-associated serological reactivity in Romanian cattle; no demonstrated T. gondii infection in the Ukrainian samples investigated and insufficient Romanian data for cross-border comparison; and extensive WNV-reactive serology in several Romanian animal species without concurrent molecular evidence of viraemia.

These findings support continuation of coordinated, risk-based and seasonally adapted surveillance. Future campaigns should focus not simply on increasing sample numbers but on improving comparability of sampling, epidemiological metadata, host and matrix selection, confirmatory testing and temporal synchronisation between the two countries. Such an approach would allow the surveillance system developed through the project to evolve from descriptive cross-border screening toward an integrated early-warning framework capable of identifying changes in pathogen circulation at the animal–human–environment interface.

CONCLUSIONS

The joint study confirmed the relevance of an integrated cross-border approach for monitoring emerging and re-emerging diseases in the Lower Danube region. The sampling and analysis activities carried out by the partners demonstrated the usefulness of information exchange, procedure harmonization, and joint interpretation of results.

The data obtained support the strengthening of regional epidemiological surveillance and highlight the need to continue cooperation between the partners, especially for diseases with a zoonotic component and for pathogens influenced by the dynamics of the environment and wildlife.

The activities carried out by PP2 – Sanitary Veterinary and Food Safety Directorate of Tulcea – achieved the main objectives established for the Romanian component of Activity A1.4 by implementing risk-based field sampling and laboratory investigations targeting diseases of major veterinary and One Health relevance in the Romanian Danube Delta and adjacent areas. The study integrated domestic animals, wildlife and species located at the wildlife–domestic animal interface and combined serological and molecular diagnostic approaches in order to assess both evidence of previous exposure and evidence of active pathogen circulation.

The Romanian results demonstrate that the investigated diseases displayed different epidemiological patterns and that their interpretation requires the integration of laboratory findings with host species, biological matrix, sampling period, geographical origin and ecological context.

For African swine fever, no ASFV genomic material was detected in any of the 65 completed real-time PCR determinations performed on swine, golden jackals and tick samples. Three domestic pigs showed ELISA reactivity, but ASFV genomic material was not detected by real-time PCR in the same animals. Therefore, the Romanian dataset did not provide direct molecular evidence of active ASFV circulation during the investigated periods. The three serologically reactive pigs nevertheless constitute a specific epidemiological signal that requires continued risk-based surveillance and appropriate epidemiological follow-up.

This interpretation is consistent with the WOAH diagnostic framework, which differentiates direct detection of the virus from detection of the immune response. WOAH recognises PCR and real-time PCR as sensitive, specific and rapid techniques for ASFV detection and notes that antibodies may develop approximately 7–10 days after infection and persist for long periods. Consequently, serological reactivity in the absence of PCR detection cannot, by itself, be interpreted as evidence of active viraemia at the time of sampling.

For avian influenza, the Romanian investigations generated no direct molecular evidence of influenza A virus infection in the tested materials. All 444 real-time RT-PCR determinations targeting the conserved influenza A matrix gene were negative. Five ELISA-reactive sera were

identified, including samples from chickens, domestic ducks and one golden jackal. The four ELISA-reactive poultry sera subsequently tested by haemagglutination inhibition were negative for antibodies against the H5N3, H5N8 and H7N1 antigens investigated. Consequently, the combined Romanian molecular and serological results do not support active H5/H7 circulation in the investigated project sample set during the sampling periods.

The findings are compatible with the WOAH diagnostic approach. Real-time RT-PCR is recognised as an important method for direct detection and characterisation of influenza A viruses, while serological ELISA assays targeting conserved nucleoprotein or matrix antigens are appropriate for group-level screening but do not, by themselves, establish the infecting subtype. The limited influenza A serological reactivity recorded in Romania should therefore be retained as an epidemiological screening signal supporting continued surveillance, particularly at the domestic bird–wild bird interface and during periods of increased migratory activity.

For leptospirosis, one of the most relevant Romanian findings was the repeated detection of serological reactivity against Leptospira interrogans serovar Hardjo in cattle. Hardjo-reactive animals were identified during both sampling periods and in several localities, including Sulina, Letea and Chilia Veche. At the same time, Leptospira genomic material was not detected in any of the 171 completed real-time PCR determinations performed on cattle, sheep, dogs, golden jackals and one rat. The results therefore indicate evidence of immune exposure within the investigated bovine population but no molecular evidence of detectable leptospiraemia in the biological materials tested at the time of sampling.

