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ESTABLISHING THE RISK OF WEST NILE VIRUS TRANSMISSION THROUGH MOSQUITO BITES USING THE DIGITAL PCR AND REAL-TIME PCR METHODS

Larisa IVĂNESCU[1], Gabriela MARTINESCU[1], Raluca MÎNDRU[1], Olimpia IACOB[1], Bianca ANDRONIC[1], Dumitru ACATRINEI[1] and Liviu MIRON[1]

e-mail: larisa.ivanescu@iuls.ro

Abstract

West Nile virus (WNV) is a re-emerging zoonotic pathogen which is a threat to both human and animal health. In Europe, there has been a marked expansion of WNV outbreaks in recent decades, causing more than 2000 symptomatic cases in 2018 alone. Winter temperatures between 2°C and 6°C were one of the strongest predictors of annual West Nile virus infections; a possible explanation for this result is that successful overwintering of infected adult mosquitoes (probably Culex pipiens) is the key to the intensity of outbreaks in the following year. The aim of the study was to compare the two diagnostic methods Real-Time PCR and dPCR, used in the detection of West NILE virus in mosquito vectors. Between April 2023 and June 2024, mosquitoes were captured from the Danube Delta area and from the north-eastern part of Romania in the city of Iasi, using the New Standard Miniature Incandescent Light Trap, model 1012. After morphological identification, mosquitoes were separated according to Culex pipiens species. Pools of 30 mosquitoes per pool were made, being tested for the presence of the West Nile virus. The advanced dPCR method was used to detect West Nile virus. Digital™ PCR (dPCR™) is an innovative technology that provides ultrasensitive nucleic acid detection and absolute quantification. It is very effective for resolving low-abundance targets, such as very small amounts of virus inside mosquito vectors.

Key words: *West Nile*, dPCR, virus quantification

INTRODUCTION

In 2020, West Nile virus outbreaks were recorded in Europe, particularly in countries like Spain and the Netherlands. However, the largest outbreak of human WNV infections in the European Union/European Economic Area (EU/EEA) occurred in 2018, when 11 countries reported 1,548 locally acquired infections [ECDC 2020]. The number of WNV infections that year exceeded the total number of cases recorded between 2010 and 2017 [Young JJ, et al. 2021]. In 2018, the total figures represented the second-highest number of recorded cases ever. Most countries reported a similar number of infections as before 2018, while Greece continued to report a high number of infections [ECDC 2020].

From the beginning of the 2020 transmission season in June until November, EU/EEA countries reported 315 human WNV infection cases through the European Surveillance System (TESSy): Greece (n=143), Spain (n=77), Italy (n=66), Germany (n=13), Romania (n=6), the Netherlands (n=6), Hungary (n=3), and Bulgaria (n=1) [ECDC 2020, WNV]. Over the last decade, a lineage 2 genetic strain of WNV has been observed spreading in Central Europe and the Mediterranean region [Bakonyi T, et al. 2013]. In Germany, this dominant EU strain was first detected in 2018 in resident birds and horses. Pietsch et al. reported an outbreak of nine locally acquired West Nile fever cases in September 2020, emphasizing the need for increased awareness of WNV presence among the public and medical professionals, as well as enhanced animal surveillance [Pietsch C, et al. 2020].

WNV lineage 2 was also reported in samples from birds and mosquitoes in the Netherlands for the first time at the end of August 2020 [Sikkema RS, et al. 2020]. An unprecedented outbreak of WNV infections occurred in the southern provinces of Spain, including Seville, Cádiz, and Badajoz, between July and September 2020, comprising 77 human cases and 137 documented equine outbreaks [ECDC 2020]. It is noteworthy that Spain had not reported any locally acquired WNV infections in humans from 2017 to 2019. Although the number of human WNV infections in 2020 was lower than in previous years, the geographical spread of WNV continued across Europe.

