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First Trans-Border Serological Evidence of West Nile Virus Infection in Horses in Romania and Bulgaria

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ABSTRACT

West Nile Virus (VNV) produces encephalitic West Nile Fever (WNF), a zoonotic disease that can affect birds, is a viral disease that can infect horses as well as other mammals, birds and humans as a result of infection by arbovirus - *Orthoflavivirus nilense*, from family *Flaviviridae*. Cases of dead affected wild birds during the summer season have been positively related to predicting WNV in human populations. We conducted the present study aiming to detect serological evidence of the WNV in horses in western and central Romania and in northern and southern Bulgaria. Previous studies conducted in neighboring countries - Serbia and Hungary, confirmed the presence of the virus in horse populations. Between July 2022 and July 2023, a total of 147 horse serums were collected from three counties in West Romania – Arad, Bihor and Timis, and one in central Romania – Sibiu. Additional sera were collected from Lovech municipality in North Bulgaria and Topolovgrad municipality in South Bulgaria. The presence of antibodies in the serum was detected using the commercial ELISA kit. In order to remove the cross-reactivity of the Usutu virus, another representative of the Mosquito-borne Japanese encephalitis virus group, an epitope-blocking ELISA kit with specificity to WNV's Glycoprotein E and absence of cross-reactivity with other Flaviviruses was used. The sera were further tested via RT-PCR for presence of the viral RNA. Seropositivity was confirmed for 45% of the sera in West Romania and for 63% of the sera in Central Romania; for 23% from North Bulgaria and 20% from South. All PCR results were negative, demonstrating an absence of the viral RNA in the horses' body at the time of sampling. The study confirms the presence of WNV in horse populations of all tested regions. The results from the different countries and areas demonstrate that there is no difference between the populations in forested areas and those in field areas.

Key words: West Nile Virus, West Nile Fever, equines, ELISA, Zoonosis, Emerging, Endemic, Romania, Bulgaria; Banat.

INTRODUCTION

Family Flaviviridae

Flaviviruses include WNV (*Orthoflavivirus nilense*), Usutu virus (*O. usutuense*), Yellow Fever virus (*O. flavi)* and Dengue virus (*O. dengue)*; *Hepacivirus* including Hepatitis C virus; *Pestivirus* – Classical Swine fever virus, and *Pegivirus* – HPgV-2. These three genera come from the family of *Flaviviridae* virus. With more than 70 viruses, the family *Flaviviridae* can be additionally classified into the mosquito-borne and tick-borne virus groups. The mosquito-borne viruses can be subdivided into two categories - encephalitic (WNV) and non-encephalitic (Yellow Fever virus and Dengue virus) (Calisher et al. 1989; Kuno et al. 1998).

The history of WNF and distribution through Romania and Bulgaria

West Nile Virus produces encephalitic West Nile Fever (WNF), a zoonotic disease that can affect birds, horses,

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other mammals, and humans. In 1937 the first reported case of WNF was in a female patient living in the West Nile area of Uganda, caused by lineage 1 of the WNV (Smithburn et al. 1940).

Suspected outbreaks of WNF in Romania were first reported in the 1950s. In Transylvania the first confirmed cases of WNV were detected in 1955, followed by a further outbreak in 1964 in Banat (Tsai et al. 1998). In 1996, Bucharest had its first registered outbreak of WNF. Over 800 neurological cases were reported, 393 were confirmed serologically, with 17 fatalities (4.1% seroprevalence) (Tsai et al. 1998). Follow-up research demonstrated seroprevalence of WNV in Romania between 1997 and 2000 with a mean of 10 human cases annually and 13% fatality (ECDC 2021; Schwarz and Long 2023). Studies covering the outbreaks during 2010-2015, discovered for the first time the presence of lineage 2 of the WNV in Romania (Wang et al. 2024).

Information regarding human outbreaks of the WNV in Bulgaria have not been found. Reports of a very small number of cases from 2016 are available. According to the data from European Centre for Disease Prevention and Control, for the period of 2016-2019 Bulgaria reported a total 25 human infectious cases, with a single fatality in 2019 (ECDC 2021). Study conducted in the period July-October 2022 in Bulgaria, using Epitome blocking ELISA resulted in 9.26% positive samples (35 from 376) (Rusenova et al. 2024).

