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Seroprevalence of West Nile Virus among Arabian Horses in Central, Eastern Anatolia, and Marmara, Turkey

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Abstract The West Nile Virus (WNV), a mosquito-borne *Flavivirus* is a common infection identified in the Middle East, Europe, West Asia, Africa, Australia and the United States. The virus can cause severe illnesses and death in horses. The current study aimed to screen West Nile virus neutralizing antibodies in Arabian horse populations from breeding farms located in Central, Eastern Anatolia and Marmara by plaque reduction neutralization test (PRNT). We sampled 165 Arabian racehorses, during March-December, 2010, in three different provinces (Eskişehir, Malatya, and Bursa) in Turkey. Screening of serum samples showed that 6.6% were positive for West Nile (WN) virus-neutralizing antibodies. The obtained data demonstrate WNV is circulating in mentioned regions among racehorses and the need to carry out a surveillance program includes WNV-focused screening tests in Turkey.

Keywords Horses, Seroprevalence, Turkey, West Nile Virus

1. Introduction

West Nile virus (WNV) was first isolated in Africa, West Nile district of Uganda, in 1937 [1]. WNV is a member of the Japanese encephalitis complex which also includes Japanese encephalitis virus, St. Louis encephalitis virus, Rocio virus, and Murray Valley encephalitis virus [2]. Although these viruses of the *Flavivirus* genus mainly infect vertebrates with arthropod vectors such as ticks and mosquitoes, the viruses have no vectors, or their hosts are only insects are also classified in this group [3]. The genome of flaviviruses encodes three structural and seven nonstructural proteins respectively; Capsid, prM/Membrane, Envelope and NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5 [2]. It is thought that non-structural proteins and their mutations play a role in virulence of WNV [4].

WNV is a re-emerging neurotropic virus and has a broad geographic distribution; have been reported in horses in several different countries in Africa, the Middle East, Asia, Southern Europe, Australia and the Americas [5]. WNV transmitted by mosquitoes of the *Culex* genus and replication cycle occurs in mosquitoes and birds which are the reservoir of WNV. WNV may also be transmitted directly to infected animals or mosquitoes by being eaten by susceptible hosts. It is known among humans that WNV can be transmitted by blood transfusion, organ transplantation and breast milk [2, 6]. Equines and humans are incidental hosts and act as dead-end hosts. In horses, WNV was first noticed in Egypt and France at the beginning of the 1960s [7]. In 1962, 76 equine cases with neurological disorders and 25–30% mortality were reported in France [8]. In the United States, WNV infection was first seen in horses in the western half of North America. It then spread to the entire North American continent and the South American continent [3].

WNV causes of mildly febrile to severely neurologic symptoms and can cause fatal infections in horses. Encephalitis, the most dangerous symptom that WNV brings to horses, occurs when the virus crosses the brain barrier [7]. A high mortality rate of up to 40% was detected in infections with neurological symptoms [5, 9]. A variety of diagnostic methods have been developed for WNV infection [8]. Molecular methods used in the laboratory are PCR, real-time PCR, and IgM, IgG antibody ELISA, which are used for the detection of RNA in acute infection [5]. Laboratory diagnosis of WNV infection is mostly serological, and the majority of the diagnostic test is ELISA (Enzyme-Linked Immunosorbent Assay). Flaviviruses, share common antigenic epitopes and create cross-reactivity that affects accurate diagnosis [10].

Therefore, gold standard plaque reduction neutralization test (PRNT) is preferable although it is laborious and time-consuming [11].

There are limited number of studies on the existence and the prevalence of WNV infection of horses in Turkey [12-14]. It is presented here up to date information and new findings on the presence of specific WNV neutralizing antibodies in Arabian racehorses in three different geographical regions of Turkey. The association of the infection with age-groups, gender, and the geographic location was investigated regarding seroprevalence.

2. Material and Methods

2.1. Cell Culture

The reference virus WN-NY99-4132 strain which was isolated in 1999 in New York from the Bronx Zoo flamingo [15] were grown, and PRNT was evaluated in the Vero E6 cell line. The cells were maintained at 37 °C under a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (Biochrom AG, Berlin, Germany), containing 100 μg/ml streptomycin, 100 IU/ml penicillin, and 10% fetal bovine serum (Biochrom AG).

