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PREVALENCE OF WEST NILE VIRUS ANTIBODIES IN INDOOR DOGS FROM AN URBAN AREA IN IAȘI, ROMANIA: INDICATORS OF VIRAL PRESENCE AND URBAN TRANSMISSION POTENTIAL

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ABSTRACT. West Nile Virus (WNV), a zoonotic mosquito-borne virus (mobovirus) originally isolated from the blood of a febrile Ugandan woman in 1937, caused substantial human disease in Europe starting in the 1990s and emerged in 1999 in The United States of America (USA) for the first time. It has become an important concern for public health due to its reemergence and frequent human outbreaks. The enzootic transmission cycle of arboviruses involves primary wild animals; however, spillover transmission is reported frequently in domestic animals. Dogs are dead-end hosts in WNV transmission epidemiology. However, detecting WNV antibodies in the dog population can indicate the virus's presence and spread in different areas. The virus is known to be endemic in parts of Romania, including Iași County. The study aimed at assessing the prevalence of anti-WNV antibodies in indoor dogs from an urban area in Iași, where all the conditions for virus transmission are met (wetland, density of wildlife hosts including birds, abundance of vectors, domestic mammal hosts and synanthropic birds). Using a commercial enzyme-linked immunosorbent assay (INGEZIM West Nile COMPAC, Ingenasa, Madrid, Spain), serum samples collected from indoor dogs between 2020–2022 were screened for WNV antibodies. The results showed an overall seroprevalence of 12.2%. Detection of specific antibodies in dogs

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suggests a possible establishment of an urban cycle for WNV or other antigenically related flaviviruses.

Keywords: dogs; seroprevalence; West Nile.

INTRODUCTION

Currently, in nature, there are more than 500 arboviruses (Arthropod-borne viruses), and few are known to have an impact on public and animal health (Nasraoui *et al.*, 2023). The enzootic transmission cycle of arboviruses involves mosquitoes as vectors and primarily wild animals as hosts, although spillover transmission is reported frequently in domestic animals (Assaid *et al.*, 2020). Among these arboviruses, West Nile Virus (WNV), a flavivirus originally isolated from the blood of a febrile Ugandan woman in 1937, causes substantial human disease (Kramer *et al.*, 2007). In the early 1990s, Europe's geographic range of human infections was associated with sporadic cases (Sambri *et al.*, 2013). Subsequently, throughout warmer regions of Europe, WNV has been responsible for disease outbreaks in humans, horses and birds (Napp *et al.*, 2018). Furthermore, in 1996, the first major epidemic of neuroinvasive infection in humans in Europe (393 hospitalised cases) (Napp *et al.*, 2018) occurred in Romania (Sambri *et al.*, 2013). In the subsequent years, the number of reported cases fluctuated, with peaks reported in 2010 (n=57) (Sirbu *et al.*, 2011) and in 2018 (n=277), when WNV infections were widely spread compared with the previous years, suggesting an increased distribution of WNV (Young *et al.*, 2021) that followed the introduction and spread of WNV lineage 2 in the country.

The natural transmission cycle of WNV involves birds with a high potential to serve as amplifying hosts and ornithophilic mosquitoes as primary vectors and other mosquito species acting as bridge vectors (Bowen and Nemeth, 2007). Occasionally, WNV infects other vertebrates (Castillo-Olivares and Wood, 2004), having a broad host range, including mammals, such as humans, horses and companion animals (Bowen and Nemeth, 2007).

In dogs, the WNV infection was first mentioned following the viral isolation from an encephalitic dog in Africa in 1977 (Buckweitz *et al.*, 2003). Subsequently, as a result of increased population density and closer proximity to humans (Austgen *et al.*, 2004), several studies considered the role of dogs in the viral transmission cycle. Although they do not actively participate in transmission as amplifying hosts, dogs were found to be susceptible to WNV infection through serological surveys (Lichtensteiger *et al.*, 2003), serving as an indicator for potential WNV presence in the human population. In this species, the clinical manifestations are poor, viraemia sets in four days post-infection, the viraemic level is low and fluctuating (Austgen *et al.*, 2004) and the length of antibody persistence is unknown (Buckweitz *et al.*, 2003).

Clinical manifestations have been reported in outdoor dogs kept in paddock-like conditions (Buckweitz *et al.*, 2003) and frequently described signs were myocarditis, encephalitis and polyarthritis (Cannon *et al.*, 2006).

Our study aimed to determine the prevalence of anti-WNV antibodies in indoor dogs from Iași County. The study's objectives focused on indoor

dogs from an urban area, considering the plasticity of viral circulation in different ecosystems, particularly in dry urban areas or forest ecosystems with flood zones (Chevalier *et al.*, 2020). It also aimed at estimating the degree of silent expansion of the virus and assessing the sentinel role of this species in light of the availability of the target population and the cohabitation or living in the area of people's homes (Davoust *et al.*, 2014).

