



PROTOCOL OF STEP-BY-STEP INSTRUCTIONS FOR IDENTIFYING THE SOURCE OF WEST NILE FEVER (DISEASE) AND PREVENTING ITS SPREAD FOR JOINT PREVENTIVE AND LIQUIDATION MEASURES (REFERENCE DOCUMENT)

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

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

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

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

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

- identifying sources and vectors of West Nile virus (WNV) along the “birds – mosquitoes – animals – humans” chain;
- assessing arboviral transmission routes and risk factors in a cross-border context;
- conducting joint entomological, ornithological, and clinical monitoring studies in line with the “One Health” principles;
- harmonizing approaches to WNV surveillance, monitoring, and control between Ukraine and Romania in the Lower Danube and Black Sea coastal area.

This Protocol draws on:

- results of joint field studies carried out under the Project;

- laboratory analyses using real-time PCR and ELISA methods (ELISA/MAC-EIA/Indirect IgG);
- the Joint Terms of References (TOR) agreed by the partners, which sets out common methods for sample collection, delivery, preparation, and testing;
- experience and findings from previous international projects;
- current joint ECDC/EFSA surveillance data on West Nile virus in Europe (monthly reports for 2024–2025) and guidance documents from WHO and WOA (OIE).

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

- Regulation (EU) 2016/429 of the European Parliament and of the Council of 9 March 2016 on transmissible animal diseases (“Animal Health Law”) — WNV in horses is a category D disease subject to mandatory notification [1];
- Commission Implementing Regulation (EU) 2020/2002 of 7 December 2020 on rules for disease notification, including WNV outbreaks in animals, via the ADIS (Animal Disease Information System) within 24 hours of confirmation of a primary outbreak, as amended by Commission Implementing Regulation (EU) 2026/501 of 5 March 2026 [2];
- Commission Delegated Regulation (EU) 2020/689 of 17 December 2019 on rules for surveillance, eradication programmes, and disease-free status [3];
- Directive 2003/99/EC of the European Parliament and of the Council of 17 November 2003 on the monitoring of zoonoses and zoonotic agents — annual aggregated WNV data are published by EFSA/ECDC as part of the “One Health Zoonoses Report” [4];
- Commission Decision (EU) 2018/945 of 22 June 2018 on the communicable diseases and related special health issues to be covered by epidemiological surveillance and relevant case definitions — includes WNV as a



human disease subject to mandatory surveillance through the TESSy system (ECDC) [5];

- Regulation (EU) 2022/2371 of 23 November 2022 on serious cross-border threats to health — the legal basis for the mandate of EU reference laboratories (ECDC), including for arboviruses (WNV, dengue, Zika, and others), introduced by a Commission decision in March 2024 [6];
- Law of Ukraine No. 1206-IX of 04.02.2021, as amended by Law No. 4718-IX of 16.12.2025, “On Veterinary Medicine and Animal Welfare” [7];
- Strategy for Ensuring Biological Safety and Biological Protection under the “One Health” principle through 2025 (Order of the Cabinet of Ministers of Ukraine No. 1416-r of 27.11.2019, as amended on 20.09.2024) [8];
- Order of the Ministry of Health of Ukraine No. 1724 of 09.10.2024, “On Approval of the List of Particularly Dangerous and Dangerous Infectious Diseases” [9];
- European Veterinary Big Data Strategy 2022–2027 [10];
- ECDC/EFSA Joint Scientific Report: Surveillance of West Nile virus infections in humans and animals in Europe [11].

Implementation of the SMS-RO-UA project supports the EU Strategy for the Danube Region (EUSDR) as it relates to environmental protection and public health. The Danube Delta area is a persistent endemic focus of WNV in Romania and a zone of elevated risk for Ukraine, as confirmed by annual ECDC data.

RELEVANCE

West Nile fever (also known as West Nile encephalitis, or “duck fever”) is an acute zoonotic arboviral disease – transmitted in nature between vertebrates by blood-feeding insects through bites, contact with the crushed remains of an insect, or its feces – with a vector-borne mechanism of transmission, caused by the eponymous West Nile virus (*Encephalitis Nili occidentalis*). In severe cases it is characterized by fever,

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inflammation of the meninges and brain tissue, damage to mucous membranes, and exanthema. West Nile virus was first identified in Uganda in 1937. It re-emerged in 1999, when seven deaths and 62 cases of encephalitis were recorded in New York — the first appearance of the virus in the Western Hemisphere. To date, West Nile virus has been detected in Africa, Europe, Asia, North America, Australia, and the Middle East.

Early outbreaks of the virus were generally self-limiting and mild. In the 1990s, West Nile virus began to be associated with severe neurological disease. According to a comprehensive literature review published in 2013, meningitis and encephalitis (neuroinvasive disease) occurred in fewer than one percent of infected patients, with a fatality rate of 10 percent among those cases. West Nile virus produces symptoms in roughly 25% of infected people; the remaining 75% have few or no symptoms at all. This is likely why the true number of WNV infections is substantially underestimated. Outbreaks are usually linked to late summer and autumn because of the mosquito life cycle and an intensified “bird-mosquito-bird” cycle. In warmer climates, cases can occur year-round.

In the United States, nearly 44,000 confirmed and probable cases of West Nile virus were recorded between 1999 and 2015, of which more than 20,000 involved neuroinvasive disease. The number of neuroinvasive cases varies considerably from year to year, ranging from 386 to 2,946. Serological studies and blood-donor screening data suggest a neuroinvasive disease rate of about 0.5% among infected patients and an infection rate of about 10% in outbreak areas — figures that extrapolate to roughly 3–5 million infections overall. In Ukraine, numerous cases of West Nile fever were recorded in 2024. WNV is transmitted through bites of mosquitoes of the genera *Culex*, *Aedes*, and *Anopheles*, which together comprise more than 1,000 species. Outbreaks were recorded in several regions of the country, but residents of Kyiv and Kyiv Oblast were hit hardest, with more than 50 cases recorded since the beginning of 2024 and more than 40 laboratory-confirmed since early August. With respect to West Nile virus infection, the Danube Delta is a high-risk zone because of the large number of vector

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mosquito species present there (particularly *Culex pipiens*) and migratory birds that can carry the virus into the area. According to national reports, human cases are recorded every summer in the Danube-adjacent counties, including Tulcea. Enzootic cycles between birds and mosquitoes help sustain the virus in the region.