[1]"Ion Ionescu de la Brad", Iasi University of Life Sciences, Faculty of Veterinary Medicine

Environmental and ecological factors responsible for WNV are complex and not yet fully understood. However, ambient temperature is known to be an important determinant due to its effect on mosquito reproduction rates and the virus's extrinsic incubation period in mosquitoes [Paz S, Semenza JC 2013]. According to the monthly climate bulletins of the Copernicus Climate Change Service, positive surface air anomalies were recorded in the southern regions of the Iberian Peninsula from July to August, as well as in northwest Europe from August to October. The temporal and spatial overlap with WNV outbreaks could be mere coincidence, but in the long term, environmental conditions tend to become more favorable for the establishment and seasonal circulation of WNV in many European regions [C3S 2020].

Most human WNV infections diagnosed in 2020 were reported in areas with already documented viral activity in previous years, indicating the virus's overwintering and local circulation. Therefore, once established, the likelihood of maintaining and the risk of re-emergence of WNV infections in affected European areas remain high.

In the 2023 West Nile virus transmission season, Europe saw a notable rise in human cases. By August, there were 1,029 confirmed cases, with Italy being the most affected, accounting for 564 cases, followed by Greece with 266 cases. Spain and Romania also reported significant numbers, with 147 and 31 cases, respectively (ECDC).

The major importance of mosquitoes in the ***Cx. pipiens*** complex in the transmission of WNV arises from the large number of viral isolates obtained from field-collected specimens: Romania (Crivei et al., 2023), Greece (Tsioka et al., 2022), Germany (Kampen et al., 2020), Italy (Savini et al., 2012), and Serbia (Petrović et al., 2021). Their vectorial competence for WNV (Vogels et al., 2015), mixed feeding behavior (Fritz et al., 2015), abundance in urban environments, ability to transmit the virus vertically from an infected female to her offspring (Dohm et al., 2002), and their role as overwintering reservoirs of the West Nile virus during the overwintering process (Kampen et al., 2021) further highlight their role. Additionally, their increasing abundance in urban environments has been implicated as a key factor in the rising transmission rates of WNV in these areas.In Europe, ***Culex pipiens*** **s.s.** occurs in two distinct behavioral and ecological forms (Di Pol et al., 2022) — an above-ground form, ***Culex pipiens pipiens***, which enters diapause during winter and primarily feeds on birds, and an underground form, **molestus**, which thrives year-round in human-made underground habitats, feeds on mammals, and can even lay eggs without a blood meal. The two forms can hybridize, leading to a complex ecological mosaic that complicates predictions about vectorial capacity and is believed to have contributed to the emergence of WNV in Europe and America in recent decades (Haba and McBride, 2022a).

For a mosquito exposed to an arbovirus to become infectious, the virus must overcome various complex and evolutionarily selective physical barriers within the mosquito's body (Kenney and Brault, 2014), such as the peritrophic membrane, the midgut barrier, and the salivary gland barrier. The midgut and salivary gland barriers are further divided into an infection barrier and an escape barrier (Vogels et al., 2017).

The midgut infection barrier (1) prevents viruses from entering or replicating in the gut cells and from spreading to other cells. The midgut escape barrier (2) stops the virus from crossing the basal lamina, which lines the midgut, preventing the virus from disseminating throughout the mosquito's body (Van den Eynde et al., 2022).

The most frequently used diagnostic method for detecting WNV is the IgM capture enzyme-linked immunosorbent assay (ELISA). The sensitivity of this test is 95%-100% for both serum and cerebrospinal fluid (Tardei G, et al., 2000). In most cases, IgM can be detected in serum and cerebrospinal fluid from the onset of the disease (communication from CDC). Specific WNV IgM antibodies are undetectable until the end of the viremic period, which can represent up to four days of illness. Elevated WNV-specific IgM in a person with encephalitis or meningitis likely indicates an infection; however, it should be noted that IgM can persist for several months to more than a year.