MATERIALS AND METHODS

Study site

Iași County is one of the migration hubs used by migratory birds on their way to the Danube Delta and the Black Sea, and also one of the most endemic areas for WNV for humans (unpublished data), animals (Crivei *et al.*, 2018; Ludu Oslobanu *et al.*, 2014) and mosquitoes (Crivei *et al.*, 2023).

Wetlands, a high concentration of animal hosts, especially birds, an abundance of vectors, domestic mammal hosts and synanthropic birds are among the ecological features in the peri-urban settings of Iasi County that make it conducive to viral transmission. A combination of woodland regions, aquatic bodies, a high density of people and hosts and the availability of artificial mosquito resting and breeding sites define the urban environment.

Sample collection

To achieve the proposed objective, between May 2020 and December 2022, 129 samples were collected from indoor dogs presented in a veterinary clinic for routine checking and without any symptomatology related to WNV. Although data regarding comorbidities, age, breed and gender were recorded, the selection of individuals for testing did not consider any of those details. As summarised in *Table 1*, the samples were collected from dogs ranging in age from 2 months to 17 years; most were female and medium-sized breeds (11–25 kg). Dogs were housed indoors singly or in pairs and were walked outdoors twice or more per day, thus being potentially exposed to mosquito bites.

All samples were taken using sterilised tools after jugular vein puncture. Serum separation was performed by centrifugation for 10 minutes at 2000 rpm; then, sera were stored at -20°C until further analysis.

Table 1 – Breakdown of the age groups and gender of dogs (after Studzinski *et al.*, 2006)

Group	Age range (years)	No. of collected samples	Gender	No. of collected samples
Puppies	<1	8	Male	58
Young	1–2.99	19	Female	71
Adult	3–5.99	15		
Middle-aged	6–7.99	19		
Old	8–9.99	14		
Senior	10–11.99	54		
No. of collected samples		**129**		**129**

Out of 129 dog samples, 90 were tested for the detection of specific antibodies against WNV. Of the analysed samples, 43 were collected from the senior group that came for a routine check in the clinic. The selection of samples was made considering the location study site (a district from Iasi City).

Authorised medical staff performed the sampling during the diagnostic procedure with the owners' consent. All the handling of the animals was done in the frame of the 205/2004 Romanian Animal Welfare Law.

Serological testing

To give an indication of anti-WNV antibodies in dogs' serum, samples were screened using a commercial enzyme-linked immunosorbent assay (ELISA INGEZIM West Nile COMPAC, Ingenasa, Madrid, Spain), previously validated in other studies (García-Bocanegra *et al.*, 2018) The kit can be used for the detection of anti-E protein antibodies produced by natural infections and uses pre-coated plates with a recombinant WNV antigen, based on competition between antibodies present in the test samples and an anti-E monoclonal antibody (mAb) conjugated to horseradish peroxidase (HRP). Competitive ELISA kits can detect virtually any immunoglobulin isotype but are usually used for IgG detection, so they are classified as tools for its detection (Beck *et al.*, 2017).

In this kit, 10 µl of sample is required for the reaction, with a final serum dilution of 1:5. Adding mAb specific to the E protein in the conjugate causes a colourimetric reaction if it is attached to the protein. If the animal is infected, the viral epitope is blocked by the specific antibodies present in the sample, and the addition of the substrate will not change the reaction product colour. Positive and negative controls were provided by the manufacturer. Using a microplate spectrophotometer (Epoch 2; Agilent BioTek), the absorbance values were recorded at 450 nm wavelength. The assay was performed according to the instructions provided by the manufacturer. The results were interpreted by calculating the percentage of inhibition (IP). Samples with a S/N ratio equal to or higher than 40% were considered positive, while if the IP was ≤ 30%, they were considered negative. The samples were tested at the Regional Centre of Advanced Research for Emerging Diseases, Zoonoses and Food Safety (ROVETEMERG), Iași.

Statistical analysis

The prevalence of WNV was estimated from the ratio of positive to the total number of tested samples, with the exact binomial confidence intervals of 95% (95% CI). The Fisher exact test was applied to evaluate the statistical significance of the results.

RESULTS

ELISA results

As a result of testing the 90 dogs' samples using the commercial INGEZIM WNV Compaq kit, anti-WNV antibodies were detected in 11 samples, with an overall seroprevalence of 12.2% [95% CI 5.46-18.99]. Regarding the gender ratio (*Table 2*), the difference between seropositive males vs females was not significant ($p < 0.05$).