GENERAL CHARACTERISTICS OF THE PATHOGEN

WNV is an enveloped virion containing a single-stranded, positive-sense RNA genome. Its nonstructural proteins perform a wide range of functions — understandably so, since the virus encodes a very limited set of proteins, each of which must fulfill several roles during infection. Several nonstructural proteins, including NS2A, NS2B, NS4A, and NS4B, have been shown to suppress one or more components of the innate immune response to viral infection [12, 13]. The West Nile virus virion is an icosahedral particle in which the capsid protein binds the RNA genome to form a nucleocapsid, surrounded by a lipid bilayer.

West Nile virus enters cells through receptor-mediated endocytosis after attaching to the cell surface. The viral polyprotein is translated and processed on intracellular membranes, yielding 10 viral proteins. The initial viral RNA is replicated by viral and cellular proteins into numerous copies used to produce new virions. Structural proteins assemble on membranes of the endoplasmic reticulum, bind to the nucleocapsid, and bud into the cytoplasm through the Golgi network [14].

Interest has grown recently in the role of partially or fully immature flavivirus particles during infection. These immature particles arise when the prM protein is not efficiently cleaved from the virion surface during maturation and budding [15]. Immature or partially mature particles of both DENV and WNV have been shown to make up as much as 40% of the total virus population in a given infection [16]. Although these particles were traditionally considered non-infectious, several recent studies have shown that immature WNV particles can be highly immunogenic and

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infectious in vitro and in vivo when bound by antibodies against the E or prM protein [17, 18]. These antibody-bound immature viral particles enter immune cells via the Fc receptor, resulting in productive infection. Further research is needed to clarify the role of immature particles in viral pathogenesis and disease, in both vectors and mammalian hosts [19].

IMPACT ON HUMAN HEALTH

Human infections are predominantly subclinical, but the associated manifestations can range from fever and myalgia to meningoencephalitis and death [20]. Encephalitis occurs in only a small proportion of patients; progression to severe neurological disease can produce acute flaccid paralysis following meningitis or encephalitis, with rapidly progressing symptoms that may affect all four limbs [21]. A severe poliomyelitis-like syndrome with a poor long-term prognosis can also occur [22]. Older adults are more susceptible to neurological damage that can be fatal, and among older survivors up to 50% may experience significant post-infection morbidity for at least a year after infection [23], with an elevated risk of death for up to 3 years after the acute illness [24]. Among people aged 70 and older, the case fatality rate ranges from 15% to 29%. Higher mortality is also seen in infected infants and immunocompromised patients [25]. Risk factors for encephalitis and death include homelessness, a history of cardiovascular disease or chronic kidney disease, hepatitis C virus infection, and immunosuppression [26, 27]. In addition, some recovering patients may develop persistent or chronic infection, detectable by urine PCR, indicating viral replication in kidney tissue [28, 29]. Although West Nile virus persistence has also been noted in several animal models, it has consistently been evident on urine analysis [19].



EPIDEMIOLOGY AND EPIZOOTOLOGY

The ability of different mosquito species to become infected with and transmit West Nile virus varies considerably. Mosquitoes of the genus *Culex* are considered the primary vector worldwide; *C. tarsalis* is the main West Nile virus vector in the western United States and can feed on a range of bird and mammal species [30, 31]. Other vectors capable of both acquiring and transmitting West Nile virus include *C. quinquefasciatus*, *C. stigmatosoma*, *C. thriambus*, *C. pipiens*, and *C. nigripalpus*. To date, more than 65 mosquito species have been shown to be infected with West Nile virus [31, 32]. In nature, West Nile virus is maintained in a cycle between mosquitoes and animal hosts. Figure 1 shows the scheme of virus transmission from mosquitoes to mammals), with birds serving as the preferred and principal reservoir [33, 34, 35, 36].

Birds of some species fall ill, show symptoms of disease, and may die, while others become infected and act as carriers without showing any signs of illness. Although house sparrows and crows are highly susceptible to WNV, they make up only a small share of the mosquito blood-meal samples analyzed and may be of limited importance for transmission. Instead, the American robin is thought to be the principal host species responsible for maintaining and amplifying WNV transmission in the United States, since the dominant vector species preferentially feed on robins [37]. Bird-to-bird transmission has been demonstrated under laboratory conditions, with several species shown to be capable of transmission by direct contact [38]. Humans are considered a “dead-end” host for West Nile virus, because the low level of viremia typically seen in mammals is usually insufficient to infect feeding mosquitoes, which breaks the transmission cycle [39]. However, the capacity of mammals to serve as competent hosts could change if mosquitoes of the genus *Aedes*, which feed predominantly on human blood, were to become the dominant West Nile virus vectors.