2.2. Stud Farms and Serum Samples

Field of study from Arabian racehorses was carried out in 3 provinces; Eskisehir (I), Malatya (II), and Bursa (III) in Turkey, from April to December 2010. Stud farms I, II and III were located in Central, Eastern Anatolia, and Marmara, respectively (Figure 1). In total, 165 serum samples were collected from animals with and without upper respiratory tract symptoms [16]. The population consisted of 17 stallions, 54 mares, 61 foals (aged 1–3 years), and 33 weaning foals without any neurological symptoms. Blood samples were taken from the jugular veins of the animals to 10ml vacuum tubes. Following that centrifuged at 3,000g for 10 min, and the samples were transferred to sterile tubes and stored at -20°C.

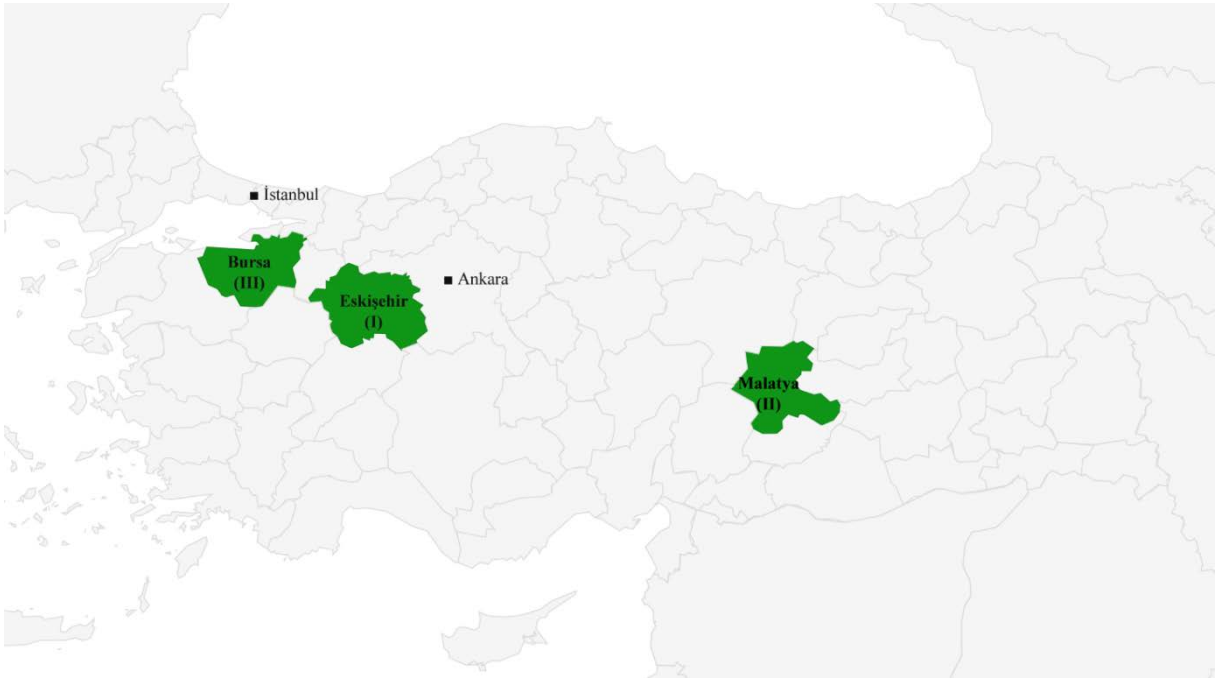

Figure 1. Location of the sampled stud farms

2.3. Plaque Reduction Neutralization Test

Vero cells were seeded at in 24-well tissue culture plates and incubated until cells are confluent at 37°C in 5% CO_2. Serum samples were inactivated at 56°C for 30 minutes, and 2-fold serial dilutions were prepared. WNV/NY99 strain was diluted in MEM (Minimum Essential Media) and equally added to each diluted serum sample. The mixture was incubated at 37°C in 5% CO_2 for an hour. The cell culture medium was then aspirated from the 24-well plates, and the virus-serum mixture as 200 µL/well was transferred from Eppendorf tubes onto the 24-well plates. For each serum dilutions, two wells were used. 24-well plates were incubated at 37°C in 5% for an hour to the non-neutralized virus could be able to absorb into cell lines. Following the incubation period, 1ml 1.6% carboxymethyl-cellulose (CMC; Sigma-Aldrich, Germany) were added to control the virus spread in a semi-solid agar. Plates were kept at 37°C in 5% and observed each day from the point of CPE. In the fourth day of the incubation without removing the CMC medium, cells were fixated with 10% formaldehyde for 20 minutes. Following the fixation; cells were stained with 0.35% crystal violet, dried and consisted plaques were counted. The assay was evaluated according to the control wells and compared with the wells includes serum samples. All experiments were performed in two replicates. Reduction of the plaques by 90% ($PRNT_{90}$) was accepted as WNV specific antibody positive for serum samples.

3. Results

Table 1. Seroprevalence of WNV antibodies in stud farms I, II, and III, according to age and sex

	Stud farm 1 (+, %)	Stud farm 2 (+, %)	Stud farm 3 (+, %)
Ab positive Mare / Total Mare	1/9 (11.1)	2/16 (12.5)	4/30 (13.33)
Ab positive Stallion / Total Stallion	1/5 (20)	0/5 (0)	0/7 (0)
Ab positive Foal / Total Foal	0/26 (0)	0/19 (0)	1/15 (6.6)
Ab positive weaning foal /Total weaning foal	0/5 (0)	0/13 (0)	2/15 (13.33)
Total	2/45 (4.4)	2/53 (3.7)	6/67 (8.95)
	10/165 (6.06)		