Table 2 – Breakdown of the gender and age of dogs that seroconverted

Gender	Seroprevalence %	95% CI	Group	Seroprevalence %	95% CI
Female	6.7	[1.51 - 11.82]	Puppies	0	
Male	5.6	[0.82 - 10.29]	Young	1.1	[-1.05 – 3.28]
			Adult	1.1	[-1.05 – 3.28]
			Middle-aged	1.1	[-1.05 – 3.28]
			Old	1.1	[-1.05 – 3.28]

Nevertheless, there was a difference in positivity in terms of age group (*Table 2*), with the odds of seroprevalence rate of 7.8% among all the tested dogs from the seniors (older than 10 years). However, the difference was not significant at ($p < 0.05$).

DISCUSSION

The present study was conducted on serum samples collected during the COVID-19 pandemic (2020–2022) and investigated the prevalence of anti-WNV antibodies and the role of dogs as sentinels for WNV.

The overall seroprevalence (12.2%) found after testing 90 dogs from urban areas in Iasi is consistent with the one reported in the Democratic Republic of the Congo (12.5%), but lower than the values obtained in Italy (55.5%, 2011; 40.43%, 2018), Morocco (62%) or other studies undertaken in Romania in 2018 (42.1%) (Crivei *et al.*, 2018).

Surveillance actions on dogs, as potential sentinels, have gained global attention (*Table 3*). In the USA, West Nile Virus detection in puppies occurred six weeks before the appearance of the first infection case in humans (Kile *et al.*, 2005).

Using an extrapolation from the horse studies, the seroprevalence in age class, seen as a potential risk factor (Epp *et al.*, 2007; Salazar *et al.*, 2004), showed variations for all age ranges, with the highest seropositivity seen in the senior group (7.8%) but the differences in this study were not significant. Although a descriptive study showed that males could be more affected than females (Epp *et al.*, 2007), in our study, we could not consider seroprevalence for sex ratios since the male dogs were neutered. The results showed a similar percentage of seroprevalence in males vs females.

Regarding the age variable, the observed seropositivity in older animals is probably connected to a higher chance for exposure to WNV.

Compared to other studies on outdoor dogs (Kile *et al.*, 2005; Lan *et al.*, 2011), we tested samples from indoor dogs and registered a lower prevalence in this study. Thus, this might be explained by the fact that the likelihood of exposure to WNV through contact with infected mosquitoes is higher outdoors than indoors. Because of the close antigenic relationship between the viruses belonging to the Japanese encephalitis complex (Petersen and Marfin, 2002), a positive ELISA sample requires confirmation to avoid false results resulting from serological cross-reactions observed in the diagnostic laboratory.

Table 3 – Literature review regarding the seroprevalence studies in dogs

Country	No. of tested samples	WNV Prevalence (%)	Bibliography
Canada	143	28	(Gaunt *et al.*, 2015)
Ciad	55	30.9	(Davoust *et al.*, 2014)
China	367	4.6	(Lan *et al.*, 2011)
Democratic Republic of Congo	24	12.5	(Davoust *et al.*, 2014)
Djibouti	47	12.8	(Davoust *et al.*, 2014)
France	71	8.4	(Maquart *et al.*, 2017)
France	104	6.7	(Davoust *et al.*, 2014)
Italy	36	55.5	(Busani *et al.*, 2011)
Ivory Coast	137	2.2	(Davoust *et al.*, 2014)
Maroc	231	62	(Durand *et al.*, 2016)
Puerto Rico	269	8.5	(Phoutrides *et al.*, 2011)
Senegal	141	4.9	(Davoust *et al.*, 2014)
Spain	810	1.3	(García-Bocanegra *et al.*, 2012)
United States of America	442	1.6 26	(Kile *et al.*, 2005)
United States of America	189	5.3	(Komar *et al.*, 2001)
United States of America	414	55.9	(Levy *et al.*, 2011)
United States of America	154	56.5	(Lillibridge *et al.*, 2004)
Turkey	114	37.7	(Ozkul *et al.*, 2006)
Missouri	169	2.36	(Buckweitz *et al.*, 2003)
Quebec, Canada	1442	3.1	(Rocheleau *et al.*, 2017)
Italy	183	40.43	(Montagnaro *et al.*, 2019)

Hence, one of the limitations of this study stems from the fact that the positive samples need confirmation using the gold-standard method, namely a seroneutralisation assay. Another limitation of the study is the low number of samples and the analysis of dogs with various conditions.

CONCLUSIONS

Detection of WNV antibodies by ELISA technique revealed an antibody prevalence of 12.2% in the tested samples (11/90), consistent with those obtained in other published studies. This result indicates that WNV is circulating in the dog population from urban areas in Iasi.

Our results indicate that dogs could be used as suitable sentinel species for monitoring general WNV circulation close to humans. In our study, sampling was made during the COVID-19 pandemic, and in this interval, no WNV human cases were reported in Iași County.

The lack of WNV reporting in humans might be due to the specific COVID restrictions. Detection of specific antibodies in dogs strongly suggests the presence and persistence of WNV infection in the studied area.

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