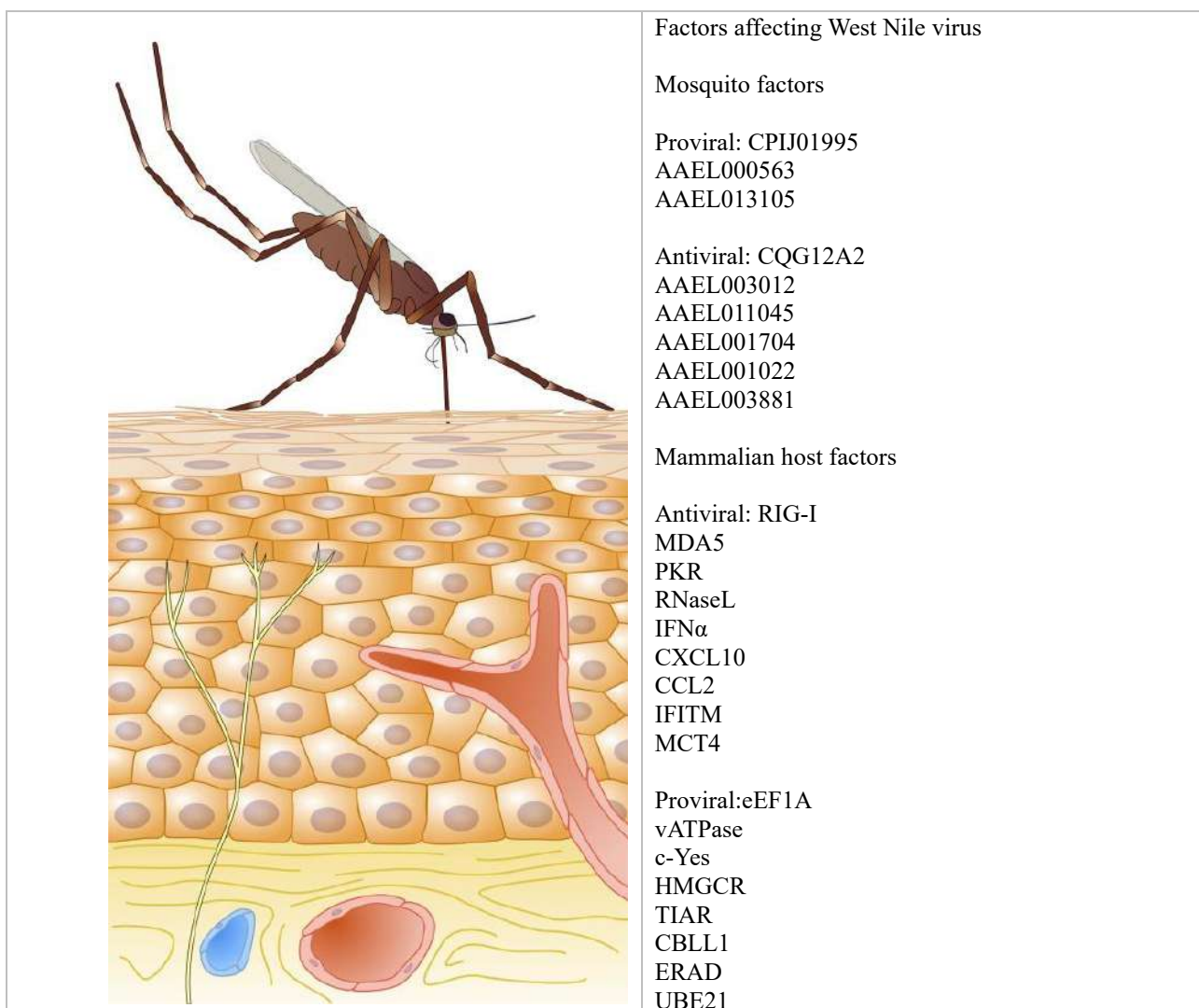


Figure 1. Scheme of West Nile virus transmission from mosquito to mammal, and host factors known to be involved in infection.

In 2024, and as of 4 December 2024, 19 European countries reported 1,436 locally acquired human West Nile virus cases with a known place of infection (Figure

2).

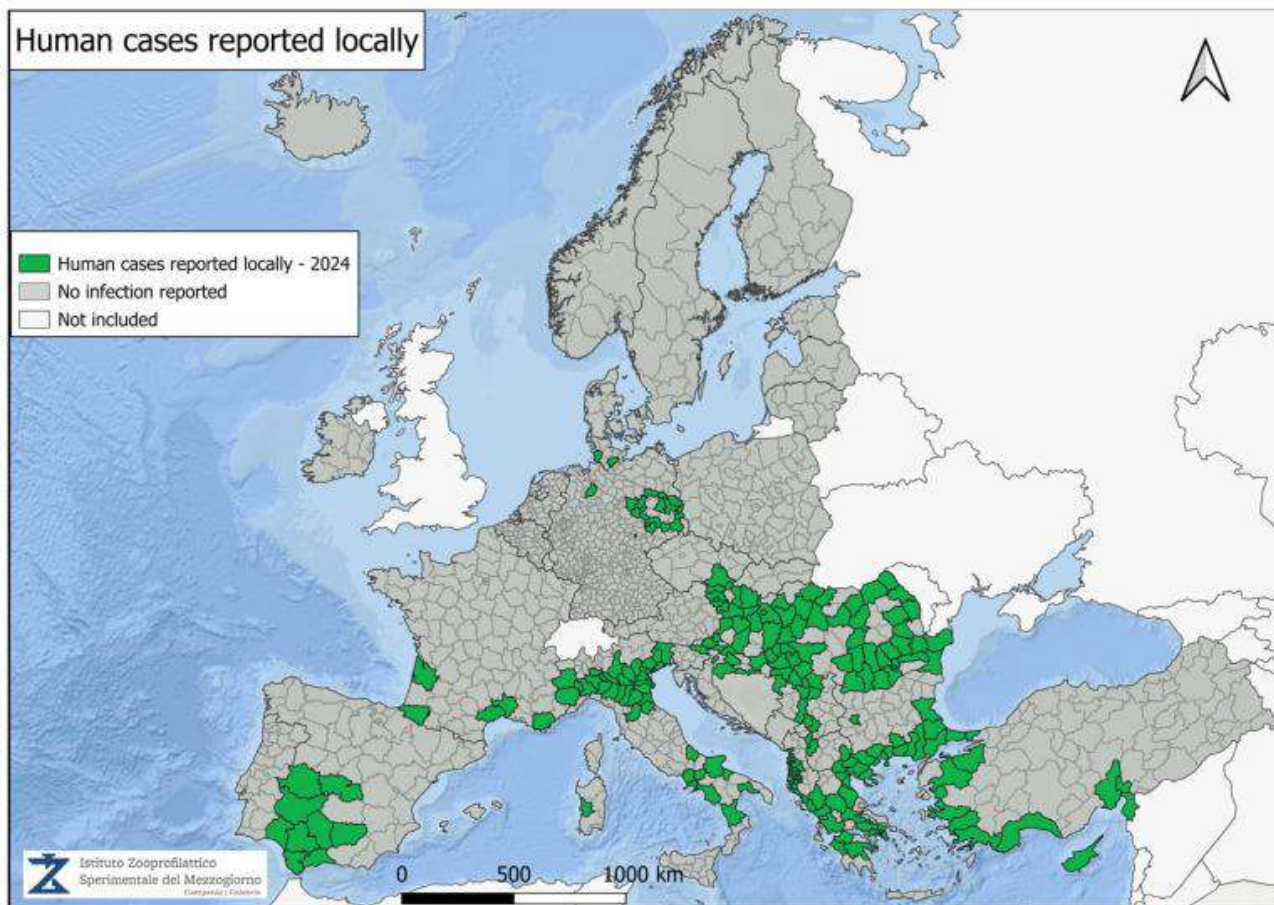


Figure 2. Distribution of human West Nile virus infection cases in Europe in 2024, within the European Economic Area (EEA). The EEA is divided into smaller territorial units according to the classification known as the Nomenclature of Territorial Units for Statistics (NUTS); in particular, NUTS3 denotes the regional level. Data are taken from EUROSTAT 2024 [41]