West Nile Virus (WNV) should be considered in the differential diagnosis for patients presenting with symptoms of viral meningitis, encephalitis, and flaccid paralysis. Surveillance systems in the United States and Europe utilize cell culture and/or real-time reverse transcriptase polymerase chain reaction (RT-PCR) to identify the virus in collected mosquitoes (Engler O, et al., 2013; Lanciotti R, et al., 2000; Hadler J, et al., 2014).

MATERIAL AND METHOD

The West Nile Virus (WN) is a mosquito-borne flavivirus with neurotropic characteristics, causing pathogenic effects primarily in humans, horses, and birds (Campbell et al., 2002). Several divergent lineages exist, with lineage 1 being the most widespread and virulent, responsible for the majority of human outbreaks. Additionally, WNV

belongs to the Japanese Encephalitis Virus (JEV) serocomplex, which includes other viruses associated with human encephalitis: JEV, St. Louis Encephalitis Virus (SLEV), Murray Valley Encephalitis Virus, and Kunjin Virus (a subtype of WNV). All flaviviruses are closely related antigenically, which explains the cross-reactive serological responses observed during serological testing (Petersen and Roehrig, 2001).

In the transmission cycle, birds serve as natural reservoirs (amplifiers), while humans and horses are incidental hosts. WNV is maintained in nature through a mosquito-bird-mosquito transmission cycle, primarily involving mosquito species from the genus Culex (Campbell et al., 2002). The importance of mosquitoes from the Cx. pipiens complex in WNV transmission is highlighted by the high number of viral isolates obtained from field-collected specimens (Tsioka et al., 2022; Savini et al., 2012; Crivei et al., 2023; Kampen et al., 2020; Petrović et al., 2021), their vector competence for WNV (Vogels et al., 2015), their abundance in urban environments, their mixed feeding behavior (Fritz et al., 2015), their ability to transmit the virus vertically from an infected female to her offspring (Dohm et al., 2002), and their role as overwintering reservoirs for the West Nile Virus during the overwintering process (Kampen et al., 2021).Furthermore, their greater abundance in urban environments has been implicated as a key factor in the increased transmission rates of WNV in these areas.

The objective of the study was to establish the prevalence of the West Nile virus in mosquitoes collected from Tulcea County (Mineri, Tulcea, Victoria, and Murighiol) during the period from April 2023 to September 2024, using RT-PCR and digital PCR methods. CDC Light Trap with dry ice as an attractant and manual aspirators were used for collecting female mosquitoes directly from human hosts. The traps were deployed overnight between 8:00 PM and 8:00 AM.

The captured mosquito species were identified using the morphological identification keys provided by Becker et al. (2010) and the interactive keys from the MosKeyTool program (Institut Pasteur, France), with only specimens from the genus Culex being tested.

DNA/RNA extractions were performed using the AllPrep DNA/RNA Mini Kit (Qiagen). The AllPrep DNA/RNA procedure integrates QIAGEN's patented technology for selective double-stranded DNA binding with RNeasy technology. Efficient purification of high-quality DNA and RNA is guaranteed without requiring additional RNase or DNase digestions. DNA/RNA extractions were conducted on 31 vector samples, with 50 specimens per sample.For the identification of the West Nile virus using RT-PCR, the CFX96 Touch Real-Time PCR system was employed. This is a powerful, precise, and flexible real-time PCR detection system. The six-channel instrument (five colors and one FRET channel) combines advanced optical technology with precise temperature control to provide sensitive and reliable detection for both simplex and multiplex reactions.

The amplification reactions contained 5 µl of QuantiNova Multiplex PCR Master Mix (Qiagen), 5 µl of DNA template, primers (0.4 µM), probes (0.25 µM), and molecular-grade water to a final volume of 20 µl.

The thermocycling conditions consisted of an initial denaturation step at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 62°C for 1 minute. Melting temperature (Tm) measurements were conducted between 65°C and 88°C at 0.5-second intervals. Fluorescent signals collected from the FAM and HEX channels were analyzed using CFX Manager Software Version 3.1 (Bio-Rad, Germany). The primers recommended by Linke et al. (2007) were used, with adaptations to the RT-PCR protocol (table 1).

