



Phylogenetic analysis of West Nile virus: first report of lineage 1 in donkey in Turkey

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Abstract

West Nile virus (WNV), a member of the *Flaviviridae*, is a major arbovirus that causes West Nile fever. Previous data showed the prevalence of the WNV serologically and molecular in Turkey, and the presence of lineage 1 in horses and humans has been reported. This is the first notification of partial phylogeny of WNV detected in donkeys in the northeast of Turkey (on the Iranian border). Blood serum samples collected from 25 donkeys without clinical symptoms were tested by RT-PCR. Sequence analysis of the sample detected as positive was performed. Multiple sequence alignments of reference sequences taken from GenBank were performed using the ClustalW method using the MEGA6 program. Partial nucleotide sequences of the capsid gene coding region revealed that the strains are closely related to viruses of lineage 1, clade 1a. According to the phylogenetic tree, the TUR/Igdir/donkey strain was included in the same cluster as the strain (KJ958922) previously obtained from horses in Turkey and the strain (GQ851658) from the Central African Republic. This study is the first report to show the circulation of WNV lineage 1 in donkeys in Turkey.

Keywords Donkey · Northeast Anatolia · Phylogeny · Turkey · West Nile virus

Introduction

Mosquito-borne viruses such as Dengue, Chikungunya, Zika, and WNV are responsible for millions of disease cases and thousands of deaths each year (Caraballo and King 2014). WNV is one of the most common arboviruses in the world and can cause death for humans (Kramer et al. 2008). The virus, first isolated in Uganda in 1937 (Smithburn et al. 1940), is considered one of the *Culex pipiens*

primary enzootic vectors (Andreadis 2012). WNV is a pathogen that affects a wide variety of vertebrates, including birds and mammals. The natural infection cycle includes birds and *Culex* mosquitoes, as well as humans and horses are considered the dead-end hosts. Transmission of WNV to susceptible individuals by the bite of an infected mosquito (Burke and Monath 2001; Blitvich 2008). Although most WNV infections are mild or asymptomatic, it can cause fever and encephalitis outbreaks in humans and horses. Less than 1% of human cases include severe symptoms such as meningoencephalitis, encephalitis, and polio-like flaccid paralysis, while 90% of cases in horses have severe neurological findings and deaths (Ward et al. 2004; Hayes and Gubler 2006; Venter et al. 2009).

This virus is a mosquito-borne pathogen belonging to the *Flavivirus* genus in the *Flaviviridae* family and is an enveloped, single-stranded, positive sense RNA virus. The genome is about 11 kb of a single open reading frame (ORF). The viral RNA is translated to produce 3 structural (C, M, E) and 7 nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) (Chambers et al. 1990; Gould et al. 1997).

WNV is biologically diverse, and 9 lineages have been proposed based on genetic differences (Pachler et al. 2014;

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Fall et al. 2017). But most of the human outbreaks with West Nile (WN) encephalitis are caused by lineages 1 and 2. Lineage 1 is quite common all over the world, and it is divided into two different clades, 1a and 1b. Clade 1a consists of strains isolated from Europe, Africa, and America. Clade 1b, also called Kunjin virus, is limited to Oceania. WN encephalitis is caused by strains belonging to lineage 1a especially in Europe, Africa, and America (Lanciotti et al. 2000; Hall et al. 2001; Bakonyi et al. 2006; Ocal et al. 2013; Fall et al. 2017). In Turkey, lineage 1 has been reported to be circulated in horses, humans, and mosquitoes such as *Ochlerotatus caspius* and *C. pipiens* (Ergunay et al. 2013; Ozkul et al. 2013). According to studies on donkeys in Turkey, Nigeria, Pakistan, Senegal, Algeria, Egypt, Palestine, and Israel, antibodies were detected from animals that were observed as healthy. In these studies, seropositivity rates were found between 1.2 and 86.2% (Baba et al. 2014; Zohaib et al. 2015; Davoust et al. 2016; Azmi et al. 2017; Kale et al. 2017; Lafri et al. 2017; Yildirim et al. 2018).