ROUTES OF INFECTION (TRANSMISSION)

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The most common route of human infection with West Nile virus is the bite of an infected mosquito of the family Culicinae. Mosquitoes become infected when they feed on the blood of infected birds carrying high levels of West Nile virus. Infected mosquitoes can then transmit the virus by feeding on the blood of humans or other animals. Humans, horses, and most other mammals do not develop high viremia and are therefore traditionally regarded as “dead-end” hosts.

West Nile virus has been detected in dead birds of at least 138 species. Birds of the corvid family (crows and blue jays) are particularly susceptible to WNV. People at elevated risk of WNV infection include those who work outdoors during periods of mosquito activity, such as farmers, foresters, landscapers, gardeners, construction workers, and other outdoor personnel. WNV is not transmitted from person to person via mosquitoes. However, in 2002, five new transmission routes were identified, described below. Their discovery undermined the theory that humans are strictly dead-end hosts for the virus.

Blood transfusion. Between August 2002 and January 2003, 20 confirmed cases of West Nile virus transmission linked to transfusion of blood products, traced to 14 infected donor units, were documented [42, 43]. Transmission of West Nile virus has been shown to occur through transfusion of red blood cells, plasma, and platelets.

Organ transplantation. Four transplant recipients who received organs from a common donor developed fever; three of them developed meningoencephalitis. West Nile virus infection was diagnosed in all four recipients. Serum and plasma samples taken from the organ donor at the time of procurement tested positive for West Nile virus by PCR and viral culture [43].

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



Transplacental transmission. In August 2002, a previously healthy 20-year-old woman at 27 weeks of pregnancy was hospitalized with symptoms consistent with West Nile virus meningoencephalitis, and specific antibodies were detected in her serum and cerebrospinal fluid samples. Five weeks later she delivered a live newborn. The infant showed serological evidence of West Nile virus infection, bilateral chorioretinitis, and magnetic resonance imaging (MRI) findings indicating severe bilateral white-matter loss in the temporal and occipital lobes [43].

Occupational infection. Infection is possible during necropsy of animals infected with the disease, when protective measures are not observed during contact with potentially infected biological material.

IDENTIFICATION OF THE SOURCE OF THE WNV PATHOGEN (LABORATORY DIAGNOSTICS)

Diagnosis of West Nile virus infection relies heavily on clinical criteria and antibody testing [44]. The incubation period of West Nile virus infection is thought to range from 2 to 14 days [28]. Diagnosis relies on detecting IgM antibodies to West Nile virus, particularly in cerebrospinal fluid (CSF). Where cross-reactivity with related flaviviruses is suspected (Japanese encephalitis virus, St. Louis encephalitis virus, yellow fever virus, and dengue virus), it can be assessed using a plaque-reduction neutralization test [28]. Replication of West Nile virus has been documented in human monocytes in vitro, and more efficiently in polymorphonuclear leukocytes; this may contribute to transmission through blood transfusion [45]. Several rapid tests have accordingly been developed for blood-donor screening using nucleic acid testing (NAT), an amplification-based transcription method that can detect West Nile virus-infected individuals before symptoms appear and can help safeguard blood supplies [238]. It should be noted that in 45% of individuals with a positive NAT result the diagnosis was subsequently not confirmed, and in one study a diagnosis of West Nile

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virus infection was ultimately established in only 4–5% of patients [46].

Antibody testing in patients follows a fairly predictable timeline: the median time from RNA detection to IgM seroconversion is 3.9 days, and from RNA detection to IgG seroconversion, 7.7 days [44]. RNA typically becomes undetectable after about 13.2 days, although its persistence beyond 40 days has rarely been observed. IgM and IgA antibody levels decline substantially, though not uniformly, while IgG levels remain elevated for more than a year after viremia is detected [44, 47, 48]. Because antibodies to West Nile virus persist *in vivo*, they cannot be used to distinguish a recent infection from one acquired earlier [19, 49].

Real-time PCR. These methods are generally rapid and reliable and can be used to detect West Nile virus in a wide range of sample types, including non-human samples such as animal tissue and mosquitoes [50]. Various technical modifications have been proposed over the past 10 years. In general, TaqMan probe-based assays are fairly sensitive (with an approximate detection limit of 0.1 PFU of viral RNA) [51], but can sometimes fail to detect genomic variants of the virus [52]. SYBR Green-based assays are as sensitive as TaqMan assays but less specific [53]. Reduced specificity can be a drawback, since it may produce false positives from unrelated viruses, but it can also be an advantage, given the broader range of West Nile virus variants it can identify [53]. Another potential limitation of real-time PCR is that it typically produces short amplicons unsuitable for sequencing and molecular characterization. Given the seasonal variability of WNV strains and their geographic distribution, new diagnostic molecular approaches have been proposed. A multiplex PCR-ligase assay (RT-PCR/LDR), based on simultaneous amplification of three different genomic regions (one within the NS2 coding sequence and two within the NS5 nonstructural gene), has minimized the risk of false-negative results caused by WNV genetic variants. Another method, based on whole-genome RT-PCR [52], overcame the failure to detect WNV isolates with genomic differences. WNV isolates have been phylogenetically grouped into 7 distinct lineages [54], with lineages 1 and 2 overlapping in some areas of Europe [53]. A new quantitative real-time PCR assay has been developed to distinguish

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between lineages 1 and 2. This assay showed an exceptionally high capacity to differentiate West Nile virus isolates obtained from different cell lines, down to a level of 2–4 genome copies [55]. West Nile virus geographically overlaps with several other arboviruses, including flaviviruses, alphaviruses, and some bunyaviruses, and as the number of newly recognized viruses grows in both tropical and subtropical regions [55, 56, 57], there is increasing need for simultaneous identification — for example, of chikungunya virus, dengue virus [58], Japanese encephalitis virus, and West Nile virus in patient samples [59]. As these methods continue to improve, early detection of multiple arboviruses is becoming a practical reality, with potential for rapid, cost-effective differential diagnosis and epidemiological surveillance.

