original article

West Nile virus seropositivity in Alanya, a coastal city in the Mediterranean region of Turkey

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BACKGROUND: West Nile virus (WNV)-related illness is a global health problem. Understanding the seropositivity rates and identifying the risk factors related to WNV in various animal species including humans is crucial for the implementation of effective prevention strategies.

OBJECTIVES: Assess the rate of seropositivity and the risk factors associated with WNV seropositivity.

DESIGN: Descriptive, cross-sectional

SETTING: Microbiology and virology departments in a veterinary college

PATIENTS AND METHODS: In a sample of healthy human participants in Alanya, located close to regions where WNV activity has been detected, anti-WNV IgG antibody detection was performed using enzyme-linked immunosorbent assays. The positive results were confirmed by virus neutralization tests (VNTs). The sample was compared with a second group of age- and gender-matched healthy subjects selected from a previous cross-sectional study.

MAIN OUTCOME MEASURES: Determination of the seropositivity and risk factors that were associated with WNV in healthy humans.

SAMPLE SIZE: 87 in current study; 356 in previous study.

RESULTS: The first group of 87, which had a high risk of encountering vector mosquitoes, had a positivity rate of 8% (7/87), whereas positivity in the second group was 4.5% (16/356; *P*=.181). In the entire sample, the anti-WNV IgG antibody was positive in 23 out of 443 (5.2%) samples by the ELISA test. Among these 23 samples, ten were confirmed as positive using VNTs. Therefore, the WNV IgG seropositivity was 2.3% (10/442). Confirmed IgG seropositivity rates were higher among male (3.8%) than female participants (0.9%; *P*=.054) and among adults aged ≥45 years (4%) than those aged 18-44 years (0.8%; *P*=.048).

CONCLUSION: This study highlights the presence of WNV infection in the research region. More comprehensive and multidisciplinary studies are required to increase our knowledge about this zoonotic infection including risk factors in line with the One Health approach.

LIMITATIONS: Small sample size.

CONFLICT OF INTEREST: None.

West Nile virus (WNV) is an enveloped virus belonging to the family Flaviviridae. The virus maintains its existence through cycles of enzootic transmission between mosquitoes and various bird species, which are the primary hosts of the pathogen. *Culex* mosquitoes, which are common in all regions of the world, are the most common vectors of WNV. The virus is mainly transmitted to humans through the bite of infected mosquitoes.[1]

West Nile virus was first isolated from a febrile patient in the West Nile Province of Uganda in 1937. Recently, the habitat of *Culex* mosquitoes has expanded rapidly due to ecosystem change. WNV disease has become endemic in many parts of the world, including Africa, the Middle East, South Asia, Australia, Europe and the United States. Over the past two decades, outbreaks of neuroinvasive WNV disease in humans and horses have been reported at increasing rates in both endemic and non-endemic areas.[1,2] Between 2010 and 2018, 2804 (73%) out of 3849 WNV cases in Europe were in the form of neuroinvasive disease.[3] West Nile viruses are divided into five genetic lineages. Genetic lineages 1 and 2 are the most common groups that cause disease in humans and horses. In our country, genetic lineage 1 was shown to be circulating first in 2011.[4] Ergunay et al reported that WNVs detected in humans, horses and mosquitoes belonged to genetic lineage 1, which is widely distributed throughout Europe, the Middle East and Africa, but is closely associated with the central African strain ArB310/67, which is distinct from Mediterranean strains.[5] Similarly, field studies conducted in various regions of Anatolia and Thrace found that mosquito species *Culex pipiens, C quinquefasciatus, C perexiguus, Aedes albopictus* and *Ochlerotatus caspius* and migratory birds were infected with genetic lineage 1 of WNV.[6-10] Recently, WNV lineage 2 was revealed from the organ samples of corvids in Istanbul, Turkey.[11]

The first human cases of WNV in Turkey were reported in its western provinces between 2010 and 2011. Forty of these cases were in the form of neuroinvasive disease, and the case death rate was 21%.[12] In serological studies conducted in various regions of Turkey, WNV seroprevalence were reported between 0.56% to 21.5% in humans, using the plaque reduction neutralization test (PRNT).[13-16] In this study, we aimed to determine the rate of seropositivity and the risk factors associated with WNV in a sample of healthy human participants in Alanya, located close to regions where WNV activity has been detected.