Life cycle and transmission

According to research data, a wide range of mosquito species have the ability to acquire and transmit WNV. It is commonly accepted that the main transmission vectors globally are the representatives of *Culex* and *Aedes.* The main vector - *Culex* mosquitoes, are known to be feeding on different avian and mammal species (Flores-Ferrer et al. 2023).

The geographic distribution of the mosquito-borne West Nile Fever largely depends on the habitat of the preferred mosquito vector, with *Culex* mosquitoes transmitting encephalitic flaviviruses mainly in the Northern Hemisphere. Transmission of WNV to horses occurs through the bite of an infected mosquito, typically the *Culex* species. The virus is maintained in a transmission cycle between birds and mosquitoes, with horses and humans being considered incidental or "dead-end" hosts. Horses are particularly susceptible to the infection, in the majority of the cases there is absence of clinical expression, and a mortality rate of up to 20% in severe cases (Schwarz and Long 2024; Wang et al. 2024).

Infections by the WNV appear to be random from geographical point of view. Using temporo-spatial analyses, the most pertinent factor is the distribution of vectors which allows for detection of potential risk zones (Kitron 2000). As a vector-borne disease, the WNF distribution is highly predictable when considering the following factors: routes of migratory birds (Lu et al. 2024; Trájer et al. 2014) and climate variations related to seasonal changes during the year (Kitron 2000). The availability of water, vegetation and temperature fluctuations influences the gathering of mosquitoes and birds (Parker 2024).

Seasonal changes influence the behavior of humans managing horses during periods of peak activity for mosquitoes (Loeb et al. 2005). Since Romania and Bulgaria are located in the Northern Hemisphere, the peak season of mosquito activity is between May and October (Napp et al. 2018).

Viral RNA and antibody detection

It was demonstrated in earlier studies, that in the initial stages of infection of WNV, the serum samples had no detectable antibodies and should be tested for presence of viral genome (Dauphin and Zientara 2007). Therefore RT-PCR is the method of choice for detection of WNV during the initial stages of infection (Varghese et al. 2023). Serological testing remains the primary diagnostic method (Dauphin and Zientara 2007). According to the World Organisation for Animal Health (founded as OIE) in their Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (2022), the "gold standard" for WNV detection is virus neutralisation which is used for titration and for confirming the presence of specific antibodies against WNV in laboratory environment (Beck et al. 2013).

In this study we used commercial epitope-blocking ELISA kits, developed for the detection of antibodies focusing on the WNV glycoprotein E (anti-prot-E IgG). The kits were proven to be highly sensitive and accurate with a very low cross-reactivity to other species of genus *Flavivirus* (Sotelo et al. 2011).

Serological studies on horse populations were conducted in neighbouring regions of Serbia and Hungary, demonstrating the notifiable presence of WNV in the region (Medić et al. 2014; Fehér et al. 2022). With the present study, we aim to reveal similar serological evidence for presence of WNV infections in horses within Romania and Bulgaria and areas with close proximity to bird migratory routes.

MATERIALS AND METHODS

Ethical statement

This research did not involve any experiments on animals. All serum samples used were collected by authorized veterinarians as a part of the annual check-up of the horses. The data were collected and analyzed using methods that adhere to ethical principles and ARRIVE guidelines for research.

Sampling

Blood sera were collected between July 2022 and July 2023 from 147 horses in four administrative zones in West Romania, one zone in Central Romania, one location in South and one in North Bulgaria (Fig. 1). Horses were sampled as part of annual check-up, were from different breeds, sex and age (between 1 and 3 years old) and none had previous vaccination against WNF. All the animals were living outdoors on open fields (no indoor housing at all) and were free from clinical symptoms for WNF or other diseases and with a normal Body Condition Score (BCS). The horses' BCS was estimated using the system, described by Lance et al. (2020).

Using ELISA serological testing

All serum samples were tested using an epitope blocking ELISA kit, commercially available under the name INGEZIM West Nile Ab COMPAC (Gold Standard Diagnostics, USA). Each sample was tested in accordance

with the manufacturer's instructions. In order to exclude intra-test variances, each sample was tested twice, using separate kit segments.