Immunohistochemistry. Detection of West Nile virus antigen using histochemical protocols in tissue from patients who died of encephalitis has been available for diagnostic purposes for many years. In modern laboratories this procedure is used only rarely, generally to strengthen confidence in a clinical diagnosis when laboratory findings are minimal [60]. Data on the diagnostic performance of this assay come mostly from animal studies, and the specificity of the method depends heavily on the specificity and quality of the antiserum used. Sensitivity can be low in many cases and often depends on the amount of West Nile virus present in the tissue and the quality of the tissue examined [60, 61].

It should be emphasized that most of the diagnostic approaches described in this section can be difficult to apply routinely for laboratory diagnosis of West Nile virus infection, owing to the lack of standardized protocols and uncertainty regarding their specificity and sensitivity.

ELISA. These methods fall into three main categories [62]: IgM antibody-capture ELISA (MAC-EIA), indirect IgG ELISA, and epitope-blocking ELISA [63]. All of these methods offer the advantage of speed and reproducibility compared with neutralizing-antibody detection methods.

MAC-EIA. This test is suitable for routine detection of immune responses arising from acute viral infection. The IgM response is usually detectable within the first week

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after West Nile virus infection, sometimes even before symptoms appear, in plasma, serum, or cerebrospinal fluid (CSF), and typically persists for up to 47 days in plasma/serum, with more prolonged persistence in CSF, up to 100–198 days after infection in viremic blood donors' plasma/serum [64]. Standard commercially available MAC-EIA kits use plates coated with antibodies to human IgM and West Nile virus antigens obtained from the brains of experimentally infected mice. Monoclonal antibodies (MAbs) directed against West Nile virus, conjugated with horseradish peroxidase, can be used as the secondary immunoglobulin [65]. The serum screening dilution is typically 1:400. This test has an estimated sensitivity of 91.7% and specificity of 99.2% [66]. The presence of virus-specific IgM in cerebrospinal fluid is generally regarded as direct evidence of infection, allowing a case to be classified as confirmed under the EU case definition [67, 68].

Indirect IgG ELISA. Detection of IgG against West Nile virus by ELISA is now well standardized, and these methods are widely used to determine immune status against West Nile virus both in patients with suspected disease and in healthy, asymptomatic populations [69]. The main limitation of these tests is, first, their limited clinical specificity because of substantial cross-reactivity with other flaviviruses. For this reason, IgG detection is recommended in combination with MAC-ELISA in all cases of suspected infection, since IgM persistence can last up to 6 weeks after disease onset [64]. All IgG-positive results obtained by this method should be further evaluated and confirmed either by a PRNT90 test or by a rise in the IgG response at later stages of infection. The combined specificity and sensitivity of the most commonly used commercial IgG ELISA methods are approximately 92–93% and 97–98%, respectively [70]. Two commercially available ELISA methods (Focus Diagnostics, Cypress, CA, USA, and Euroimmun, Lübeck, Germany) have been used to assess infection dynamics by measuring IgG avidity during symptomatic West Nile virus disease. In sera obtained within the first 30 days after infection onset, IgG avidity was below 40%, whereas an avidity index above 55–60% correlated with infections lasting at least 180 days [71].



Determining IgG avidity is likely to provide additional useful data for clinically distinguishing recently acquired from previously acquired West Nile virus infections.

Epitope-blocking ELISA. A technical variant of ELISA for IgG identification is the so-called epitope-blocking ELISA. This method is based on competition between the test serum and specific monoclonal antibodies to West Nile virus for binding to West Nile virus antigens, and it was originally developed for diagnosis and prevalence studies in animals, since it is not species-dependent [72]. This technique has recently been evaluated for diagnosing human infection. The results obtained showed that the method is applicable only in populations not previously exposed to flaviviruses other than West Nile virus, which leads to a lack of correlation with PRNT90 results.

Use of immunofluorescence assays in routine WNV diagnosis largely depends on the number of samples to be evaluated and on how well laboratory staff are trained to interpret test results. Most such tests are developed “in-house” using WNV-infected cell cultures. As a result, this technique suffers from a lack of standardization when results are compared between laboratories, and would benefit considerably from external quality assessment (EQA) [73].

CLINICAL MANIFESTATIONS AND HEMATOLOGICAL CHANGES IN BLOOD PARAMETERS IN WEST NILE VIRUS INFECTION

Symptomatic patients frequently show neurological manifestations, often accompanied by diffuse myalgia and musculoskeletal pain, which can mimic other infectious or inflammatory diseases [23]. Clinically apparent disease is more common in individuals, particularly older people and patients with significant comorbidities, including cardiovascular and immunological disorders [22]. Conversely, patients with oligosymptomatic or asymptomatic infection may present with nonspecific symptoms such as fever, headache, back pain, myalgia, and anorexia lasting several days, which complicates early clinical diagnosis [74, 22].

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As with other viral infections, West Nile virus can cause lymphocytopenia and broader immune dysregulation [75]. This potentially predisposes patients to secondary bacterial or fungal infections. In this context, some patients – particularly those who initially had mild or minimally symptomatic disease – may subsequently develop signs and symptoms consistent with sepsis, rather than the classic rash associated with arboviral infections [26].

In a typical clinical course, after an incubation period of 2 to 15 days, neurological signs and symptoms may appear as a result of direct viral damage to the central nervous system. Meningoencephalitis is observed more often than isolated meningitis and can be associated with acute flaccid paralysis due to damage to the anterior horn cells of the spinal cord [22, 27].