Serum samples of 165 horses from three breeding farms (Eskişehir, Malatya, Bursa) were sampled. Based on the results of the PRNT, it was found that 10 of 165 (6.06%) horses had specific antibodies against WNV (Table 1). In all three stud farms, mare groups seroprevalence were detected as positive for WNV antibodies with varying rates, 11.1%, 12.5%, 13.33% respectively (Table 1). In stud farm III, WNV antibodies were detected in all animals except stallions. The stud farm I, stallions showed the higher prevalence compared to other groups with 20% ratio. In two farms (stud farm I-II), there were no antibodies detected against WNV in foals and weaning foals. Besides, stud farm III showed 6.6% and 13.33% seroprevalence in foals and weaning foals respectively. Stud farm III had higher seroprevalence with 8.95% in three farms (Figure 2). Most of these equines were healthy thoroughbreds, and gammaherpesviruses have been investigated in these horses previously [16].

4. Discussion

Almost 10% of experimentally infected horses are known to show clinical disease. WNV infection does not cause disease frequently in horses [17]. It is known that horses in show and racing industry in the world could be exposed to WNV infection because they are a convenient source for feeding mosquitoes [18]. In this report, a total of 165 Arabian racehorses, consist of different sex and ages were screened in Central, Eastern Anatolia, and Marmara of Turkey. It is reported that female horses were 2.9 times more probable to die compared to males [19]. In our investigation, female horses showed highest ratios in their farms against WNV antibodies, and all three mare groups were detected as positive in on all three stud farms (Figure 2). This may perhaps be due to a close relationship between mare and foal, which includes birth, giving care and lactation. It may be that the developing immune system of the foal, making it susceptible to diseases and may be increasing the frequency of mother's encounter with infection. Also, it could be because of the situation that while fewer stallions are held in separate units, a large number of mares are being kept all together with their weaning foals. Presence of infection in the mare is also crucial for the offspring. It was reported that WNV that is known to be able to cause abortion in sheep is also possible to transmit through the placenta in horses and may cause abortion [20]. In this study, foals were found to be positive for the WNV antibody on a farm where mares carry antibodies against WNV, while antibody presence was not detected for those of the other two who also showed antibody presence in mothers. It has been reported that WNV vaccination of mares in late pregnancy has a functional consequence on foal WNV titer [21]. In this study, the percentage of positive antibodies against WNV disease in the weaning foals was found to be higher than the colts. Although it is not known whether or not the mothers are vaccinated, it is thought that the obtained antibodies against WNV may depend on the maternal antigens. It should also be remembered that it may be caused by postnatal infection.