The value for Inhibition Percentage (IP) was calculated per each serum, using the formulation provided by the ELISA kit manufacturer. According to the manufacturer's instructions, samples are considered Positive when IP is ≥40%, and Negative when IP ≤30%. We used the Sunrise reader model from Tecan Austria GmbH.

Serological testing was conducted in two laboratories: the ELISA laboratory unit at the University of Life Sciences in Timișoara, Romania, and the Laboratory of Serology at IEAMPAM – Bulgarian Academy of Sciences, Bulgaria.

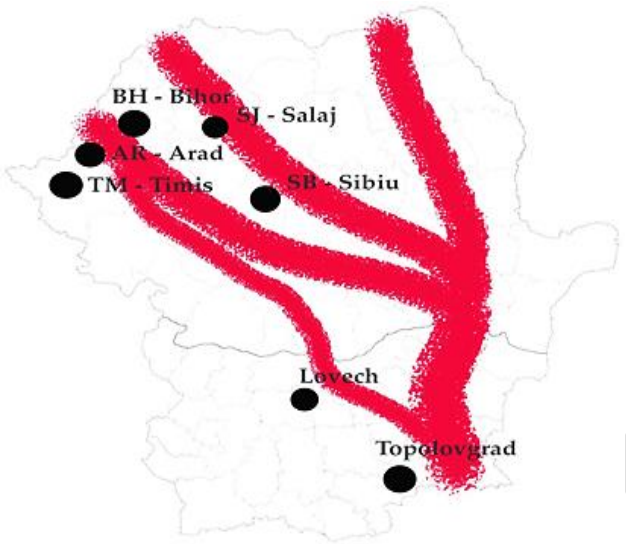

Fig. 1: Map of Romania and Bulgaria showing in red birds' migration routes, according to the Global Register of Migratory Species and in black - the areas where serum sampling was performed.

RNA extraction and RT-PCR

The serum samples were stored at -80°C prior to RT-PCR (Reverse Transcription Polymerase Chain Reaction). RNA from the equine samples was extracted using QIAmp Viral RNA Mini kit (Qiagen, Germany), following the manufacturer's instructions. Extracted RNA was stored at -80°C before testing. The samples were screened for WNV lineages 1 and 2 using a one-step TaqMan RT-PCR assay targeting a 144bp of a conserved region of the capsid gene (Linke et al. 2007). The oligonucleotide primers used for RT-PCR (Linke et al. 2007), have the following sequences:

-ProC-F1: CCTgTgTgAgCTgACAAACTTAgT, position: S (Same)
-ProC-R: gCgTTTTAgCATATTgACAgCC, position: AS (AntiSense) and
-ProC-TM: 6FAM-CCTggTTTCTTAgACATCgAgATC TXCgTgCp, position: AS.

All RT-PCR tests were performed at the reference laboratory of the National Centre for Infectious and Parasitic Diseases in Sofia, Bulgaria.

RESULTS

As seen in Table 1, 147 horses were tested using the epitope-blocking ELISA in our study, 51 had antibodies against WNV specific glycoprotein E in the serum. All positive samples were negative for the presence of WNV RNA by further testing using RT-PCR. The highest seroprevalence was detected in Sibiu County, which could be explained by the large wetland and forest areas providing favourable conditions for vector propagation (Ukawuba and Shaman 2018). The results for Timis County were unexpectedly high, unlike those from previous studies in the neighbouring region of Vrsac (Serbia) (Medić et al. 2014). The percentage of positive results was in direct correlation with the geographical location. The decreasing number of positive samples southward could potentially be explained by the different climate conditions in those specific areas.

