



RESEARCH ARTICLE

Investigation of West Nile virus infection in brown bears (*ursus arctos*) in Turkey

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Türkiye'deki boz ayılarda Batı Nil Virüsü'nün araştırılması

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Öz

Amaç: Bu çalışmada, Türkiye'de Sarıkamış/Kars yöresindeki doğal parkta, serbest dolaşımda bulunan boz ayılarda Batı Nil virüsünün (BNV) varlığı/oranı serolojik ve virolojik olarak araştırıldı.

Gereç ve Yöntem: Bu amaçla 11 adet boz ayıdan alınan kan örneklerinde BNV spesifik antikor varlığı commercial competitive enzyme-linked immunosorbent assay (C-ELISA) yöntemi ile tarandı. Toplanan kan örneklerinden elde lökosit örneklerinde BNV nükleik asidinin belirlenmesi amacıyla da Reverse Transkripsiyon-Polimeraz Zincir Reaksiyonu (RT-PZR) tekniği kullanıldı.

Bulgular: Örneklerin hiçbirinde BNV nükleik asidi ve BNV spesifik antikor tespit edilmedi.

Öneri: Türkiye'de serbest dolaşımdaki vahşi memeli hayvanlar ve boz ayılarda varlığı/oranı bilinmeyen BNV enfeksiyonunun ileriki dönemlerde daha ayrıntılı araştırılması gerektiği düşünülmektedir. Bu araştırma Türkiye'de serbest dolaşımdaki boz ayılarda BNV enfeksiyonu ile ilgili yapılan ilk çalışmadır.

Anahtar kelimeler: Batı Nil virusu, Boz ayı, C-ELISA, RT-PCR

Abstract

Aim: This study is a serological and virological examination of the West Nile Virus (WNV) in free-ranging brown bears (*Ursus arctos*) in Sarikamis district of Kars province of Turkey.

Materials and Methods: For this purpose, blood samples from 11 brown bears were tested for antibodies against WNV using a commercial competitive enzyme-linked immunosorbent assay (C-ELISA). Reverse transcription polymerase chain reaction (RT-PCR) technique was used to determine the presence of West Nile Virus nucleic acid.

Results: Seropositivity rate was detected to be zero % and West Nile virus nucleic acid was not found in samples.

Conclusion: The significance of WNV infection in brown bears and other free-ranging mammals in the Turkey is unknown at this time and will require further investigation. This is the first report of WNV infection in brown bears in Turkey.

Keywords: Brown bears, ELISA, RT-PCR, West Nile virus



Introduction

West Nile Virus (WNV) can cause a variety of neurological symptoms such as mild febrile illness, meningitis, encephalitis or death in humans, horses, dogs, birds and wild animals in America, Asia, Africa, Europe and particularly in countries bordering the Mediterranean Sea (Fauquet et al 2005). Taxonomically, WNV is a member of the Japanese encephalitis virus complex of the family Flaviviridae, genus Flavivirus, which also includes Japanese encephalitis virus, St. Louis encephalitis virus (SLEV), Murray Valley encephalitis virus (MVEV), and others.

These viruses are mosquito borne, primarily transmitted by *Culex* sp, and have wide, overlapping distributions throughout the world (Mackenzie et al 2002). The virus is an enveloped virus with a single-stranded, positive sense, 11-kb RNA genome that transcribes a single polyprotein, cleaved by host and viral proteases into structural and nonstructural viral proteins (Chambers et al 1990). Three genetic variants of WNV have been demonstrated in Russia and these isolates showed genetically high similarity to those reported from the United States and Israel (Lvov et al 2004).

Laboratory diagnosis includes the determination of antibodies using enzyme-linked immunosorbent assay (ELISA) (Dauphin and Zientara 2007), plaque reduction neutralization (PRNT) assay (Lanciotti et al 2000, Ozkul et al 2006) and virus isolation (Lanciotti et al 2000).

While PRNT is still considered the gold standard for specific diagnosis, ELISA is now routinely used. Padilla et al (2009) reported ELISA has a higher specificity (9.4%) and sensitivity (84.9%) for WNV infection. RT-PCR has been used to develop highly sensitive and specific assays for the identification of WNV in human serum, CSF, brain tissue, mosquito pools, and avian tissues (Porter et al 1993).

The objective of this study was to investigate the WNV infection as serologically and virologically in brown bears (*Ursus arctos*) in Sarikamis district of Kars province in the north-east Turkey. This is the first report of WNV infection in brown bears in Turkey.

Material and Methods

Animals and blood samples

This study evaluated the materials obtained from the study which aims to place GPS/GPRS collar to the bears captured from its natural environment in Sarikamis/Kars-Turkey region by Forestry and Water Affairs Ministry, General Directorate of Nature Protection and National Parks (25 December 2009 "valid for five years", The protocol of protection of the wildlife in the East Anatolia) and Kuzey Doga Society.