This distinction is important because WOAH identifies MAT as the standard serological test for leptospirosis while recognising ELISA as useful for screening, including assessment of Hardjo infection in cattle. WOAH also notes that PCR performance depends on the biological matrix, stage of infection, sample processing and quality-control procedures. Consequently, negative molecular results in blood do not necessarily exclude previous exposure or a chronic renal or genital carrier state. The Romanian findings therefore support further serological characterisation, including MAT where appropriate, consideration of vaccination history, and the inclusion of urine, renal or genital samples in targeted investigations where epidemiologically justified.

For toxoplasmosis, the datasets available for the present Romanian update did not contain PP2 serological or molecular investigations for Toxoplasma gondii. Consequently, no Romania-specific laboratory conclusion regarding T. gondii circulation can be derived from the datasets included in the present report. This absence of comparable Romanian results should be considered when interpreting the joint cross-border findings and when planning subsequent sampling campaigns.

For West Nile virus infection, the Romanian component identified the most extensive serological signal among the diseases investigated. Of 432 serum samples examined by

competitive ELISA, 168 were positive and eight doubtful, with reactivity recorded in several domestic and wildlife species. However, WNV RNA was not detected in any of the 140 real-time RT-PCR determinations. Therefore, the Romanian data demonstrate substantial WNV-reactive serological evidence within the targeted project sample set but provide no direct molecular evidence of detectable viraemia at the time of sampling. Because the sampling scheme was targeted and the sampled populations differed between periods, the observed proportions cannot be interpreted as population prevalence or as evidence of a quantified temporal increase in infection.

WOAH describes WNV as a mosquito-borne flavivirus maintained predominantly in a bird–mosquito–bird transmission cycle and recognises the importance of migratory birds in viral dispersal. It also recognises RT-PCR for detection of viral nucleic acid and several serological methods for detection of antibodies, while warning that serological cross-reactions may occur with related flaviviruses such as Usutu virus. Accordingly, the Romanian ELISA results should be regarded as WNV-reactive serological evidence and, for epidemiologically important samples, should ideally be complemented by specific neutralisation methods such as PRNT or VN.

Overall, the Romanian component confirms the value of combining molecular detection, serological surveillance, domestic-animal monitoring and wildlife-interface investigations. The results did not provide molecular evidence of active ASFV, influenza A, pathogenic Leptospira or WNV circulation in the tested Romanian biological materials at the time of collection, but they identified several epidemiologically relevant serological signals: ASFV reactivity in a limited number of domestic pigs, influenza A group-level reactivity in poultry and one golden jackal, repeated Hardjo-associated reactivity in cattle, and extensive WNV-reactive serology across several species.

These findings demonstrate that surveillance based exclusively on direct pathogen detection would provide an incomplete epidemiological picture. Conversely, serological reactivity must not be equated automatically with active infection. The complementary use of both approaches, interpreted according to WOAH principles and together with epidemiological metadata, provides a substantially stronger basis for risk assessment.

The activities implemented by DSVSA Tulcea also strengthened the operational capacity for integrated surveillance in the Romanian Danube Delta by expanding sampling to several host categories, incorporating wildlife and wildlife–domestic animal interfaces, applying harmonised laboratory procedures and generating structured epidemiological information that can be used as a baseline for subsequent monitoring campaigns.

The Romanian results therefore support the continuation of risk-based, seasonal and multi-species surveillance, with particular attention to migratory bird periods, mosquito activity, livestock–wildlife contact areas and wetland ecosystems. Future investigations should also

strengthen confirmatory testing and optimise the selection of biological matrices according to the epidemiology of each pathogen.

Beyond the disease-specific findings summarized above, the work carried out by Partner 3 (Odesa State Agrarian University) made a substantive contribution to the overall objectives of the joint study. By organizing systematic sampling across the full range of ecological zones relevant to transboundary disease circulation on the Ukrainian side — river branches, freshwater lakes, wetlands, agricultural land, and settlements in direct contact with wild avifauna — and testing all collected material under BSL-2 biosafety conditions in line with the joint Terms of Reference, Partner 3 ensured that the Ukrainian dataset was both methodologically comparable with the Romanian results and ecologically representative of the highest-risk segment of the cross-border area, the Danube Delta itself.