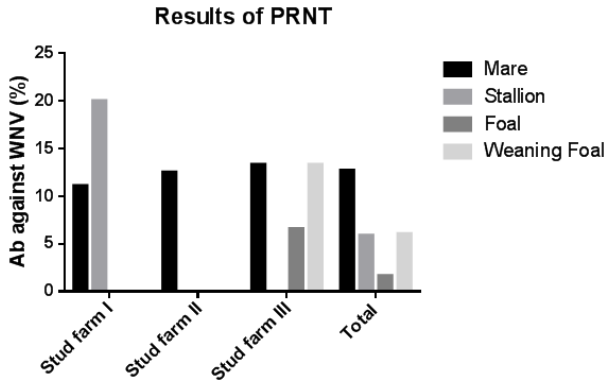

Figure 2. Seroprevalence of WNV-specific antibody in percentages in three breeding farms (Eskişehir, Malatya, Bursa) as detected by PRNT

Serological surveys are essential to screen prevalence of diseases in a population. Studies were done previously showed WNV presence and prevalence with 17% and 2.5% to 37.7% in humans and animals in Turkey, respectively [22, 14]. Although the virus has been detected in the variety of regions, more studies are needed to determine the presence, prevalence, transmission routes and regular follow-up. The rate of work done on horses is even lower, and its role in WNV cycling in Turkey and control of horses' health is essential [23].

Although WNV infections do not often cause clinical illness, climatic changes and temperature can influence the mosquito reproduction and therefore virus replication. Europe and North USA have a moderate climate which increases heat and insect counts in summer, following that encephalitis cases are encountered [24]. Turkey, located on the continent of Europe, bordered by eight countries and surrounded by Mediterranean, Aegean, and the Black Sea. Both because of its geographical location and climate conditions, as well as the migration of migratory birds, Turkey creates a favorable environment for the growth of arthropods and the emergence of viral infections. In our research; despite geographical locations were different for each sampled region; temperature conditions were approximately same during the sampling period. According to Turkish State Meteorological Service's statistical data; the stud farm I in Eskişehir (sampled in March), stud farm II in Malatya (sampled in November), stud farm III in Bursa (sampled in December) had 5.2, 7.7, 7.4 average temperatures, 65%, 53%, 69% humidity and 393mm, 420mm, 646mm rainfall annually, respectively.

In stud farm I, in January 2011 WNV positive cases were reported, and RNA detection was done successfully [13]. Two weaning foals first showed flu-like symptoms; following those neurological symptoms like weakness in the back limbs, motion troubles and excitability were observed. Our sera samples were taken one year before from this case, and 4.4% (2 of 45) WNV specific antibodies were detected. After a year, Ozkul et al. (2013) found 31.6% (57 of 180) of positive antibodies against WNV in the same stud farm by PRNT. In just a short period of 1 year, infection rate increased by 14%. While investigating the factors that cause infections; regular testing of WNV in the horses will shed light on the reasons for this increase. Another factor that will play a role in preventing this increase is vaccination. It is known that due to climatic conditions and an increased ratio of the infection; horses should be vaccinated against WNV virus. It is thought that in high mosquito populated regions vaccination can be done every six months against WNV virus [25]. The results obtained from a study by Ward et al. it has been shown that vaccinations can reduce the risk of death by at least 44%, even if they are not adequately administered every six months before WNV infection in a region [9]. The fighting with the vectors is an important place to take the disease under control. In arboviral infections such as WNV, methods such as residual insecticide spraying, removal of potentially mosquito spawning areas close to the human environment and use of insecticide mosquito and window curtains or screens are used [26].

5. Conclusions

Three economically essential stud farms that raise Arabian racehorses were investigated by PRNT and antibodies detected against WNV. WNV is an endemic disease in horses in Turkey and should be screen periodically with serological and nucleic acid-based assays.

Ethical Approval

Animal sampling was conducted follow in the approval of Ankara University Animal Experiments Local Ethical Committee (Number: 2009/53, 2009-223, 2009-266).

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