The aim of this study is to determine the phylogeny and lineage of WNV in Turkish donkeys. A phylogenetic tree was constructed from capsid gene coding region for the WNV strain which was found positive as a result of RT-PCR testing in donkey blood sera obtained from Iğdır province in northeastern Anatolia region of Turkey.

Materials and methods

Samples

Blood sera were collected from a total of 25 donkeys from the province of Iğdır (39°55'10.7"North and 44°02'44.5" East) the northeast of the country. One of the important territorial features of the region is the wide use of odd-toed animals (e.g., horses and donkeys) for haulage, transportation, and in agriculture, and thus, one or two donkeys are raised on every farm in the study area. The equines used in this study comprised healthy appearing donkeys older than 1 year of age and unvaccinated against the WNV infection. The presence of WNV nucleic acid in blood serum samples was investigated by RT-PCR method. Sequence analysis was performed on the one serum sample found positive as a result of RT-PCR. Blood serum samples were collected by jugular vein puncture into vacuum tubes with clot activator from randomly selected donkeys. The samples were brought to the laboratory under cold chain storage. After clotting at room temperature for 15–30 min and centrifugation at 3000 g, at 4 °C for 10 min, sera were carefully harvested and stored at –20 °C until analysis.

Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was isolated from blood sera by using TRIzol reagent (Sigma). Total RNA was treated with RQ1 DNase I (Promega). Reverse transcription (RT) was performed according to the manufacturer's directions (Fermentas) using 1 unit of MMLV reverse transcriptase with 5 µg of total RNA and oligo dT22 primer. The 25 µl PCR mixture contained were performed using 1 µL of cDNA template, 0.25 µL (5u/µL) of Taq polymerase, 2.5 µL (100 ng) of primers, 1.5 µL (25 mM) dNTP, 2.5 µL 10×PCR buffer, and 1.5 µL MgCl₂. The RT-PCR assay was used to detect of the nucleic acid of West Nile virus. WNV-specific primers were designed as follows: WN233 (5'-TTGTGTTGGCTC TCTTGGCGTTCTT-3') and WN640c (5'-CAGCCGACA GCACTGGACATTCATA-3') (Lanciotti et al. 2000; Farfan-Ale et al. 2009). PCR reactions were performed: 94 °C for 3 min, 30 cycles at 94 °C for 45 s, 50 °C for 45 s, and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min.

Purification from the gel and sequence analysis of PCR products

In the result of PCR, the base size to the products which was found to be positive was identified by using appropriate markers (generally λ DNA EcoRI/HindIII). The band, which of size is 408 bp, was taken by cutting. The purification was performed according to kit manual by using QIAquick Gel Extraction Kit and kit's protocol. Sequence analysis of the purified product was performed in Anadolu University.

Bioinformatics analysis

Blast analysis of obtained sequences was performed by NCBI data base. The species which determined to its lineage was compared as vertical with the registered species of gene bank by using programs of CLUSTALW, GeneTool, PepTool, and Interproscan, and by using these sequences were created phylogenetic tree by MEGA 6.

Results

Sequence analysis was carried out according to one donkey sample that was identified as WNV-positive. A phylogenetic analysis was carried out by sequencing 408 bp (233–616) of the gene coding region to create a capsid protein of WNV. A total of 29 sequences obtained from the GenBank database and reported in previous studies were used. The K2+I model (Kimura-2 parameter) was determined as the best