PATIENTS AND METHODS

The climatic conditions of the Mediterranean region provide a favorable environment for the life cycle of *Culex* species. This research was conducted with the approval of Clinical Research Ethics Committee of Alanya Alaaddin Keykubat University (registration no. 10354421-2020/16/31; approval date December 13, 2020) in Alanya district, located in the Mediterranean region of Turkey (**Figure 1**). According to the Address-Based Population Registration System of the Turkish Statistical Institute, Alanya had a population of 333 104 individuals in 2020.

This descriptive cross-sectional study involved a first group of people aged 18 years or older, including veterinarians, agricultural engineers, farmers/shepherds and people living near streambeds, who have a high risk of encountering vector mosquitoes. Informed consent forms were signed by all individuals who volunteered to participate. Questionnaires were completed during face-to-face interviews, and 10 mL of venous blood sample was drawn from each participant under sterile conditions and stored at −80°C until analysis. A second group consisted of age- and gender-matched healthy subjects selected from a previous cross-sectional study (This former study was approved by the Clinical Research Ethics Committee is unpublished as yet. University, registration no. 13054421/2019/4; approval date March 04, 2019) conducted in the same district. Retained sera from this previous study were stored at −80°C in our hospital.

Anti-WNV IgG antibody detection was performed using enzyme-linked immunosorbent assays (ELISA; Euroimmun, Germany) according to the manufacturer's recommendations. A standard curve was drawn using reference sera, which was used to evaluate the participants' optical densities. Serum samples with antibody levels of <16 RU/mL, 16–22 RU/mL and ≥22 RU/mL were considered negative, borderline and positive, respectively.

Virus neutralization tests (VNTs) were used to confirm the ELISA anti-WNV IgG-positive results (≥16 RU/mL).[17] Anti-WNV antibody titers of 1:5 or higher were considered positive. The tests were performed in the Biosafety Laboratory of the Faculty of Veterinary Medicine, Department of Virology. Heat-inactivated (30 min at 56°C) serum samples were diluted with Dulbecco's Modified Eagle's Medium (Greiner, Germany), starting from the reference 1:5 dilution, followed by twofold dilution performed in four steps. An equal volume of 100 TCID50 (Tissue Culture Infectious Dose50) dose