Table 1: Locations of testing, number of samples and positive results for WNV in horses

County	Tested sera	Positive sera	Percentage positive
Romania	74	35	47
AR - Arad	16	9	56
BH - Bihor	18	7	39
TM - Timis	15	9	60
SB - Sibiu	8	5	63
SJ - Salaj	17	5	29
Bulgaria	73	16	22
North (Lovech mpty)	48	11	23
South (Topolovgrad mpty)	25	5	20
Total	147	51	35

DISCUSSION

WNV has clinical representation in 8 to 10% of the cases of infected horses and it has a 20% mortality amongst those with clinical signs (Schwarz and Long 2024; Wang et al. 2024). The horses tested in the present study had a subclinical infection without symptomatic indicators. Keeping in mind the short viremia for WNF, RT-PCR tests are reliable only during the active stages of infection. The use of serological methods for detecting antibodies resulted in a higher surveillance value. To detect the prevalence of infection, epitope blocking and competitive or indirect ELISA are proven to be the most reliable methods (Beck et al. 2017; OIE 2022).

Our results demonstrated the widespread presence of the WNV antibodies among the tested horses. 47%, or almost every second horse from the Romanian sample and 22%, or a fifth from the Bulgarian, had direct contact with the virus. None of the tested animals had lived in another area for the last 12 months or more.

The results from Romania showed there was a higher percentage of cases in the counties with bigger concentration of forests and water sources. Timis, Sibiu and Arad have almost twice as much serologically positive horses in comparison to Bihor and Salaj. Hereby, we can demonstrate that the availability of wetlands and forests preferred by birds for roosting or nesting in the areas where horses reside, affects positively WNV distribution.

The profile of the two selected areas in Bulgaria is similar – the southern region has favorable moderate temperatures with the presence of wetlands and forests, and the northern region has plenty of wetlands and includes the Danube River. Resulting in a similar percentage of seropositive horses from both areas.

Table 2: Demographic data for Romania (Institutul Național de Statistică) and Bulgaria (National Statistical Institute)

Zone	Total population	Urban		Rural	
Romania (up to 01-Dec-2021)		Count	%	Count	%
AR - Arad	409072	223351	55	185721	45
BH - Bihor	549752	266353	48	283399	52
TM - Timis	650544	400673	62	249871	38
SB - Sibiu	375992	245922	65	130070	35
SJ - Salaj	217895	83775	38	134120	62
Total	2203255	1220074	54	983181	46
Bulgaria (up to 31-Dec-2022)					
North (Lovech mpty)	113356	70158	62	43198	38
South (Topolovgrad mpty)	8615	4466	52	4149	48
Total	121971	74624	57	47347	43

Regarding the human cases of West Nile Fever in Romania and Bulgaria and whether they are related to the results in equines

Since the first outbreak of WNF in Romania (Tsai et al. 1998), every year there are cases of symptomatic patients in the human population. Located on a main migratory route and in proximity to endemic regions for birds in Serbia and Hungary, Romania and Bulgaria are at a high risk for annual growth in WNF cases in humans. Horses, birds and other animal species affected by WNV, could be surveyed during the summer seasons and the data obtained could provide valuable information regarding the distribution trends of WNF in the area. County Timis is in close proximity to the Serbian region of Vrsac, and is now known to have a higher level of WNV seroprevalence due to the presence of two big rivers flowing through Timis County – Bega and Timis, which puts the county at higher risk of increased infection growth. The presence of rivers, lakes and other environmental components favourable for mosquitos' propagation and distribution are present in all other areas studied. The Danube river passes through South Romania and North Bulgaria, and in South Bulgaria the mountains provide annual median temperatures ranging between 1.4°C and 20.7°C (Marinova et al. 2017).

In Bulgaria human cases of WNF appear sporadically: during 2010, 2011, 2013, 2014, 2021, and in 2022 no cases of infection in humans were registered; there were two cases in 2012, 2015 and 2016; a single occurrence in 2017 and 2020; six in 2018 and four cases in 2019. The highest percentage of human WNV infections were observed in Burgas County, located in the southern zone of sampling in Bulgaria (ECDC 2021).

We could hypothesize the big difference between the cases in Romania and Bulgaria is due to different demographic distribution. With a much lower population, Bulgaria has the most infections concentrated in urban areas, at a distance from mountains or water areas. The northern part of the country, at a close proximity to the Danube River, has a very low population density, with the exception of the towns of Pleven and Ruse (Table 2).