Across the two sampling periods, close to 1,160 biological and environmental samples were collected and tested on the Ukrainian side alone, covering all five priority diseases identified for the project. This volume of harmonised, quality-controlled data provides a solid empirical foundation not only for the present report, but for future joint surveillance planning, and demonstrates the research capacity of a regional academic institution operating within a clearly defined technical framework, delivering research output at a scale and standard suitable for cross-border epidemiological cooperation.

Equally important is the institutional legacy of this work. The cooperation model established between Partner 3 and the Romanian partner — built on a shared Terms of Reference, synchronized sampling calendars, and joint interpretation of results — offers a replicable template for future transboundary disease monitoring in the Lower Danube region, extending in principle to Moldova as a third neighboring state sharing the same ecological corridor. The continuation of this cooperation, and the further development of the laboratory and field capacity built during the project, should be regarded as a direct and lasting outcome of Activity 1.4, alongside the specific epidemiological findings reported above.

1. Based on the results of the laboratory analyses in Ukraine, the presence of the ASF pathogen was not established in the test animals; however, persistence of the influenza A virus pathogen was detected, indicating an additional antigenic burden that may be a factor reducing the resistance of the pigs' organism.
2. The Ukrainian studies established the circulation of low-pathogenic isolates in wild waterfowl populations, which is a normal situation but indicates the need to intensify monitoring in the Danube Delta and adjacent territories, as well as to conduct research for the in-depth determination of the molecular-genetic characteristics of the low-pathogenic influenza viruses detected.
3. Circulation of non-pathogenic leptospiras has been laboratory-confirmed in Ukraine in a number of water bodies of the Odesa region, demonstrating the potential risks of pathogenic leptospirae reproduction in these biotopes. No positive results were found for

the presence of leptospirosis pathogen DNA (PCR) or specific antibodies to it (serology) among farm and domestic animals, but monitoring of this infection should be continued in the region, given the existing cross-border risks and historical trends.

4. Studies conducted to detect the toxoplasmosis pathogen on Ukrainian project territory found no positive results for the presence of toxoplasmosis pathogen DNA (PCR) or specific antibodies to it (serology) among farm and domestic animals, but monitoring of this infection should be continued in the region, given the existing cross-border risks and historical trends.

The work performed is of significant importance for ensuring the veterinary wellbeing not only of the Odesa region, but of the entire cross-border Ukraine-Romania-Moldova region. The results obtained made it possible to lay the groundwork for objective monitoring of the circulation of especially dangerous infection pathogens; assessment of epizootic risks for the region based on scientifically substantiated data; the development of recommendations for the prevention of cross-border diseases; the strengthening of international cooperation in veterinary medicine; and the implementation of the “One Health” approach.

Overall conclusions of Activity A1.4 – Joint research of emerging diseases in the Romania–Ukraine cross-border area

Activity A1.4 demonstrated the practical and scientific value of an integrated cross-border surveillance system for emerging and re-emerging diseases in the Lower Danube region. The joint work performed by PP2 – Sanitary Veterinary and Food Safety Directorate of Tulcea – and PP3 – Odesa State Agrarian University – combined field sampling, laboratory diagnosis, wildlife surveillance and environmental investigation within a common methodological framework. The use of common Terms of Reference, coordinated sampling periods, harmonised laboratory principles and joint interpretation created a functional basis for comparable epidemiological assessment across the Romania–Ukraine border. The original report similarly identifies methodological harmonisation, information exchange and joint interpretation as major outcomes of the activity.

The joint results demonstrate that pathogen circulation in the cross-border territory is heterogeneous and disease-specific, reflecting the complex interaction between domestic animals, wildlife, vectors, aquatic ecosystems and seasonal environmental conditions.

For African swine fever, neither the Romanian nor the Ukrainian molecular investigations demonstrated ASFV DNA in the biological or environmental materials examined during the project periods. In Romania, however, three domestic pigs showed ASFV serological reactivity without concurrent PCR detection. In Ukraine, ASFV DNA was not detected in animal blood, feed or the collected blood-sucking arthropods. Taken together, the joint results provide no direct molecular evidence of active ASFV circulation within the project samples, while the Romanian serological findings justify continued targeted surveillance in susceptible suid populations.