Table 1.

Specific primers used for *West Nile* virus detection

WNV	**ProCF1**: CCTGTGTGAGCTGACAAACTTAGT **ProC-R**: GCGTTTTAGCATATTGACAGCC **ProC-TM**: CCTGGTTTCTTAGACATCGAGATCTXCGTGCP	5′-UTR	Linke și colab., 2007

For the detection of the *West Nile* virus using dPCR, the QIAcuity One system from Qiagen was used. The dPCR Microbial DNA Detection Assay portfolio utilized digital PCR nanoplate technology.Digital PCR (dPCR) is a cutting-edge technology that provides a more precise and sensitive approach for the absolute quantification of DNA or RNA. Instead of measuring the cumulative signal of a reaction, dPCR divides the sample into multiple compartments, enabling the

individual analysis of molecules.In dPCR, the sample is diluted and partitioned into a very large number of small compartments (droplets, microvolumes, or chambers). Each compartment contains either a single target DNA molecule or none. After PCR amplification, the fluorescent signal from each compartment is analyzed to determine the presence or absence of target molecules.

The dPCR program utilized was the one provided with the QIAcuity One-Step Advanced Probe PCR Kit from Qiagen (table 2).

Table 2.

dPCR program used

Number of repetitions	Temperature °C	Duration
1 x	50	40 min
1 x	95	2 min
40 x	95 60	5 s 30 s

RESULTS AND DISCUSSIONS

A total of 31 pools, representing 1,550 specimens classified under the *Culex* genus, were tested for the presence of the West Nile virus. Out of the 31 pools, only one pool tested positive using both methods employed. This indicates a prevalence rate of 0.31% for the West Nile virus in Tulcea County.

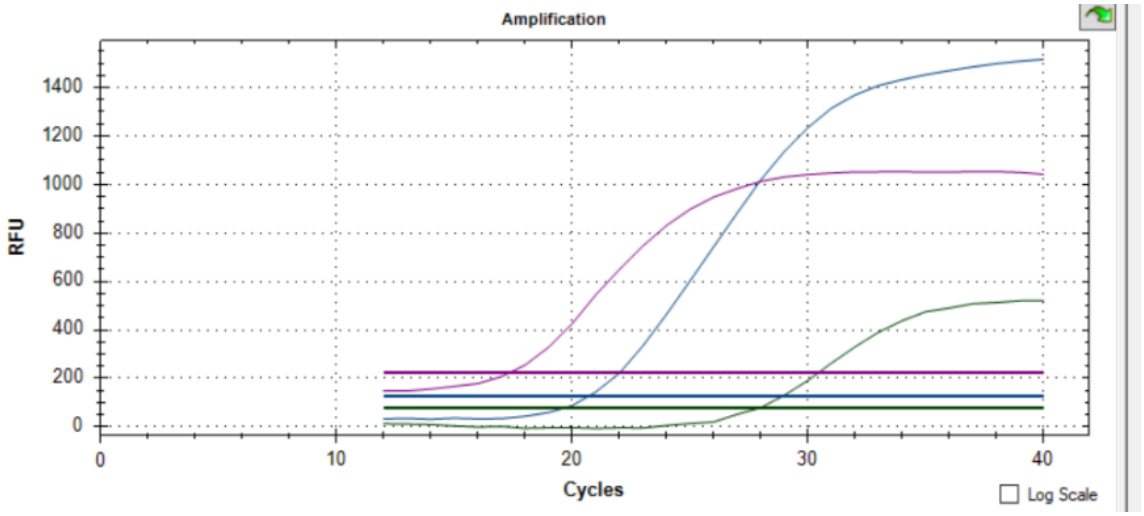

Figure 1. **Amplification curves and melting temperature for the *West Nile* virus**

In the pool identified as positive for the presence of the West Nile virus, a high viral load was observed with an early migration curve (Fig. 1) and a CT value of 20.63 (Fig. 2), indicating a strongly positive sample.

