

Short Communication

The First Investigation of West Nile Virus in Horses Using Real Time RT-PCR in Middle Black Sea Region in Turkey

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Abstract

Background: West Nile Virus (WNV) is a mosquito-borne disease that can cause fatal infection in mammals including humans, dogs, horses, birds and reptiles. Although West Nile Virus is an asymptomatic infection, especially it can cause neurologic disorders in humans and horses. The aim of this study was to investigate virological presence of WNV in horses in the Black Sea Region of Turkey using real time RT-PCR (rRT-PCR).

Methods: Totally, 120 horse sera were collected equally from 4 provinces in Middle Black Sea Region of Turkey and investigated for WNV presence by Taqman based rRT-PCR.

Results: WNV nucleic acid was not detected in any horse serum sample.

Conclusion: Although obtained result indicated no evidence of WNV-RNA in horses, Black Sea Region of Turkey is one of the suitable places for the WNV infection. For this reason, our research will continue for the determination of the viruses in vectors and susceptible animals such as horses, dogs, etc.

Keywords: Horses, RT-PCR, Sera, West Nile Virus

Introduction

West Nile virus (WNV) is a positive sense single stranded RNA virus of the *Flavivirus* genus in family *Flaviviridae* (Monath and Heinz 1996) and is classified in the Japanese Encephalitis serocomplex group together with St Louis Encephalitis (SLE), Murray Valley encephalitis (MVE), Japanese encephalitis (JE) and Kunjin (KUN) viruses (Petersen and Roehrig 2001). West Nile virus is an important pathogen for vertebrates including humans, horses, dogs birds and reptiles (Castillo-Olivares and Woods 2004). Birds are considered the main reservoir hosts of WNV, and migratory birds play an important role in its spreading (Rappole et al. 2000). The natural cycle of WNV typically involves ornithophilic *Culex* mosquitoes feeding on avian hosts (Apperson et al. 2004). Horses are highly susceptible to

WNV infection and mortality in these animals can be observed during WNV outbreaks (Castillo-Olivares and Wood 2004). The latest outbreaks of WNV were characterized by an increased proportion of neurological disease in both humans and horses (Petersen and Roehrig 2001). Mortality rates among clinically affected horses have been estimated around 38%, 28%, 44% and 42% during outbreaks in the USA, France (2000), Morocco and Italy (1998) respectively (Tber Abdelhaq 1996, Cantile et al. 2001, Murgue et al. 2001, Ostlund et al. 2001).

West Nile virus has a wide geographical distribution that includes countries of Europe, Asia, Africa, Australia, and America (Hubalek and Halouzka 1999, Savage et al. 1999, Hayes et al. 2005). Since the summer of 1999 during which WNV was introduced in New York

City, the distribution of WNV has expanded to include 46 states of the United States, 7 provinces of Canada, Mexico, and probably a number of the Caribbean islands (Kramer et al. 2001, Blitvich et al. 2004). Serological evidence of arboviral infection based on the detection of haemagglutination-inhibiting (HAI) antibodies was reported in Turkey in the 1970s (Ari et al. 1972, Radda et al. 1973). Subsequently, serological identification of WNV was reported in humans and different mammalian species including; sheep, ass-mule, cat, cattle (Meco 1977, Ozkul et al. 2006, Ozer et al. 2007). On the other hand, there is only one virological datum reported in Turkey (Arpacı et al. 2009)

The presence of WNV in neighboring countries (Bulgaria and Greece) of Turkey was reported in mammalian species and mosquitoes (Katsarov et al. 1980, Koptopoulos et al. 1980). Additionally wetland ecosystems such as river deltas or flood plains and migration routes of migratory birds are mainly the appropriate foci of WNV infections (Komar 2000, Ozer et al. 2007) and Middle Black Sea Region of Turkey involves river deltas and migratory routes of birds.

All these data suggest that WNV might be present in our region, so the aim of this study was to investigate the presence of WNV in horses, which is the most susceptible animal to infection in the Black Sea Region of Turkey using real time RT-PCR.

Materials and Methods

Sera

Blood sera samples were collected from randomly selected 120 horses from 4 different provinces (Samsun, Sinop, Amasya and Tokat) of Central Black Sea Region, Turkey between March and August 2007 (Fig. 1) and trans-

ported to the laboratory at 4 °C. All sampled horses were older than 1 year old. The blood samples were centrifuged at 2000 rpm for 10 min at 4 °C. The sera were subsequently separated into vials (Eppendorf Germany), then inactivated at 56 °C for 30 min and stored at -20 °C until tested.

RNA extraction and TaqMan based rRT-PCR assay

Viral RNA was extracted from 350 µl of the horse sera by using the MagNA Pure LC RNA Isolation Kit- III (Roche, Mannheim, Germany). Purified nucleic acid was eluted in 50 µl elution buffer and stored at -70 °C until used. The samples were tested by real time RT-PCR for WNV-RNA detection using the oligonucleotide primers and Taqman probe targeting WNV E gene designed by Lanciotti et al. (2000). The TaqMan probes were labeled at the 5' end with the FAM reporter dye and were labeled at the 3' end with the quencher dye TAMRA (Table 1). For the TaqMan assay, 2 µl of RNA combined with 50 pmol of each primer and 10 pmol of the FAM- and TAMRA-labeled probe in a 50 µl total reaction volume by using the OneStep RT-PCR Kit (Qiagen, Hilden, Germany) and amplified in a LightCycler@2.0 Real Time PCR system (Roche Diagnostics, Germany). The following cycling times and temperatures were applied: 1 cycle of 42 °C for 30 min and 94 °C for 2 min and 40 cycles of 94 °C for 30s, 55 °C for 30s, and 72 °C for 20s.