For avian influenza, the cross-border comparison produced an important ecological distinction. No influenza A RNA was detected in the Romanian molecular dataset, whereas the Ukrainian investigations demonstrated influenza A RNA in samples associated with migratory wild birds and aquatic environments. Subsequent testing of the Ukrainian positive samples did not identify H5 or H7, and the findings were interpreted in the report as circulation of low-pathogenic influenza A viruses. During the spring period, influenza A RNA was again detected in wild-bird-associated samples involving mallard, ruff and dunlin, while no influenza A RNA was detected in the corresponding soil samples.

This result is particularly relevant to the ecology of the Danube Delta because WOAH identifies aquatic birds as an important natural reservoir of influenza A viruses and notes that many viruses circulating in these populations are of low pathogenicity. The Ukrainian detections, together with the absence of H5/H7 identification, do not indicate circulation of H5/H7 viruses within the samples concerned, but they demonstrate that the wild-bird–wetland interface functions as an important surveillance target. The results therefore support continued synchronised surveillance on both sides of the border during major migration periods.

For leptospirosis, the two national datasets reveal complementary epidemiological information. Romania showed repeated Hardjo-associated serological reactivity in cattle without PCR detection of Leptospira DNA. In Ukraine, pathogenic Leptospira DNA and specific antibodies were not detected in the tested animals, while two environmental water samples contained DNA belonging to the genus Leptospira, interpreted in the report as most probably originating from non-pathogenic species. These findings illustrate the value of combining animal and environmental surveillance and also demonstrate why serological, molecular and ecological evidence must be interpreted together rather than independently.

For toxoplasmosis, the Ukrainian testing did not identify T. gondii DNA or specific antibodies in the examined animal samples, and environmental water samples were also negative. Comparable PP2 results were not available in the Romanian datasets used for the current version of the report. Consequently, the available evidence does not allow a balanced bilateral assessment of T. gondii circulation, and future project-related surveillance would benefit from comparable sampling and testing on both sides of the border.

For West Nile virus, the Romanian findings provide a substantial serological indication of exposure across several animal species, although WNV RNA was not detected in the materials examined. This pattern is epidemiologically compatible with the WOAH description of WNV infection, in which virus circulation is maintained mainly through the mosquito–bird cycle, vertebrate viraemia may be transient, and serological evidence can persist beyond the period in which viral RNA is detectable. Because serological cross-reactivity with other flaviviruses is possible, the results should be interpreted as WNV-reactive evidence unless confirmed using a more specific neutralisation method.

Taken together, the results demonstrate that no single species, sample type or diagnostic method is sufficient to characterise epidemiological risk in the Lower Danube ecosystem. The project therefore validates the rationale of the One Health approach, in which information from livestock, wildlife, vectors and environmental matrices is integrated to identify epidemiological signals that would remain undetected if these components were investigated separately.

An important outcome of Activity A1.4 is also the establishment of a common technical framework for future surveillance. The Ukrainian component alone covered close to 1,160 biological and environmental samples across the two periods, while systematic Romanian sampling generated extensive molecular and serological datasets from domestic animals and wildlife. More importantly than the absolute number of samples, the activity demonstrated that institutions from the two countries can implement coordinated sampling, exchange technical information and interpret laboratory results within a common epidemiological framework.

At the same time, the results confirm several methodological limitations that must be taken into account. Sampling was targeted and risk-based rather than probabilistic; host species, sample numbers and matrices were not identical between countries or sampling periods; environmental sampling was more extensive on the Ukrainian side; and Romania-specific toxoplasmosis results were unavailable for the current analysis. Therefore, positivity rates reported within the project describe the investigated samples and must not be interpreted as estimates of disease prevalence in the general animal population or in the entire cross-border region.

Despite these limitations, the activity achieved an essential objective: the creation of a common epidemiological baseline for future cross-border monitoring. The findings identify concrete signals requiring continued attention—ASF serological reactivity in Romanian pigs, influenza A circulation associated with Ukrainian wild birds and aquatic habitats, Hardjo-associated serological exposure in Romanian cattle, environmental Leptospira detection in Ukraine, and widespread WNV-reactive serology in the Romanian multispecies dataset.