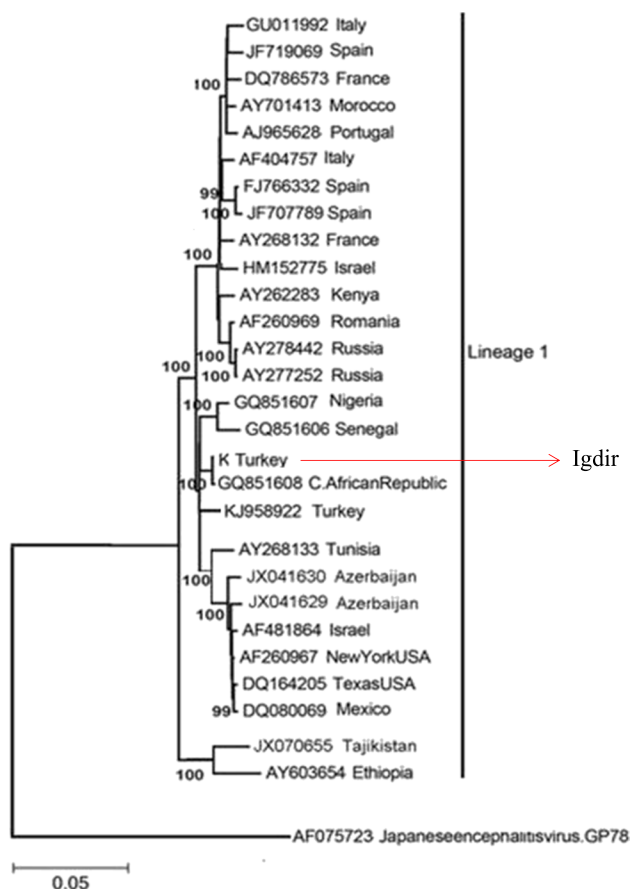


Fig. 1 A rooted phylogenetic tree analysis of West Nile virus (WNV). The phylogenetic analyses were conducted using the neighbor-joining method in MEGA 6. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the analyzed. There were in the final dataset coming from the total of 28 different WNV. Red arrow indicates WNV isolate from Igdir. Japanese encephalitis virus (JEV, GenBank accession: AF075723) was used as an outgroup to root the trees

DNA/protein model (Nei and Kumar 2000; Kumar et al. 2018). The phylogenetic tree that was created using the NJ method is shown in Fig. 1. According to the phylogenetic tree, the TUR/Igdir/donkey strain was included in the same cluster as the strain (KJ958922) previously obtained from horses in Turkey and the strain (GQ851658) from the Central African Republic, and 5 separate clusters were formed. A total of 28 strains obtained from different geographical regions and different species of the world showed similarities between 96.1 and 100% (Fig. 2). The phylogenetic tree formed was not divided into branches according to geographical distribution and host affiliation.

Strains of WNV lineage 1 have a widespread geographic distribution in Africa, Europe, the Middle East, and North America, forming 3 clades. In this study, the TUR/Igdir/donkey strain was classified as lineage 1 and clade a.

Discussion

WNV is a mosquito-borne virus that has been the subject of many studies in Turkey and has been reported to cause infections in humans and horses (Ozkul et al. 2006; Kalaycioglu et al., 2012; Ergunay et al. 2013; Ozkul et al. 2013; Ergunay et al. 2017). In addition, the presence of WNV circulation in donkeys has been demonstrated by molecular and serological studies (Kale et al. 2017; Yildirim et al. 2018). In this study, based on capsid protein, partial phylogenetic analysis of WNV isolated from donkey was performed and this strain was found to be in lineage 1, which was reported to have circulated in previous studies in Turkey (Ocal et al. 2013; Ergunay et al. 2013; Ozkul et al. 2013).

A previous study reported the presence of WNV in the country not only in regions with a subtropical climate such as the Aegean and Mediterranean regions, but also in eastern Anatolia with a cold climate for a significant part of the year (Ergunay et al. 2014). The WNV strain determined in this study was found in the province of Igdir in the Eastern Anatolia Region and is located on the border of Armenia and Iran. It has been determined that *Cx. pipiens* mosquitoes collected in Iran have an important role in the transmission of WNV (Bakhshi et al. 2020). *Cx. pipiens* is known to have circulated in eastern Anatolia (Kars and Igdir provinces) (Gunay et al. 2015) and nearby provinces (Akiner et al. 2019). It is conceivable that the source of WNV identified in this study may be these mosquitoes.