Well	Fluor	Target	Content	Sample	Cq
C02	Cy5		Unkn	WNV_T47	17.28
C02	FAM		Unkn	WNV_T47	20.63
C02	HEX		Unkn	WNV_T47	27.84

Figure 2**. Positive RT-PCR sample with high viral load (CT 20.63) from a *Culex* pool**

Table 3.

Identification of the West Nile virus using dPCR

	Sample/NTC/Control	Reaction Mix	Target			Conc. [cp/µL]		CI (95%)	Partitions	Threshold
			Name	IC	Control Type	dPCR reaction	Undiluted sample	dPCR reaction	Valid Positive Negative	
F1	47TI	WNV _QIAcuityOneStepAdvanced 2 Total volume: 40 µL	WNV	-	-	13638.0	-	28.3%	23244 23243 1	53.90
			IC	IC	-	102.2	-	4.8%	23229 1686 21543	21.04
G1	48TI	WNV _QIAcuityOneStepAdvanced 2 Total volume: 40 µL	WNV	-	-	0	-	-	18407 0 18407	53.90
			IC	IC	-	0	-	-	18407 0 18407	35.96
H1	NTC_WNV	WNV _QIAcuityOneStepAdvanced 2 Total volume: 40 µL	WNV	-	-	0	-	-	23844 0 23844	53.90
			IC	IC	-	140.5	-	4.0%	23830 2376 21454	14.66

The West Nile virus was identified using the QIAcuity One system and the QIAcuity One-Step Advanced Probe PCR Kit (Qiagen). The dPCR method allowed for precise and absolute quantification of viral RNA by partitioning the sample into numerous small compartments within a nanoplate. Each compartment was analyzed individually to detect the presence or absence of the target viral RNA.

The results from dPCR confirmed the presence of the West Nile virus in one of the tested pools, supporting the findings from the RT-PCR analysis. The advanced sensitivity and specificity of dPCR ensured reliable detection, even in cases of low viral loads.In the analyzed sample, 13,638 copies/µl were recorded, indicating a strongly positive sample and confirming the RT-PCR result (table 3).

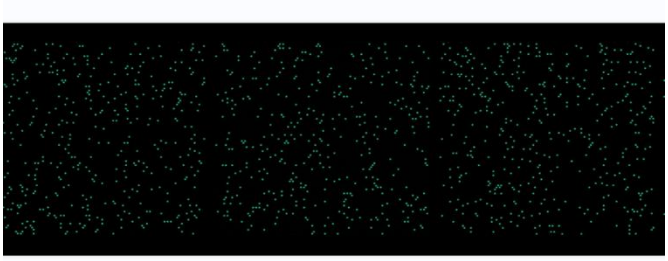

Figure 3. **Positive partitions marked by fluorescence for *West Nile virus***

Based on the viral load, positive partitions marked by fluorescence can be observed. These are evenly distributed, indicating accurate sample reading and a properly executed cycle (fig. 3).

CONCLUSIONS

The study detected West Nile virus (WNV) in one pool out of 31 tested, indicating a prevalence of **0.31%** in Tulcea County during the study period.

Both **Real-Time PCR** and **digital PCR (dPCR)** successfully identified the same positive pool, confirming the reliability of these methods. Digital PCR provided absolute quantification and demonstrated enhanced sensitivity for detecting low viral loads. A high viral load of **13,638 copies/µl** was detected in the positive sample, showing the robustness of dPCR technology for precise measurement. WNV outbreaks are influenced by climatic conditions, such as winter temperatures between 2°C and 6°C, which aid in the overwintering of infected mosquitoes and contribute to the intensity of subsequent outbreaks. The study highlights the vectorial competence of *Culex pipiens*, which plays a crucial role in WNV transmission due to its abundance, feeding behavior, and ability to serve as a reservoir during winter.The combined use of RT-PCR and dPCR ensures accurate detection of WNV in mosquito populations, crucial for effective surveillance and outbreak managemen

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