Conclusion

In summary, the study for the first time presented through serological evidence, the spread of West Nile Virus infection amongst horse populations in Romania and Bulgaria. Correlated with the occurrence of infection in humans. Using horse populations could be a way to develop, perform and maintain a predictive surveillance program.

Conflicts of interest: The authors declare no conflict of interest.

Author's contributions: Plamen M. Kirov and Ionica Iancu participated equally in all stages of the current research. Elitsa Panayotova performed all related to the PCR testing steps – samples preparation and testing. Rusko Petrov, Hristo K. Hristov, Abedulkadir Abudalleh and Alexandru Gligor contributed in samples collection on the field. Mirela Imre – ELISA testing and results analysis. Radostina Alexandrova and Viorel Herman provided guidance for the study design and editing of the manuscript.

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REFERENCES

Beck C, Jimenez-Clavero MA, Leblond A, Durand B, Nowotny N, Leparc-Goffart I, Zientara S, Jourdain E and Lecollinet S, 2013. Flaviviruses in Europe: Complex circulation patterns and their consequences for the diagnosis and control of West Nile disease. International Journal of Environmental Research and Public Health 10: 6049-6083. https://doi.org/10.3390/ijerph10116049

Beck C, Lowenski S, Durand B, Bahuon C, Zientara S and Lecollinet S, 2017. Improved reliability of serological tools for the diagnosis of West Nile fever in horses within Europe. PLoS Neglected Tropical Diseases 11: e0005936. https://doi.org/10.1371/journal.pntd.0005936

Calisher CH, Karabatsos N, Dalrymple JM, Shope RE, Porterfield JS, Westaway EG and Brandt WE, 1989. Antigenic relationships between flaviviruses as determined by cross-neutralization tests with polyclonal antisera, Journal of General Virology 70: 37-43. https://doi.org/10.1099/0022-1317-70-1-37

Dauphin G and Zientara S, 2007. West Nile virus: recent trends in diagnosis and vaccine development. Vaccine 25: 5563-5576. https://doi.org/10.1016/j.vaccine.2006.12.005

ECDC, 2021. European Centre for Disease Prevention and Control. West Nile virus infection. In: ECDC. Annual epidemiological report for 2019. Stockholm.

Fehér OE, Fehérvári P, Tolnai CH, Forgách P, Malik P, Jerzsele Á, Wagenhoffer Z, Szenci O and Korbacska-Kutasi O, 2022. Epidemiology and Clinical manifestation of West Nile Virus infections of equines in Hungary, 2007-2020. Viruses 14: 2551. https://doi.org/10.3390/v14112551

Flores-Ferrer A, Suzán G, Waleckx E and Gourbière S, 2023. Assessing the risk of West Nile Virus seasonal outbreaks and its vector control in an urbanizing bird community: An

integrative R_0-modelling study in the city of Merida, Mexico. PLOS Neglected Tropical Diseases 17(5): e0011340. https://doi.org/10.1371/journal.pntd.0011340

Kitron U, 2000. Risk maps: transmission and burden of vector-borne diseases. Parasitology Today 16(8): 324-325. https://doi.org/10.1016/s0169-4758(00)01708-7

Kuno G, Chang GJ, Tsuchiya KR, Karabatsos N and Cropp CB, 1998. Phylogeny of the genus Flavivirus. Journal of Virology 72: 73-83. https://doi.org/10.1128/JVI.72.1.73-83.1998

Lance AB, Amanda MB, Kelsey JN, John LP, Logan DH, Trent JM, Travis CT, Zane MT, Austin HV and Ty EL, 2020. Relationship between live body condition score and carcass fat measures in equine. Translational Animal Science 4: txaa179. https://doi.org/10.1093/tas/txaa179

Linke S, Ellerbrok H, Niedrig M, Nitsche A and Pauli G, 2007. Detection of West Nile virus lineages 1 and 2 by real-time PCR. Journal of Virological Methods 146: 355-358. https://doi.org/10.1016/j.jviromet.2007.05.021

Loeb M, Elliott SJ, Gibson B, Fearon M, Nosal R, Drebot M, D'Cuhna C, Harrington D, Smith S, George P and Eyles J, 2005. Protective behavior and West Nile virus risk, 2020. Emerging Infectious Diseases 11:1433-1436. https://doi.org/10.3201/eid1109.041184