According to phylogenetic analyses, there are 9 lineage identified belonging to WNV. Lineage 1 is widely distributed in Africa, Asia, and America and is known to cause infections in humans. However, it has been reported for Europe that lineage 2 can cause human infections (Zehender et al. 2017). In this study, the partial WNV sequence characterized by donkeys was included in lineage 1 and clade a, with different strains previously obtained in the world and the strain previously characterized in horses in Anatolia. The strain previously identified in horses in Turkey (KJ 958,922) and the strain detected in the Central African Republic was determined to be close (Ergunay et al. 2015). It was reported that the WNV strain detected in Iran in 2009 was 99% similar to the strain detected in the Central African Republic (Shah-Hosseini et al. 2014). The TUR/Igdir/donkey strain identified from the donkey was found to be very close to the strains of Turkey and Central African Republic and the same cluster was included in this study. Turkey's WNV strains that circulated in Eastern Europe, the Mediterranean, and the Middle East are known to be genetically different strains (Ergunay et al. 2015). However, the strain identified in the donkey in Igdir province, which borders neighboring Iran and Iran, and the strains previously identified in Turkey, may be considered to be African in origin. The most important

Strains	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
1-TUR/Igdir/donkey																													
2-GQ851608-C.A.F	100																												
3-KJ958922-Turkey	100	100																											
4-HM152775-Israel	97,4	97,4	97,4																										
5-AF481864-Israel	99,1	99,1	99,1	97,1																									
6-AF260967-NYUSA	99,4	99,4	99,4	97,4	99,7																								
7-DQ164205-TexasUSA	99,4	99,4	99,4	97,4	99,7	100																							
8-DQ80069-Mexico	98,8	98,8	98,8	96,8	99,1	99,4	99,4																						
9-GQ851607-Nigeria	99,1	99,1	99,1	96,5	98,9	99,1	99,1	98,6																					
10-AY268133-Tunisia	98,5	98,5	98,5	96,5	98,9	99,1	99,1	98,6	98,3																				
11-AF260969-Romania	98,5	98,5	98,5	97,6	97,7	97,9	97,9	97,4	97,7	97,0																			
12-GQ851606-Senegal	97,3	97,3	97,3	94,6	97,1	97,3	97,3	97,1	98,3	96,4	95,8																		
13-AF404757-Italy	98,2	98,2	98,2	97,9	97,4	97,6	97,6	97,1	97,4	96,7	98,5	95,6																	
14-GU011992-Italy	97,9	97,9	97,9	97,6	97,1	97,3	97,3	96,8	97,1	96,4	98,2	95,3	99,1																
15-AY701413-Morocco	98,5	98,5	98,5	98,2	97,7	97,9	97,9	97,4	97,7	97,0	98,8	95,9	99,7	99,4															
16-F1766332-Spain	98,5	98,5	98,5	98,2	97,7	97,9	97,9	97,4	97,7	97,0	98,8	95,9	99,7	99,4	100														
17-JF707789-Spain	98,2	98,2	98,2	97,9	97,4	97,6	97,6	97,1	97,4	96,7	98,5	95,6	99,4	99,1	99,7	99,7													
18-JF19069-Spain	97,9	97,9	97,9	97,6	97,1	97,3	97,3	96,8	97,1	96,4	98,2	95,3	99,1	99,4	99,4	99,4	99,1												
19-AJ965628-Portugal	98,5	98,5	98,5	98,2	97,7	97,9	97,9	97,4	97,7	97,0	98,8	95,9	99,7	99,4	100	100	99,7	99,4											
20-DQ786573-France	96,4	96,4	96,4	96,1	95,6	95,8	95,8	95,9	95,6	94,9	96,7	93,7	97,6	97,9	97,9	97,9	97,6	97,9	97,9										
21-AY262283-Kenya	97,7	97,7	97,7	96,8	96,8	97,1	97,1	96,8	96,8	96,2	97,9	95,3	97,7	97,4	97,9	97,9	97,7	97,4	97,9	95,8									
22-AY268132-France	97,4	97,4	97,4	97,1	96,5	96,8	96,8	96,2	96,5	95,8	97,6	94,7	97,9	97,6	98,2	98,2	97,9	97,6	98,2	96,1	96,8								
23-AY277252-Russia	98,2	98,2	98,2	97,3	97,4	97,6	97,6	97,1	97,4	96,7	99,7	95,6	98,2	97,9	98,5	98,5	98,2	97,9	98,5	96,4	97,7	97,9							
24-AY278442-Russia	98,2	98,2	98,2	97,3	97,4	97,6	97,6	97,1	97,4	96,7	99,7	95,6	98,2	97,9	98,5	98,5	98,2	97,9	98,5	96,4	97,7	97,9	100						
25-AY603654-Ethiopia	96,1	96,1	96,1	95,2	96,5	96,7	96,7	96,8	95,9	95,8	95,2	94,6	95,5	95,2	95,8	95,8	95,5	95,2	95,8	94,3	94,3	94,6	94,9	94,9					
26-JX041629-Azerbaijan	96,7	96,7	96,7	95,5	97,1	97,3	97,3	96,8	96,5	96,4	95,2	94,6	95,5	95,2	95,8	95,8	95,5	95,2	95,8	93,6	94,6	94,6	94,9	94,9	96,5				
27-JX-041630-Azerbaijan	96,7	96,7	96,7	95,5	97,1	97,3	97,3	96,8	96,5	96,4	95,2	94,6	95,5	95,2	95,8	95,8	95,5	95,2	95,8	93,6	94,6	94,6	94,9	94,9	96,5	100			
28-JX070655-Tajikistan	96,7	96,7	96,7	95,2	97,1	97,3	97,3	97,4	97,1	96,4	95,8	95,9	96,1	95,8	96,4	96,4	96,1	95,8	96,4	94,9	94,9	94,6	95,5	95,5	98,3	96,5	96,5		
29-AF075723 (JEV)	48,1	48,1	48,1	49,4	48,1	48,1	48,1	48,8	48,1	48,1	50,5	49,9	48,0	48,0	48,6	48,6	48,0	48,6	48,6	48,6	51	50,6	50,5	50,5	48,1	48,1	48,1	48,1	