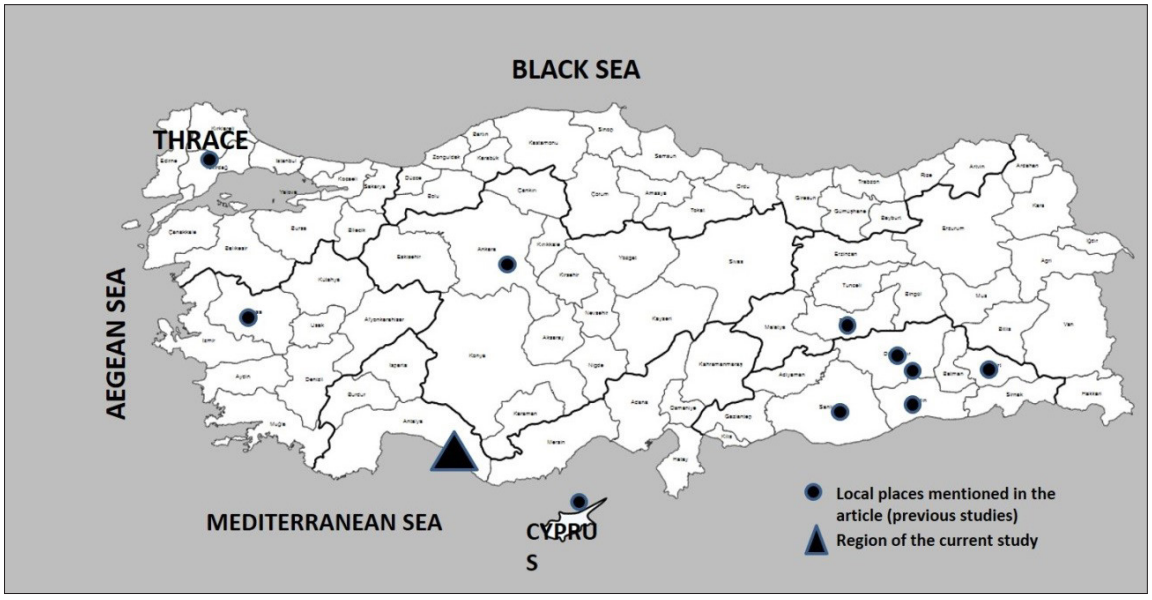

Figure 1. Locations of the current and previous studies.

(1:10.000) of the WNV NY99 strain was added at each dilution step and incubated at 37°C for 1 hour. Vero E6 cells were then added to the mixture and incubated at 37°C. The test was evaluated as soon as a 100% cytopathic effect was seen in the wells reserved for virus control. The serum dilution that blocked virus growth by 50% was recorded as the mean antibody titer.

Statistical analysis was performed using the SPSS software version 23.0 (IBM SPSS Corporation, Armonk, NY, United States). The normality analysis of continuous data was evaluated by histogram and Kolmogorov-Smirnov methods. Mean and standard deviation values were used for normally distributed variables. Median and interquartile range were used for variables not normally distributed. Differences between the groups were assessed using the chi-squared test or Fisher's exact test, as appropriate. Values of $P<.05$ were considered statistically significant.

RESULTS

The first group of 87 recruited for this study, which had a high risk of encountering vector mosquitoes, had a positivity rate of 8% (7/87), whereas the second group was 4.5% (16/356; $P=.181$). Using VNTs, anti-WNV antibody positivity was detected in 10 (2.3%) of the total of 443 samples. The positivity rate in the first group was 2.3% (2/87), while that in the second group was 2.2% (8/356; $P=.999$). ELISA anti-WNV antibody positivity was detected in 23 (5.2%) of the 443 samples. The female-to-male ratio of the entire study population was 1.1 (231/212), and the median age (IQR) was 42.0 (21) years (range: 18–91 years).

Sixty-eight participants reported having high blood pressure, 37 reported diabetes, 36 reported heart disease, five reported autoimmune diseases. None of the participants had a history of blood transfusion, organ transplantation, chronic kidney failure or immunodeficiency.

Comparison of risk factors for the WNV seropositivity was performed in 443 people. Confirmed WNV seropositivity rates were higher among male (3.8%) than female participants (0.9%; $P=.054$) and among adults aged ≥45 years (4%) than those aged 18-44 years (0.8%; $P=.048$). Positivity rates were also higher, albeit not significantly, among people living in wetlands (6.7%) and retired people (12%; **Table 1**). The characteristics of the VNT anti-WNV IgG-positive participants are presented in **Table 2**.

DISCUSSION

Although WNV infection is usually asymptomatic in humans, it can cause a fatal neurological disease, especially in elderly and immunocompromised individuals.[18,19]

The presence of the West Nile virus in various mosquito species, especially of the genus *Culex*, and bird species has been documented in field studies conducted in various regions of Turkey.[6-11,20] In this study, in line with the density and distribution of the *Culex* genus documented in previous studies, WNV seropositivity was tested in healthy individuals in the Alanya region.

Table 1. Relationships between independent variables and anti-WNV antibody positivity.