In an exploratory logistic regression analysis, a lower mean corpuscular hemoglobin (MCH) and a lower monocyte percentage were both associated with a septic phenotype in univariate models, and MCH remained independently associated with sepsis in a simplified multivariate model that included MCH alone. These findings suggest that subtle changes in red-cell indices and monocyte counts may reflect a more pronounced systemic inflammatory response, bone-marrow dysfunction, or inflammation-related anemia in patients who develop sepsis in the context of neuroinvasive West Nile virus infection. Although specific data on red-cell indices in West Nile virus infection are limited, similar associations between red-cell parameters, inflammation, and adverse outcomes have been reported in bacterial sepsis and other severe infectious diseases [76]. Patients with a septic phenotype showed a numerically higher neutrophil-to-lymphocyte ratio (NLR), systemic immune-inflammation index (SII), and related composite indices, along with a relatively lower lymphocyte percentage, indicating a more pronounced inflammatory process. Platelets have also been proposed as potentially informative in West Nile virus infection; for example, platelet distribution width has recently been suggested as a potential prognostic/diagnostic marker. In addition, we identified a potential role for two laboratory indicators — the neutrophil-to-lymphocyte ratio (NLR), as reported for other

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viral infections, and mean corpuscular hemoglobin (MCH) — which may help distinguish patients with neurological manifestations from other patients [77].

In horses, clinical signs are comparable to those in humans, but mildly symptomatic forms are rarely observed after natural infection. Some horses develop only mild lethargy with a brief fever, and such cases usually go unrecorded. Alongside signs such as weakness, anorexia, loss of appetite, and lethargy, ocular disease has been reported, although blindness is considered one of the rarer clinical signs. The most frequently observed signs include enterocolitis, colic, rectal prolapse, and lameness, which can take various forms, including laminitis, pain in the cervical and thoracic spine, anemia, glossitis, jaundice (indicating liver involvement), and urination disorders. Colic-like behavior often appears first and persists for a short period before serious neurological disorders develop; this may be a behavioral disturbance indicating CNS involvement, but it can also present as genuine colic with abdominal pain caused by damage to the autonomic nervous system.

Birds, especially corvids, play a decisive role in the epidemiology of the disease. They are the most susceptible species and show symptoms resulting from damage to multiple organs, including the liver, spleen, kidneys, heart, and especially the CNS. Affected birds usually die within 24–48 hours [78].

Two experiments have also been conducted in pigs regarding West Nile virus infection. Viremia was observed in pigs under both experimental conditions, but seroconversion occurred in only one of them. No clinical signs were recorded in either experiment, both of which were aimed at assessing pig susceptibility to West Nile virus. In both experiments mentioned above [79, 80], no data on gross pathology or histopathology were obtained.

In sheep, natural West Nile virus infection associated with neurological clinical signs has been observed repeatedly. Gross CNS pathology was not evident, whereas pulmonary edema was often the most significant lesion observed at necropsy. Non-suppurative meningoencephalitis and poliomyelitis were, in some cases, the most characteristic histopathological findings. Neurohistopathology was characterized by

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lymphoplasmacytic infiltration and perivascular lymphoplasmacytic cuffing. The brain could also show gliosis and cell necrosis [81].

REDUCING THE RISK OF HUMAN AND ANIMAL INFECTION WITH WEST NILE FEVER

Numerous definitions of integrated pest management share one common principle: appropriate control methods should be used to keep pest populations at a level that does not cause unacceptable harm. For West Nile virus, this means applying preventive measures that stop the intensity of virus transmission from reaching a level that leads to outbreaks of human disease. This is often stated as the goal in mosquito-control management plans, and, as noted above, the literature contains many examples documenting the effect of control measures on vector abundance. *Culex pipiens* larval numbers in catch basins can be effectively reduced through various insecticide treatments, which is associated with reduced adult mosquito abundance [82]. Weather (temperature and precipitation) affects the dynamics of West Nile virus transmission across different ecosystems in the United States and can, to a degree, help predict transmission levels [83, 84, 85]. Mosquito-based epidemiological surveillance systems also provide useful indicators of human risk, such as the West Nile virus infection rate in mosquito populations or a vector index reflecting the abundance of West Nile virus-infected mosquitoes [86, 87]. There is strong evidence that, when applied with sufficient intensity, adult mosquito control measures can markedly reduce entomological indicators of WNV transmission activity, leading to fewer human WNV cases compared with situations where emergency control measures were not applied [88].

Vaccine development has been one of the main lines of effort against West Nile virus since the disease first appeared. Four vaccines have been licensed for horses by the United States Department of Agriculture, and the West Nile virus vaccine is

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considered a core vaccine by the American Association of Equine Practitioners (AAEP). Core vaccines protect against diseases of potential public-health significance that are endemic to a given region. Two inactivated whole-virus vaccines are currently available, along with a modified live recombinant canarypox vaccine carrying the premembrane and envelope genes of West Nile virus, and a chimeric vaccine combining a West Nile virus antigen with an inactivated flavivirus backbone. All available vaccines confer protective immunity and have been licensed on the basis of meeting one or more of the following criteria: helping prevent viremia; helping reduce viremia, encephalitis, and clinical disease; helping prevent disease, viremia, encephalitis, and mortality; and helping reduce the severity of clinical disease.

At present, no vaccine against West Nile virus has been approved for use in humans [89].

Prevention of human infection with West Nile fever.

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

Mosquitoes are usually most active at dawn (the first light of day) and at dusk (just before dark).

Cover exposed skin during these times.

When outdoors, wear:

- Long trousers and loose, long-sleeved shirts, socks, and a hat.
- Try wearing mosquito netting over your hat to protect your head.
- Wear light-colored clothing (dark colors attract mosquitoes).
- When outdoors, use insect repellents (sprays and lotions) containing repellents or icaridin.

How can you reduce mosquito breeding sites around your home?

- Mosquitoes lay eggs in standing water (water that is not moving or flowing).

Eliminate standing water around your home, in particular:

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- drain or dry out water in old tires, rain barrels, children's toys, flowerpots, and children's pools;
- clean gutters regularly to prevent blockages where water accumulates;
- turn boats and fishing gear on their side to drain them;
- change the water in outdoor pet bowls and bird baths at least twice a week;
- fit screens on windows and doors to keep mosquitoes out of your home.

How should you handle dead animals and wild birds?

If you find a dead animal or bird, never touch the body with bare hands. Always wear rubber gloves when handling a dead bird or animal. Report dead birds you suspect may be infected with West Nile virus. If you hunt wild animals or skin them, remember that West Nile virus can be transmitted through blood-to-blood contact.