Lu L, Zhang F, Oude Munnink BB, Munger E, Sikkema RS, Pappa S, Tsioka K, Sinigaglia A, Del Molin E, Shih BB, Günther A, Pohlmann A, Zieger U, Beer M, Taylor RA, Bartumeus F, Woolhouse M, Aarestrup FM, Barzon L, Papa A, Lycett S and Koopmans MPG, 2024. West Nile virus spread in Europe: Phylogeographic pattern analysis and key drivers, PLOS Pathogens 20(1): e1011880. https://doi.org/10.1371/journal.ppat.1011880

Marinova T, Malcheva K, Bocheva L and Trifonova L, 2017. Climate profile of Bulgaria in the period 1988-2016 and brief climatic assessment of 2017. Bulgarian Journal of Meteorology and Hydrology 22: 2-15.

Medić S, Van den Hoven R, Petrović T, Lupulović D and Nowotny N, 2014. Serological evidence of West Nile virus infection in the horse population of northern Serbia. Journal of Infection in Developing Countries 8: 914-918. https://doi.org/10.3855/jidc.3885

Napp S, Petrić D and Busquets N, 2018. West Nile virus and other mosquito-borne viruses in Eastern Europe. Pathogens and Global Health 112: 233-248. https://doi.org/10.1080/20477724.2018.1483567

OIE, 2022. World Organisation for Animal Health (OIE). Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2022.

Parker N, 2024. Exploring the role of temperature and other environmental factors in West Nile virus incidence and prediction in California counties from 2017–2022 using a zero-inflated model. PLoS Neglected Tropical Diseases 18(6): e0012051. https://doi.org/10.1371/journal.pntd.0012051

Rusenova N, Rusenov A, Chervenkov M and Sirakov I, 2024. Seroprevalence of West Nile Virus among equids in Bulgaria in 2022 and assessment of some risk factors. Veterinary Science 11: 209. https://doi.org/10.3390/vetsci11050209

Schwarz ER and Long MT, 2023. Comparison of West Nile Virus disease in humans and horses: Exploiting similarities for enhancing syndromic surveillance. Viruses 15(6): 1230. https://doi.org/10.3390/v15061230

Smithburn KC, Hughes TP, Burke AW and Paul JH, 1940. A neurotropic virus isolated from the blood of a native of Uganda. American Journal of Tropical Medicine 20: 471-492. https://doi.org/10.4269/ajtmh.1940.s1-20.471

Sotelo E, Llorente F, Rebollo B, Camuñas A, Venteo A, Gallardo C, Lubisi A, Rodríguez MJ, Sanz AJ, Figuerola J and Jiménez-Clavero MÁ, 2011. Development and evaluation of a new epitope-blocking ELISA for universal detection of antibodies to West Nile virus. Virological Methods 174: 35-41. https://doi.org/10.1016/j.jviromet.2011.03.015

Trájer AJ, Bede-Fazekas Á, János B and Anna P, 2014. Seasonality and geographical occurrence of West Nile fever and distribution of Asian tiger mosquito. Quarterly Journal of the Hungarian Meteorological Service 118: 19-40.

Tsai TF, Popovici F, Cernescu C, Campbell GL and Nedelcu NI, 1998. West Nile encephalitis epidemic in southeastern Romania. Lancet 352: 767-771. https://doi.org/10.1016/s0140-6736(98)03538-7

Ukawuba I and Shaman J, 2018. Association of spring-summer hydrology and meteorology with human West Nile virus infection in West Texas. Parasites Vectors 11: 2002-2016. https://doi.org/10.1186/s13071-018-2781-0

Varghese J, De Silva I and Millar DS, 2023. Latest Advances in Arbovirus Diagnostics. Microorganisms 11(5): 1159. https://doi.org/10.3390/microorganisms11051159

Wang HR, Liu T and Gao X, 2024. Impact of climate change on the global circulation of West Nile virus and adaptation responses: a scoping review. Infectious Diseases of Poverty 13: 38. https://doi.org/10.1186/s40249-024-01207-2