The overall scientific conclusion of Activity A1.4 is therefore that the Lower Danube and Danube Delta should be approached as a single epidemiologically interconnected ecological system, even though pathogen detection patterns may differ locally and temporally between the Romanian and Ukrainian territories. Wildlife movements, migratory bird routes, wetlands, vector populations and hydrological connections operate independently of administrative borders and require coordinated surveillance.

The project has consequently provided 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 the partners would transform the infrastructure and

cooperation established through Activity A1.4 into a sustainable surveillance mechanism for emerging and re-emerging diseases in the Lower Danube region.

In this respect, one of the most important long-term results of Activity A1.4 is the transition from isolated national observations toward a shared Romania–Ukraine surveillance framework based on coordinated field investigation, comparable laboratory methods, joint interpretation and the One Health concept. This framework represents a relevant foundation for the prevention, early detection and coordinated management of future cross-border epidemiological threats.

RECOMMENDATIONS

The findings obtained within Activity A1.4 support the continuation and further development of coordinated surveillance of emerging and re-emerging diseases in the Romania–Ukraine cross-border area. Future activities should preserve the common methodological framework established during the project while increasing the comparability of the Romanian and Ukrainian datasets, strengthening confirmatory testing, and integrating information from domestic animals, wildlife, vectors and environmental matrices.

The recommendations below derive from the epidemiological signals identified during the two sampling periods and from the diagnostic principles described in the relevant WOAH standards.

Continuation of coordinated cross-border surveillance

Coordinated monitoring of African swine fever, avian influenza, leptospirosis, toxoplasmosis and West Nile virus infection should be continued beyond the current sampling periods. Surveillance should retain a risk-based and seasonally adapted approach, with sampling intensity focused on periods and locations where the probability of pathogen introduction, circulation or detection is highest.

Particular attention should be given to the ecological continuity of the Lower Danube and Danube Delta. Wetlands, migratory bird routes, wildlife movement, vector activity and hydrological connections function independently of administrative borders. Consequently, surveillance programmes on the Romanian and Ukrainian sides should be planned as complementary components of a common epidemiological system rather than as independent national exercises.

Whenever operationally possible, future Romanian and Ukrainian campaigns should use synchronised sampling periods, comparable host categories and equivalent biological matrices. This would allow more robust interpretation of differences between territories and between successive sampling periods. The cooperation model already established through common Terms of Reference, synchronised calendars and joint interpretation provides a suitable basis for this continuation.

Strengthening methodological harmonisation and comparability

The common sampling and laboratory framework developed within the project should be maintained and progressively refined. Future campaigns should use harmonised definitions for sample categories, epidemiological units, positive, negative, doubtful and inconclusive results, together with common procedures for recording relevant epidemiological metadata.

For each sample, the epidemiological database should, as far as possible, include host species, age, sex, holding or sampling location, date of collection, clinical status, vaccination history where relevant, contact with wildlife, environmental characteristics and laboratory method used.

The same disease should preferably be investigated using comparable diagnostic algorithms in both countries. Where different assays or kits are used, their intended purpose, validation status, analytical characteristics and interpretation criteria should be documented so that differences in laboratory methodology are not mistaken for epidemiological differences.

Direct pathogen detection and serology should continue to be regarded as complementary methods. Molecular methods provide information concerning pathogen nucleic acid in the tested material at the time of sampling, whereas serological methods primarily provide evidence of previous exposure or immune response. The two categories of results should therefore not be interpreted interchangeably.

African swine fever

ASF surveillance should remain focused primarily on domestic pigs, wild boar and other susceptible suids, with investigations of other wildlife species or arthropods retained mainly as supplementary information regarding the wildlife–domestic animal interface.

WOAH distinguishes direct agent detection from serological detection and identifies PCR and real-time PCR as highly sensitive, specific and rapid techniques for ASFV detection. At the same time, antibodies may appear approximately 7–10 days after infection and can persist for long periods.

Accordingly, the three Romanian pigs that were ELISA-reactive but PCR-negative should remain subject to epidemiological follow-up within the competent surveillance system. Where justified by the epidemiological context, serologically reactive or otherwise suspect results should be investigated using appropriate supplementary or confirmatory procedures rather than being interpreted solely on the basis of a single screening result.

Continuation of active and passive surveillance in domestic swine and wild boar populations is warranted, particularly in locations characterised by previous ASF occurrence, frequent wildlife–domestic animal interaction or movements of susceptible animals.