To protect yourself from diseases an animal may carry:

- Always wear gloves when handling dead animals.
- Always make sure any open wounds on your hands are covered.
- Wash your gloved hands, then wash your bare hands, each time you touch a dead animal.

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

Other preventive measures worth noting include:

- Raising awareness among medical practitioners about West Nile virus, so that it is considered in the differential diagnosis of travelers returning from affected areas.
- Providing information to travelers heading to affected areas on how to reduce their risk.
- Ensuring the country has adequate laboratory diagnostic capacity.

CONTROL MEASURES



When cases of serous meningitis, meningoencephalitis, or milder forms of viral infection presenting with general intoxication syndrome without pronounced catarrhal-exudative features are identified, and WNV is suspected, an urgent report is recommended to be submitted to the Department of Public Health and/or similar country's institutions.

For all patients with the above diagnoses, as well as other manifestations of disease suspected to be WNV, paired blood serum samples – taken during the acute phase of illness and again 10–14 days later – are sent to virology laboratories. Collection and transport of material from sick individuals and suspected cases must follow the applicable sampling rules.

Epidemiological investigation of a West Nile fever focus includes interviewing the patient, collecting epidemiological history, laboratory testing, reviewing documentation, observing the focus, and completing a focus investigation form that records the following additional information gathered during the investigation:

- whether the patient has previously had any of the conditions listed above, and in what year;
- whether the patient has been under dispensary observation (or previously tested for WNV), and since what year;
- type of residential area (private, state-owned), presence of basement mosquitoes, presence of a body of water or forest strips near the home;
- contact with birds (wild, domestic) and rodents;
- the suspected source of infection;
- whether the patient traveled to a countryside house or rested in natural settings during the preceding 14 days.

A brief description of the area and possible mosquito contact is to be compiled, covering the conditions of exposure (the circumstances and situations the patient was in during the presumed period of infection, with dates and locations of stay over the preceding 2 weeks):

- if the suspected infection is linked to a mosquito bite, note the conditions and location of exposure (also noting the address, based on the patient's account);
- record the mosquito species present and their abundance per premises, based on entomological monitoring at the patient's place of residence (in both residential and non-residential premises);
- record the presence of bodies of water, their characteristics, and approximate area within a 3 km radius of the focus, and whether WNV cases previously linked to that body of water have been recorded there;

Among the measures for eliminating an epidemic focus, once a WNV patient is identified, the focus and the adjacent area are immediately surveyed for the presence of vector mosquitoes. The areas requiring treatment are determined by specialists.

Destruction of mosquitoes indoors and at breeding sites (basements, bodies of water) is carried out by organizations licensed to perform pest-control and disinfestation work, regardless of ownership form, immediately upon detection of a disease focus. In addition, during the period of mass mosquito breeding (April–August), comprehensive mosquito-control treatment of potentially hazardous bodies of water and building basements is carried out based on entomological indicators and in line with a comprehensive plan.

If the focus is located in a small settlement, treatment covers every yard, including residential and non-residential premises, farm buildings, and possible resting sites of vector mosquitoes. Where small bodies of water of no economic significance exist near the settlement and are colonized by mosquito larvae, they are treated at the same time, or filled in.

In larger settlements, treatment covers the basements of multi-story buildings and bodies of water with no economic significance.

Where WNV cases are identified in an area where the disease has previously been recorded, the scope of measures depends on the intensity of transmission and must be decided on a case-by-case basis.



If 5 or more WNV cases are registered in an area, and the situation is worsening relative to the baseline, the epidemiological situation is treated as an emergency, and the full range of anti-epidemic measures provided for in such cases is implemented.

Active case-finding is carried out, including in general (non-infectious) inpatient facilities, among patients who sought medical care with complaints of high fever, headache, and other manifestations of viral infection, including meningitis and encephalitis, with clinical material sent for WNV antibody testing.

Centers of hygiene and epidemiology, together with health-management authorities, shall organize consultant teams that travel to infection foci and help carry out case detection, treatment, and the organization of anti-epidemic measures on site. At the same time, work is carried out to prepare infectious-disease hospitals for a possible surge of patients requiring intensive care. Medication stocks are assessed, and urgently replenished if necessary.

Measures are taken to reduce populations of synanthropic birds that feed vector mosquitoes in settlements, by reducing the availability of food of anthropogenic origin (clearing unauthorized waste dumps), destroying nesting sites, and preventing birds from using buildings and structures for breeding (sealing off access).

The choice and justification of priority areas within the complex of sanitary-hygienic and anti-epidemic measures for WNV prevention are based on the results of epidemiological surveillance in the area, and are accompanied by the development and implementation of a comprehensive plan of preventive and anti-epidemic measures.

The content of the comprehensive plan, approved at the national and regional level, must include preventive and anti-epidemic measures, with an indication of the scope of work, deadlines, responsible officials, and the specific names of consulting specialists, together with their workplace and home addresses (telephone numbers). The priority areas of such a plan are:

- 1) Treatment and prevention:
 - identifying WNV patients (passive and active methods);



- collecting material and testing it in the laboratory for specific WNV antigens and antibodies;
 - symptomatic treatment of patients;
 - epidemiological investigation of the focus;
 - verifying that no other cases exist within the focus;
 - dispensary follow-up of patients.
- 2) Anti-mosquito and anti-tick measures (including entomological monitoring):
- counting mosquito and tick numbers;
 - determining infection rates in mosquitoes and ticks, taking seasonal variation into account;
 - monitoring mosquito and tick breeding sites and changes in their area;
 - preventing the formation of new bodies of water that serve as mosquito breeding sites, and reducing the area of existing ones;
 - clearing reed thickets along the banks of bodies of water, and especially along roadside ditches;
 - treatment of bodies of water (chemical, biological, and technological methods);
 - preventing mosquito and tick bites (protective clothing, repellents, and the like);
 - treating basements with insecticides, with evaluation of effectiveness;
 - acaricidal treatment of cattle and small ruminants.
- 3) Reducing populations of synanthropic birds that feed vector mosquitoes in settlements, by eliminating their food sources (clearing unregulated waste dumps), destroying nesting sites, and similar measures.
- 4) Public health education through:
- mass media;
 - leaflets;
 - information sheets and similar materials.