Variable	Enzyme-linked immunosorbent assay			Virus neutralisation test		
	No. tested	Antibody-positive (%)	*P* value	No. tested	Antibody-positive (%)	*P* value
Sex						
Female	231	3.5	.087	231	0.9	.054[a]
Male	212	7.1		212	3.8	
Age						
18–44 years	244	2.9	.015	244	0.8	.048[a]
≥45 years	199	8.0		199	4.0	
Education						
Illiterate	12	16.7	.261	12	0	.781
Basic literacy	15	6.7		15	0	
Primary school	172	5.8		172	3.5	
Secondary school	31	6.5		31	3.2	
High school	112	6.3		112	1.8	
University	88	1.1		88	1.1	
Occupation						
Retired	25	16.0	.135	25	12.0	.100
Self-employed	148	6.1		148	2.7	
Farmer/shepherd	40	10		40	2.5	
Government official	47	5.9		47	2.1	
Homemaker	125	2.4		125	0.8	
Student	19	0		19	0	
Agricultural engineer	7	0		7	0	
Veterinarian	5	0		5	0	
Dwelling type						
Flat	341	5.3	.999[a]	341	2.1	.999[a]
House	91	4.4		91	2.2	
Building material						
Reinforced concrete	429	5.1	.999[a]	429	2.1	.999[a]
Wood	3	0		3	0	
Potable water source						
Tap water	230	6.5	0.188	230	3.0	.423
Bottled water	122	3.3		122	0.8	
Home water filter	34	0		34	0	
Spring water	31	9.7		31	3.2	

Table 1 (cont.). Relationships between independent variables and anti-WNV antibody positivity.

Variable	Enzyme-linked immunosorbent assay			Virus neutralisation test		
	No. tested	Antibody-positive (%)	*P* value	No. tested	Antibody-positive (%)	*P* value
Proximity to wetlands						
No	401	4.5	.058[a]	401	1.7	.124
Yes	30	13.5		30	6.7	
Mosquito net						
No	340	5.3	.999[a]	340	2.1	.999[a]
Yes	91	4.4		91	2.2	
Exposure to ticks						
No	386	5.2	.999[a]	386	2.3	.607[a]
Yes	45	4.4		45	0	
Outdoor activities						
No	306	4.9	0.765	306	2.0	.723[a]
Yes	125	5.6		125	2.4	
Hunting						
No	402	5.0	0.652	402	2.2	.999[a]
Yes	29	6.9		29	0	
Exposure to pets or livestock						
No	297	5.1	0.940	297	2.0	.999[a]
Yes	134	5.2		134	2.2	

[a]Determined using Fisher's exact test (two-tailed).

Table 2. Characteristics of the anti-WNV positive participants according to virus neutralisation tests (n=10).

Age/Sex	Occupation	Proximity to wetlands	Mosquito net	Outdoor activities	Exposure to pets or livestock	Chronic disease	IgG antibody titer	
							ELISA	VNT
67/M	Retired	No	No	No	No	No	>200	1:20
58/M	Self-employed	No	No	No	Yes	Yes	>200	1:20
71/F	Homemaker	Yes	No	Yes	No	Yes	>200	1:10
64/M	Retired	No	No	No	No	Yes	195.60	1:10
49/M	Farmer/shepherd	Yes	Yes	Yes	Yes	No	190.32	1:10
38/F	Self-employed	No	No	No	No	No	146.81	1:10
77/M	Self-employed	No	No	Yes	Yes	Yes	144.63	1:5
61/M	Retired	No	Yes	No	No	No	97.92	1:5
59/M	Government official	-	-	-	-	-	87.79	1:10
42/M	Self-employed	No	No	No	No	HT	19.47	1:10

ELISA: Enzyme-Linked ImmunoSorbent Assay; VNT: Virus neutralisation tests; M: Male; F: Female

To the best of our knowledge, only one case of WNV encephalitis (43-year-old male) has been reported so far in the Alanya region, where this study was carried out.[21] In our study, a total of 443 healthy individuals were included. As a result, we calculated the WNV seropositivity of 5.2% by ELISA, and it was confirmed as 2.3% by VNTs.