For arthropod investigations, interpretation should take account of the fact that WOAH specifically identifies soft ticks of the genus Ornithodoros as recognised ASFV reservoirs and vectors in epidemiological systems where these ticks occur. Consequently, results obtained from non-speciated ticks should be considered supplementary unless the vector species and its epidemiological relevance are established.

Avian influenza

Avian influenza surveillance should be intensified around wild aquatic birds, migratory bird concentrations and wetlands, particularly during autumn and spring migration.

This recommendation is strongly supported by the Ukrainian findings, where influenza A RNA was repeatedly detected in samples associated with migratory mallard, dunlin and ruff

populations and in aquatic environmental samples, whereas H5 and H7 were not detected. The report therefore identifies the wild-bird–aquatic environment interface as an important component of influenza A circulation in the project area.

WOAH identifies aquatic birds as a major natural reservoir of influenza A viruses and notes that many viruses circulating within these populations are of low pathogenicity. The detection of influenza A RNA without H5/H7 identification should therefore lead to continued surveillance and, where technically feasible, further molecular characterisation rather than being regarded as an endpoint.

Future sampling should prioritise:

- recently collected oropharyngeal/cloacal material from appropriate bird species;
- fresh wild-bird faecal material from well-characterised bird populations;
- carcasses or organs from dead or clinically affected wild birds where available;
- domestic poultry holdings located close to wetlands or areas with intense wild-bird activity;
- selected aquatic environmental samples where their epidemiological relevance can be clearly linked to bird concentrations.

For molecular surveillance, generic influenza A detection should be followed by H5/H7-specific investigations whenever influenza A RNA is detected. WOAH recognises direct molecular detection, including real-time RT-PCR, as an important approach for influenza A diagnosis and characterisation.

Serological ELISA should continue to be used as a screening tool, but positive reactions should be interpreted as influenza A group-level reactivity unless supported by appropriate subtype-specific testing. WOAH notes that ELISA assays targeting conserved nucleoprotein and matrix antigens are widely used for influenza A antibody detection.

Where isolates or sufficiently suitable RNA-positive materials are obtained, additional molecular characterisation, sequencing and phylogenetic analysis would provide important information concerning subtype, genetic relationships and possible movement of viruses through migratory routes.

Leptospirosis

The repeated Hardjo-associated ELISA reactivity identified in Romanian cattle warrants further targeted investigation. Future surveillance should integrate ELISA screening with MAT and direct agent detection using biologically appropriate matrices.

WOAH identifies MAT as the standard serological test for leptospirosis and notes that ELISA may be useful for antibody detection, including assessment of serovar Hardjo infection in cattle. Therefore, MAT using a panel of serogroups epidemiologically relevant to the region should be

incorporated, where available, to improve interpretation of the Romanian bovine serological signal.

Vaccination history should be systematically recorded because vaccination against the serovar of interest may affect interpretation of some ELISA results.

The biological matrix used for molecular investigation should also be adapted to the presumed phase of infection. WOAH notes that leptospiral nucleic acid can be demonstrated in tissues and body fluids by PCR but that diagnostic performance depends strongly on sample processing, tissue or fluid type and host species.

Where chronic infection or carriage is suspected, urine, kidney or genital material should therefore be considered in addition to blood. WOAH emphasises that leptospires may persist in the kidney, urine or genital tract of clinically normal animals and that a negative urine result at a single sampling point does not necessarily exclude chronic renal carriage.

Environmental surveillance should also be continued in selected wetlands and standing-water bodies. However, detection of Leptospira-genus DNA should be followed, wherever possible, by methods capable of distinguishing pathogenic from non-pathogenic leptospires. In the current Ukrainian dataset, two water samples contained Leptospira-genus DNA interpreted as most probably belonging to non-pathogenic species, whereas pathogenic Leptospira DNA was not demonstrated.

Toxoplasmosis

Comparable surveillance for Toxoplasma gondii should be strengthened on both sides of the border. The current joint interpretation is limited because Romanian PP2 laboratory results were not available in the datasets used for the present report, while the Ukrainian animal and environmental samples tested were negative.

Future campaigns should therefore aim to use comparable host species, sample types, sampling periods and diagnostic methods in Romania and Ukraine so that a genuinely bilateral epidemiological assessment can be performed.