Blood samples were collected from randomly selected 11 free-ranging brown bears (*Ursus arctos*) aged >4 year old in Sarikamis district of Kars province of Turkey. Appropriately captured bears were taken under anesthesia with zolazepam+tiletamine (Zoletil®, Virbac, Netherland) and blood samples were taken from vena cephalica. For serological purposes, serum obtained from the centrifugation of taken samples was stored at -20°C until used in the study.

The blood samples, collected for virological purposes, were collected in tubes with anticoagulant (EDTA) and centrifuged at 1500g for 10 min, the buffy coat fraction was removed by capillary pipette and resuspended in 2 ml of phosphate buffered saline (PBS, 0.01 M, pH 7.2) containing penicillin (100 units/ml), streptomycin (0.1 mg/ml) and amphotericin B (2.5 µg/ml). Then, they tested for WNV nucleic acid by reverse transcription polymerase chain reaction (RT-PCR).

Competitive Enzyme Linked Immunosorbent Assay (C-ELISA)

West Nile Competition Multi-species ELISA kit (ID Screen®, Product code: West Nile Competition Multi-species (WNC), IDvet, Grabels, France) were used to detect anti-pr-E antibodies against the WNV.

Tests were performed according to the manufacturer's directions. Briefly, 50 µl of test sera, and controls diluted at 1:2 in dilution buffer were added to each well. Following 90 min incubation at 21°C, all wells were washed three times and anti-pr-E antibody peroxidase (HRP) conjugate was added in all well as a 100 µl. Washings were performed again after 30 min incubation at 21°C. In the final step, 100 µl substrate solution tetramethylbenzidine (TMB) was added to each well and incubated for 15 min at 21 °C and reaction was stopped by adding 100 µl 0.5M H2SO4.

The OD of each well was read using an ELISA reader at a wavelength of 450 nm. According to the kit procedure: the test is validated if the mean value of the negative control optical density (ODnc) is greater than 0.700 and the mean value of the positive control optical density (ODpc) is less than 30% of the ODnc (ODpc / ODnc < 0.3).

The S/N percentage (S/N%) for each sample was calculated by the following formula:

$$S/N\% = (OD_{\text{sample}} \div OD_{\text{nc}}) \times 100$$

Samples presenting a S/N percentage: greater than 50% are considered negative, less than or equal to 50% and greater than 40% are considered doubtful, less than or equal to 40% are considered positive.



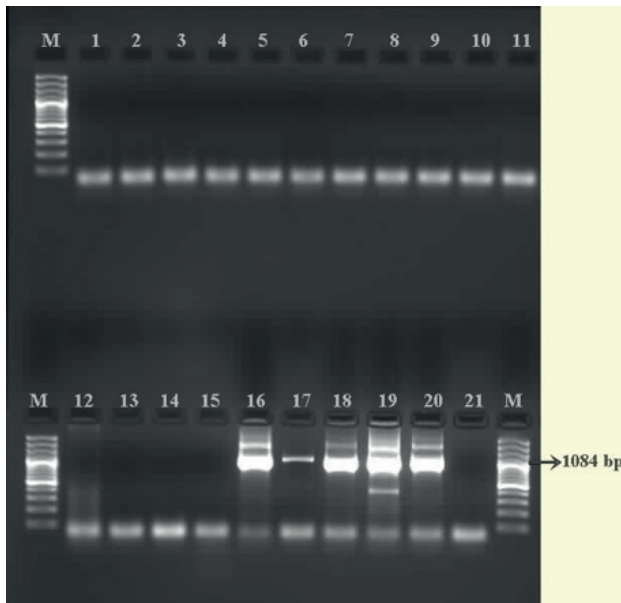


Figure 1. Samples by RT-PCR

Reverse transcription polymerase chain reaction (RT-PCR)

RNA was extracted from leukocyte samples by using the QIAamp Viral RNA Mini Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer’s recommendation. Reverse transcription and amplifications were performed in a continuous RT-PCR method by using the QIAGEN OneStep RT-PCR Kit (Qiagen GmbH), according to the manufacturer’s recommendation.

PCR was performed using the primer pairs (to amplify a wide range of mosquito-borne flaviviruses): 5'-TACAA-CATGATGGGVAARAGAGAGA-3' (nt position 9031-9055 of WNV GenBank accession no. NC 001563) and 5'-AG-CATGTCTTCYGTBGTCATCCAYT- 3' (nt position 10115-10091), resulting in a 1.084-bp amplification product (Weissenböck et al 2002).