Flaviviruses are a family of enveloped single-stranded RNA viruses. Among the most notable mosquito-borne flaviviral human pathogens are the hemorrhagic fever viruses, dengue and yellow fever, and neurotropic viruses, such as West Nile, Japanese encephalitis, Saint Louis encephalitis, and Zika. Accordingly, flaviviruses have the potential to cross-react and influence the specificities of various serological tests, including ELISA. The PRNT is regarded as the most specific serological test for accurate serological identification of flaviviruses. Specifically, the 90% endpoint PRNT (PRNT90), known as the most stringent PRNT, is considered the standard protocol for the serodiagnosis of flavivirus infections. However, it has been demonstrated that VNTs can efficiently replace PRNT for quantitation of WNV antibodies.[17]

Consistent with our study, Ergunay et al reported a WNV positivity rate of 1.6% (19/1200) by ELISA and 0.8% (10/1200) by PRNT.[15] In another study, WNV reactivity was 0.99% (25/2516) using ELISA and 0.56% (14/2516) using PRNT.[16] Our seropositivity rate was higher than the results of these two different studies conducted in healthy blood donors in Central Anatolia (**Figure 1**).[15,16]

Our results are supported by a study conducted in Mersin province, which is close to Alanya and is a coastal city on the Mediterranean Sea. In their study, conducted on a cohort of blood donors, Ergunay et al reported a WNV seroprevalence of 12.1% using the PRNT. In the same study, the seroprevalence in horses was 8.2%, and entomological surveys identified the presence of the virus in various mosquito species known to be vectors.[6] This finding clearly indicates the presence of WNV activity in the Mersin province. Therefore, in cases of febrile illness and viral central nervous system infections occurring during the months when vector mosquitoes are active in our region, WNV should be considered as a potential etiological agent.

The serological presence of WNV in Turkey was demonstrated in studies conducted in the 1970s. However, in studies conducted during these years, high seropositivity rates obtained by the hemagglutination inhibition method may have been due to cross-reactions between flaviviruses.[1] In 1977, Meco et al reported that seropositivity in healthy individuals was between 38% and 47.8% in five provinces in southeastern Anatolia, using the hemagglutination inhibition method.[22]

In addition, studies conducted in individuals living in WNV-endemic regions or in high-risk groups reveal higher seropositivity rates compared to studies conducted in healthy individuals. In the Aegean region, following the peak of epidemics, the seropositivity reported as 21.5% using the neutralization test.[12] The other study, which assessed the presence of antibodies in serum samples collected either from patients with unidentified encephalomyelitis or from transplantation patients with no evidence of infection, reported 20.4% seropositivity using the PNRT.[14]

The risk factors for West Nile virus infection include older age, male sex and underlying conditions, such as cancer, cardiovascular disease, diabetes and immunodeficiency. Living in rural areas, spending time in nature either recreationally (e.g., hunting) or occupationally (e.g., farming, shepherding and gardening) are additional risk factors.[1,15] In this study, the WNV seropositivity, as determined by VNTs, was significantly higher among male than female and among people aged ≥45 years than those aged 18-44 years. We found that WNV seropositivity increased with age, especially among males. Although the differences were not statistically significant, higher seropositivity was observed more among retired people than occupational groups. Unfortunately, data on occupations before retirement were not available. Contrary to our expectations, no association was found between WNV seropositivity and the dwelling type, building material, proximity to wetlands, mosquito net usage, outdoor activities including hunting, or exposure to pets or livestock. The reason for this can be attributed to the small sample size, which is a limitation of our study.

It is also noteworthy that five of the 10 VNT anti-WNV positive people (**Table 2**) had histories of chronic diseases, such as hypertension, diabetes, chronic heart failure and epilepsy. However, none of the seropositive individuals had a history of blood transfusion or organ transplantation, which are known to increase the risk of WNV transmission.[23,24]

In conclusion, with the introduction of WNV antibodies from serum samples in Alanya, this study highlights the presence of WNV infection in humans in our region. It is most likely that VNW infection is underdiagnosed and underreported. Our results should be a reminder in differential diagnosis. More comprehensive, multidisci-

plinary studies are required to increase our knowledge about this zoonotic infection including risk factors in line with One Health approach which recognizes the interconnectedness of the health of animals and humans with disruptions in the environment.

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