Priority host categories should be selected according to the objectives of the joint surveillance protocol and should be accompanied by relevant epidemiological information on animal origin, management conditions and potential exposure pathways.

Environmental investigations should be incorporated only where the selected matrices, sampling method and analytical method are appropriate for the epidemiological question being addressed.

West Nile virus surveillance

The strong WNV-reactive serological signal identified in Romania warrants the development of a more integrated surveillance approach combining vertebrate serology, molecular testing, wild-bird surveillance and mosquito surveillance.

The 168 ELISA-positive and eight doubtful Romanian samples should not be interpreted as population prevalence estimates because the study used targeted sampling and heterogeneous host populations. Nevertheless, the multispecies distribution of serological reactivity represents a relevant epidemiological signal. WNV RNA was not detected in the 140 real-time RT-PCR determinations, indicating absence of detectable viraemia in the tested samples at the time of collection rather than demonstrating absence of virus circulation in the wider ecosystem.

WOAH describes WNV as a mosquito-borne flavivirus maintained primarily through the bird–mosquito–bird cycle and recognises migratory birds as important contributors to geographical dispersal. Accordingly, future sampling should be coordinated with seasonal mosquito activity and periods of high migratory bird abundance.

Where epidemiologically relevant, ELISA-reactive samples should be subjected to more specific confirmatory testing. WOAH recognises virus neutralisation and plaque-reduction neutralisation tests and also cautions that antibodies to related flaviviruses, including Usutu virus, may generate cross-reactions in some serological assays.

Consequently, PRNT/PRN or VN should be considered for representative positive samples, particularly when results are intended to support epidemiological conclusions concerning WNV-specific exposure.

Future surveillance should also include mosquito collection and identification in selected high-risk sites, particularly wetlands, settlements and livestock areas situated close to aquatic habitats. Combining vector data with bird and mammalian serology would provide a more complete picture of WNV transmission dynamics.

Strengthening the One Health component

The activity demonstrated that domestic animals, wildlife, vectors and environmental samples provide different but complementary information. Future surveillance should therefore further operationalise the One Health approach rather than treating animal and environmental data as separate components.

Joint datasets should allow epidemiological links to be explored between:

- domestic animal exposure,
- wildlife activity,
- migratory bird movements,
- vector occurrence,

- climatic and seasonal conditions,
- hydrological characteristics and pathogen detection in environmental matrices.

Where appropriate, collaboration should also be strengthened with public-health, environmental, wildlife-management and academic institutions so that relevant animal, vector and environmental signals can be interpreted in a broader epidemiological context.

The objective should be the development of an integrated early-warning framework capable of identifying concordant signals across different surveillance components before large-scale outbreaks occur.

Improvement of sampling design

Future sampling plans should clearly distinguish between risk-based surveillance and studies intended to estimate prevalence.

The current project generated highly valuable targeted surveillance data, but differences in host composition, sampling intensity, geographical coverage and matrices mean that observed positivity percentages should not be extrapolated to the wider animal populations.

If future activities aim to estimate population prevalence, statistically defined sampling frames, sample-size calculations and representative selection procedures should be established in advance.

For risk-based surveillance, sampling should instead concentrate intentionally on locations and populations with the highest probability of pathogen introduction or circulation. The purpose of each sampling component should therefore be explicitly defined before field implementation.

Strengthening wildlife and environmental surveillance

Wildlife should remain a major component of future investigations because several target pathogens are influenced directly or indirectly by wild-animal populations.

Priority should be given to:

- migratory and resident aquatic birds for influenza and WNV surveillance;
- wild boar for ASF surveillance;
- selected wild mammals at livestock–wildlife interfaces;
- rodents and other epidemiologically relevant hosts for leptospirosis;
- vectors such as mosquitoes where vector-borne pathogen surveillance is undertaken.

Environmental samples should be collected where they answer a defined epidemiological question. Water sampling proved useful within the Ukrainian component, including the detection of influenza A RNA and Leptospira-genus DNA. Environmental testing should therefore continue in selected wetlands and aquatic systems, but laboratory detection must be

interpreted cautiously because detection of nucleic acid does not necessarily demonstrate viability, infectivity or transmission to susceptible hosts.