The presence of WNV-vector mosquitoes creates a potential risk of West Nile fever cases and of new natural foci emerging within the country.

The **set of measures** for mosquito control includes:

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- preventive and ongoing sanitary oversight, which are the main tools for preventing the formation of mosquito breeding sites in settlements and are aimed at preventing the flooding of basements in residential and public buildings, as well as improving the condition of local areas;
- carrying out sanitary-technical and extermination measures.

During the summer-autumn period, anti-mosquito and anti-tick treatment of bodies of water and the grounds of children's health facilities, recreation areas, and tourist routes must be carried out before each intake of visitors, in the necessary volumes.

When treating open bodies of water and basements, only insecticides approved for use in the country must be used.

For treating basements and building entrances against adult mosquitoes, various agents approved in the country are used — pyrethroids and organophosphate insecticides. All insecticides must be applied according to their accompanying instructions and used only for their intended purpose. Treatment must be carried out with strict observance of safety precautions when working with pesticides.

In addition, to destroy mosquito larvae in surface and enclosed bodies of water with no fishery significance, preparations from various classes of chemical compounds can be used (organophosphates, carbamic acid derivatives, pyrethroids, neonicotinoids), as well as agents based on entomopathogenic bacteria and insect growth regulators; microbiological preparations made from the delta-endotoxin of the entomopathogenic bacterium *Bacillus thuringiensis* H-14 var. *israelensis*, which act as selective intestinal-action insecticides. When ingested by mosquito larvae, these preparations cause toxicosis and subsequent death; insect growth regulators are also used.

These preparations may take the form of pyrotechnic smoke cartridges, tablets, emulsion concentrates, wettable powders, microemulsions, aqueous solutions, and water-soluble granules and briquettes.



Only microbiological preparations may be used for treating bodies of water that have fishery significance.

For individual and collective protection against mosquitoes, repellents approved for use in the country must be used. For protecting adults, top- and first-category products can be used in the form of sprays, lotions, and aerosol or non-aerosol formulations.

Counts of adult mosquitoes and larvae are made on the day of, or immediately before, treatment, and then again 5–7 days after treatment.

Control of ticks is comprehensive in nature and must cover the insect's entire life cycle, with the goal of reducing their numbers in areas affected by the virus.

Farm-level tick-control measures:

- rotating pastures for each herd after the period of peak tick infestation (June);
- surface treatment of land to destroy engorged females and their egg clutches;
- destroying corvid nesting sites in forest shelterbelts near pastures, followed by chemical treatment of the area;
- barrier treatment of forest shelterbelts;
- improving the condition of farm grounds.

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

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

Physical precautions:

- do not crush ticks with bare hands;
- if a tick has attached, apply an oily substance to it and, after 10–15 minutes, remove it, gently turning it to one side using soft tweezers or gloved hands;
- wear tight-fitting clothing in pastures and forest shelterbelts. Trousers must be tucked into socks and boots;

Only pesticides approved for use in the country should be used as acaricides, in accordance with their instructions for use and applicable safety precautions.



Personal protective equipment against ticks that is approved for use must be used according to its instructions for use. Acaricidal and acaricidal-repellent products should be applied only to clothing; repellent products may be applied to the skin.

CONCLUSIONS AND OUTLOOK

1. WNV is a persistent endemic disease in the cross-border region, with a tendency toward range expansion. According to joint ECDC/EFSA surveillance data, recent years have seen outbreaks emerge in new EU regions that had not previously recorded WNV. This confirms the ongoing geographic expansion of the virus under changing climate conditions.

2. The Danube Delta is an epidemiologically critical zone for WNV in Europe. The concentration of migratory waterfowl (the main WNV reservoir), the exceptional diversity and abundance of *Culex* mosquito populations, and human tourism and fishing activity in the area together create a distinctive epidemic environment that cannot be effectively assessed and controlled by a single country alone.

3. There is no approved vaccine against WNV for humans. This fundamentally distinguishes WNV from several other diseases on the project's list and raises the importance of vector control, personal protection, and early laboratory confirmation as the only tools available for protecting people. Vaccines for horses, by contrast, are licensed and provide effective protection for this “sentinel” species.

4. Seasonal serological and entomological dynamics are a key monitoring parameter. WNV transmission in the region peaks in July–September and correlates with peak *Culex pipiens* activity. Detection of WNV in mosquitoes during winter requires careful interpretation, as it may reflect residual antibodies rather than active transmission.



Priorities for further development of West Nile virus surveillance in the cross-border region are shaped by the current EU regulatory framework, project findings, and global trends:

1. Connection to the ADIS system and integration with TESSy (ECDC). Under Commission Implementing Regulation (EU) 2020/2002 (as amended by 2026/501), confirmation of a primary WNV outbreak in animals is subject to notification within 24 hours. The results of the SMS-RO-UA project provide a methodological basis for connecting national laboratories to the ADIS system. In parallel, integration with TESSy would allow human WNV case data from Ukraine to be incorporated into the pan-European ECDC surveillance database.

2. Predictive modeling using climate data and AI. A documented link exists between climate warming, the expanding range of vectors (*Culex pipiens*, *Culex modestus*), and the increasing frequency of WNV outbreaks. The project's GIS platform (D.1.4.3) provides a basis for developing predictive models that combine data on bird migration, air and water temperature, and mosquito population dynamics, in line with the European Veterinary Big Data Strategy 2022–2027.

3. Development of a cross-border outbreak response plan. The current situation — in which Romania records confirmed human WNV cases every year, while Odesa Oblast is a zone of elevated risk — calls for a joint operational plan between the competent authorities of the two countries. Such a plan, involving joint entomological surveys, mutual notification, and synchronized vector-control measures, is a logical continuation of the results of the SMS-RO-UA project.

4. Population-level protection through vector control and public education. Since no vaccine against WNV is approved for human use, mosquito population control and behavioral prevention remain the primary means of protection.

5. Incorporating WNV monitoring into veterinary medicine education curricula. The materials in this Protocol, together with the Practical Guide to Assessing Emerging Diseases (D.1.4.5), are recommended for integration into training programs. Particular attention should be given to the clinical diagnosis of WNV in horses and

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birds, as well as to rules for handling dead wild birds, the primary “sentinel” objects of surveillance

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