Results

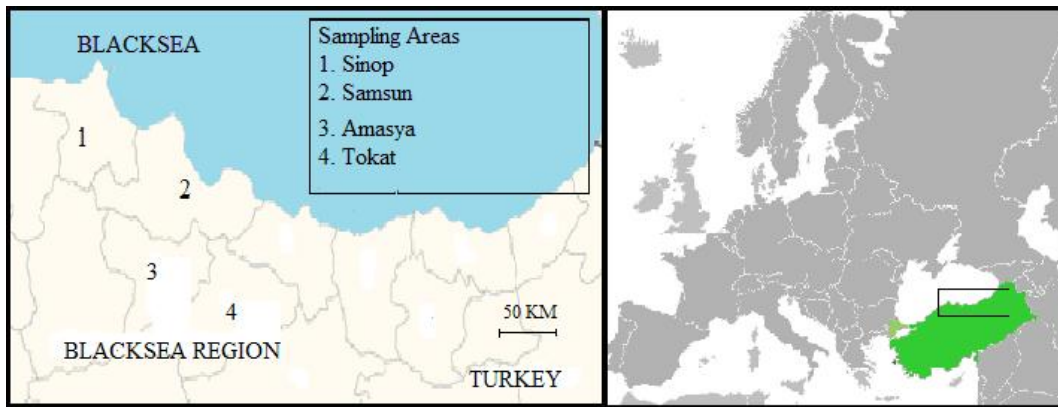
Samples tested using Taqman-based Real Time RT-PCR for the presence of WNV-RNA but all samples were negative (Table 2).

Table 1. Oligonucleotide primers and probe used in the TaqMan-based rRT-PCR assay

Primer	Genome position	Sequence (5'-3')	rRT-PCR product size (bp)
WNENV-forward	1160–1180	TCAGCGATCTCTCCACCAAAG	70
WNENV-reverse	1209–1229	GGGTCAGCACGTTTGTTCATTG	
WNENV-probe	1186–1207	TGCCCCGACCATGGGAGAAGCTC	

Table 2. Distribution and results of serum sample according to provinces

Provinces	Number of Tested horse Sera	Results of Real Time RT-PCR for WNV
		Number of Positive Sera
Samsun	30	-
Sinop	30	-
Amasya	30	-
Tokat	40	-
TOTAL	120	-

**Fig. 1.** Location of provinces where sera collected from horses in the Black Sea Region of Turkey

Discussion

The transmission cycle of WNV involves several species of mosquitoes (primarily *Culex* spp) and various species of birds. Mosquitoes become infected with WNV when they feed on a bird carrying the virus in its blood. After 10 to 14 days, the virus can be transmitted to another bird, person, or other animal such as horse, dog, cats that the mosquito bites. During blood feeding the mosquito injects the virus, contained in its saliva, into the bird, animal, or person and may cause illness. Horses and humans, are considered dead-end or incidental

hosts for the virus because they do not produce enough virus to reinfect a mosquito and maintain the transmission cycle. However, they are capable of showing clinical symptoms (Bunning et al. 2002).

Various test methods can be used for the diagnosis of WNV. Virus isolation can be attempted from cerebrospinal fluid (CSF), blood or tissues in cell cultures such as Vero, RK-13 cells or mosquito cell lines (Ostlund et al. 2001). Real Time RT PCR methods were designed to specifically detect WNV. This meth-

od has demonstrated greater sensitivity than traditional RT-PCR methods and gives advantages over classical virological methods (Porter et al. 1993, Lanciotti et al. 2000).

The presence of WNV has been reported with virological and serological study results in many European countries (Hubalek and Halouzka. 1999, Zeller et al. 2004). In Turkey, WNV had been identified only serologically until 2009 (Meco 1977, Ozkul et al. 2005, Ozer et al. 2007). Subsequently, Arpaci et al. (2009) reported the first time virological identification of WNV from graft-versus-host disease patient using RT-PCR in Turkey. However WNV has not been virologically identified from animal and vectors in Turkey.

In this study, WNV-RNA was not detected in screening horses in a Middle Black Sea Region of Turkey. However, the reasons of these negative results can depend on many factors such as the quantity of the virus in the material; region's environmental features i.e. having cold weather conditions in winter which provides unsuitable circumstances for the presence of mosquitoes all around the year in Northern Turkey.

In conclusion, although obtained result indicated no evidence of WNV-RNA in horses, Black Sea Region of Turkey is one of the suitable places for the WNV infection. For this reason, our research will continue for the determination of the viruses in vectors and susceptible animals such as horses, dogs, e.g.

Acknowledgments

The authors declare that there is no conflict of interests.

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