Reverse transcription was performed for 30 min at 50°C. Following an initial denaturation for 15 min at 95°C, the reaction mixture was subjected to 45 cycles of heat denaturation at 94°C for 30 s, primer annealing at 60°C for 30 s, and DNA extension at 72°C for 1 min, completed by a final extension of 10 min at 72°C. Twenty microliters of each PCR product were analyzed on 1.2% Tris acetate- EDTA-agarose gel containing ethidium bromide (Sigma, USA). The WNV obtained from the Bornova Veterinary Control Institute was used as positive control.

Results

Competitive Enzyme Linked Immunosorbent Assay (C-ELISA)

A total of 11 serum samples were tested for WNV specific antibodies using C-ELISA. All animals were negative for an-

tibodies against to WNV.

Reverse transcription polymerase chain reaction (RT-PCR)

West Nile Virus nucleic acid was not found in samples by RT-PCR (Figure 1).

Discussion

WNV is a mosquito-borne flavivirus and human, equine, and avian neuropathogen. The virus is indigenous to Africa, Asia, Europe, and Australia, and has recently caused large epidemics in Romania, Russia, and Israel. During the summer of 1999, WNV was first detected in the Western Hemisphere in the northeastern U.S. (Petersen and Roehrig 2001).

The first serological evidence of WNV infection in Turkey was reported in the 1970s based on the detection of haemagglutination-inhibiting (HI) antibodies (Ari 1972, Radda 1973, Meco 1977). One study reported WNV seroprevalence in the western region of Turkey as 6% and 1.5%, in humans and sheep respectively (Ari 1972). Five years later, seroprevalence as high as 40% was reported in randomly selected human sera collected in southeast Turkey, using the HI assay (Meco 1977). Recently, Ozkul et al (2006) were carried out a serosurvey in mammalian species by PRNA.

Positivity rates for the animals varied and were as follows: Ass-mules 2.5%, cattle 4%, dogs 37.7%, horses 13.5%; sheep 1% and humans 20.4%. Albayrak and Ozan (2013) checked blood samples from 70 each cattle, horse, sheep, goat and water buffalo in northern Turkey for the existence of WNV antibodies using competitive ELISA (c-ELISA). The c-ELISA revealed that the seropositivity for WNV in goat was 2.85%. Ergunay et al (2014) reported that seroprevalance for WNV infection was 9.9% in duck, 12.5% in horse, 1.9% in sheep and 12.1% in human using PRNT to identify WNV antibodies in blood serum samples collected from 423 duck, 389 horse, 102 sheep and 266 human in 15 provinces in Turkey.

Reservoir-vector-climate trio was important at the epidemiology for the all mosquito-borne viruses. Sarikamis district of Kars province of Northeastern Turkey where the study was carried out has large forest lands therefore it is suitable for the survival of wild animals. This district is located at 40° 20'17"N and 42° 34'23"E and has 2060 m. altitude. Average annual values of heat, humidity and rainfall of Sarikamis are 3.4°C, 70% and 506.7 mm3, respectively. In addition, annual heat changes are more dramatic in this region.

Climate conditions of western, central and southern parts of Turkey were more suitable for mosquitoes than northern part of Turkey. This is the first report of WNV infection in brown bears in Turkey. None of the bear samples were positive for antibodies to WNV by ELISA in this study. WNV-



neutralizing antibodies were detected in three (6%) of the 51 black bears sampled by PRNT by Farajollahi et al (2003). In Croatia, Madic et al (1993) detected WNV- neutralizing antibodies in 4 brown bears of the 15 using hemagglutination-inhibition (HI) test.

The nested and real-time Reverse Transcription polymerase chain reaction (rRT-PCR) methods have been successful molecular tools for the confirmatory diagnosis of WNV infection by identifying the viral genome (Dutton et al 2009, Albayrak and Ozan 2010, Ergunay et al 2014). They have allowed the detection of WNV RNA in a variety of clinical samples such as blood, tissues and mosquito specimens.

The RT-PCR has the advantages of speed, specificity, and sensitivity for the detection of WNV RNA. Ergunay et al (2014) tested plasma samples from 256 horses and 266 people in Mersin, Adana and Muğla provinces for the presence of WNV RNA using nested and rRT-PCR and WNV RNA was detected in a total of 31 samples in the study. Albayrak and Ozan (2010) did not detect WNV RNA in wild bird samples. Similarly, WNV RNA was not detected in brown bear samples in this study.

Conclusion

In conclusion, no antibody response was detected against WNV in brown bears in Sarikamis district of Turkey. In addition, WNV nucleic acid was not detected in samples of brown bears. Although mosquito species known to transmit mosquito-borne infections have been observed (Dik et al 2006), there is no report of acutely infected humans and animals in Turkey.

This may suggest that WNV infection is not present in Northeastern Turkey. The mosquitoes in this region may not carry WNV. This study showed that WNV might not become a risk potential for animals in Northeastern Turkey.

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