Laboratory quality assurance and biosafety

Laboratory testing should continue under documented quality-assurance procedures, including appropriate positive and negative controls, internal amplification controls where applicable, validated interpretation criteria and traceable sample handling.

Each laboratory should ensure that molecular assays are validated for their intended matrix and host species, particularly when methods are applied to unusual wildlife species or environmental samples.

Biosafety and biocontainment measures should continue to be determined according to pathogen-specific risk assessment and the nature of the laboratory procedure. This is particularly important for avian influenza investigations, for which WOAH explicitly requires appropriate biosafety and biosecurity measures and recommends specialised containment for certain virus subtypes and procedures.

Joint database and epidemiological data exchange

A common Romania–Ukraine epidemiological database should be maintained and progressively developed.

The database should contain standardised laboratory results together with essential epidemiological and geographical metadata and should allow results to be compared between countries, sampling periods, host species, matrices and pathogens.

Procedures should be established for rapid exchange of information when relevant laboratory findings are obtained, particularly:

detection of ASFV; detection of influenza A followed by H5/H7 identification; evidence suggesting pathogenic leptospiral circulation; detection of WNV RNA or significant vector activity; or other unexpected epidemiological signals.

Where technically feasible, spatial representation of results using GIS should be incorporated in order to identify clusters, interfaces between wildlife and domestic animals and locations requiring intensified surveillance.

Molecular characterisation and future research

Where positive molecular samples are obtained in future campaigns, additional genetic characterisation should be encouraged.

For influenza A, sequencing and phylogenetic analysis would allow better characterisation of circulating viruses and comparison with strains detected elsewhere along migratory routes.

For ASF, molecular characterisation of confirmed positive samples would support tracing of virus introduction and spread.

For leptospirosis, isolation and typing, where technically possible, would improve knowledge of locally circulating serovars and maintenance hosts.

For WNV, molecular characterisation of RNA-positive material would assist in identifying circulating lineages and evaluating possible epidemiological links across the cross-border area.

The resulting sequence and epidemiological information should, where applicable and permitted, be shared between partners and relevant reference laboratories.

Training, biosecurity and awareness

Continued professional training should accompany laboratory surveillance. Veterinarians, laboratory staff, field personnel, farmers, hunters and other stakeholders should receive targeted information on recognition of relevant clinical signs, correct sample collection, transport, biosecurity and reporting procedures.

Specific awareness activities should be adapted to each disease. For example, ASF communication should focus on swine biosecurity and reporting of wild-boar mortality; avian influenza communication should address poultry keepers and personnel encountering dead wild birds; leptospirosis awareness should include occupational and environmental exposure; and WNV communication should include vector-related prevention and surveillance.

This training component should be integrated with the One Health objectives of the project and with the joint methodological protocols developed by the partners.

Sustainability of the cross-border surveillance mechanism

The collaboration established through Activity A1.4 should be maintained after completion of the current joint research cycle. The project has already created a practical structure based on common Terms of Reference, coordinated sampling, laboratory cooperation and joint scientific interpretation, while the Ukrainian component alone generated close to 1,160 biological and environmental samples covering all five priority diseases.

Future cooperation should therefore build on this infrastructure rather than restart surveillance independently. Periodic technical meetings between PP2 and PP3, joint review of epidemiological signals, coordinated annual or seasonal sampling plans and exchange of laboratory experience would support continuity.

The surveillance model could subsequently be expanded to other relevant institutions in the Lower Danube region where epidemiologically justified, while preserving common methodological principles and interoperable data collection.

Final recommendation

The principal recommendation resulting from Activity A1.4 is to transform the surveillance framework developed during the project from a series of discrete sampling campaigns into a sustainable, risk-based, cross-border One Health surveillance system.

Such a system should integrate molecular diagnosis, serological surveillance, wildlife monitoring, vector surveillance and environmental investigation; use harmonised methodologies and comparable epidemiological metadata; apply disease-specific confirmatory algorithms consistent with WOAH principles; and ensure regular exchange and joint interpretation of epidemiological information between the Romanian and Ukrainian partners.

The results obtained during the current activity demonstrate that the epidemiological situation of the Lower Danube cannot be adequately characterised through isolated investigations of individual animal species or individual national territories. Continued coordination between Romania and Ukraine is therefore essential for early detection of emerging and re-emerging disease signals, more accurate assessment of transboundary risks and timely implementation of preventive measures.

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