Fig. 2 Detailed nucleic acid similarity rates for WNV nucleotide sequences used in phylogenetic tree

reason for this can be considered to be migratory birds. Because when the flight routes of birds are looked at, it is seen that migratory bird routes passing through Turkey follow the African route.

WNV is an RNA virus and RNA viruses do not have proofreading mechanisms, so significant mutation and genomic changes are observed. From this point of view, viruses can vary according to the where they are detected, and the phylogenetic tree can be clustered according to the host affiliation and geographical distribution. However, WNV was not divided into branches according to the host or geographic region in which it was identified in both previous studies (Fall et al. 2017; Ozkul et al. 2013) and in this study.

This study is the first report to show the circulation of WNV lineage 1 in donkeys in Turkey. It is important to identify this infection agent in Igdir province in the eastern region of Turkey. It is thought that forward-looking surveillance studies for a comprehensive analysis of viral dispersion in this region, covering the entire mosquito season, and research that will reveal more clearly the status of WNV infection in other species, will benefit in determining the circulation of the infection.

Author contribution YY and VY conceived and designed research. YY, VY, and KY conducted experiments. CÖ and AO contributed new reagents or analytical tools. YY and AAÇ analyzed data. YY and AAÇ wrote the manuscript. All authors read and approved the manuscript.

Data Availability Not applicable.

Code availability Not applicable.

Declarations

Ethics approval Approval was obtained from the animal testing local ethics committee of University Erciyes. The procedures used in this study adhere to the tenets of the Declaration of Helsinki.

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflict of interest The authors declare no competing interests.

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