First evidence of West Nile virus lineage 2 circulation in Turkey

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Veterinaria Italiana 2016, **51** (2), 77-81. doi: 10.12834/VetIt.838.4169.1
Accepted: 13.09.2015 | Available on line: 24.02.2016

Keywords
Horse,
Lineage 2,
Molecular characterization,
Turkey,
West Nile virus.

Summary
In August 2014, a West Nile virus (WNV) strain belonging to lineage 2 was detected in the brain tissues of a 9 year old mare euthanised after showing severe clinical signs in Bursa region, Turkey. Phylogenetic analyses of 290 bp of NS3 coding region clustered the Turkish strain together with the 2010-2012 Greek isolates. Either IgG and IgM or IgG only WNV antibodies were detected in 2 and 11 horses, respectively, which were in the outbreak surrounding. No WNV RNA was detected in pools of 50 individuals of *Culex pipiens* (n = 2), *Ochlerotatus caspius* (n = 2), and *Culex theileri* (n = 1) collected in the infected premises.

Prima segnalazione della circolazione del virus della West Nile appartenente al lineage 2 in Turchia

Parole chiave
Caratterizzazione molecolare,
Equidi,
Lineage 2,
Turchia,
West Nile virus.

Riassunto
Nell'agosto del 2014 nella regione di Bursa, in Turchia, un grave quadro clinico associato ad una sindrome neurologica in un cavallo di nove anni di età è stato segnalato alle autorità sanitarie del Paese. La severità delle manifestazioni cliniche ha indotto i veterinari alla soppressione dell'animale. Nel tessuto cerebrale prelevato nel corso dell'esame necroscopico è stato rilevato il virus della West Nile appartenente al lineage 2. L'analisi filogenetica effettuata sulla sequenza nucleotidica di un frammento di 290 bp nella regione codificante la proteina non strutturale 3 (NS3) del genoma virale ha dimostrato una stretta correlazione tra il virus turco e i ceppi virali che hanno circolato in Grecia nel periodo 2010-2012.

West Nile virus (WNV) is a mosquito-borne *Flavivirus* belonging, together with other important neuro-invasive viruses, to the Japanese encephalitis antigenic complex of the family *Flaviviridae* (Heinz and Stiasny 2012). According to phylogenetic analyses, WNV strains have been arranged in 8 lineages (Pachler *et al.* 2014). Isolates of lineages 1 and 2 are nowadays by far the most widespread. Lineage 1 strain circulation has been reported in North America, North Africa, Europe, and Australia; strains of lineage 2 spread outside their historical geographic range between Southern Africa and Madagascar only recently. In 2004, a lineage 2 strain was recorded for the first time in Hungary (Bakony *et al.* 2006), where it became endemic before crossing the Austrian border in 2008-2009 (Wodak *et al.* 2011). Since then WNV lineage 2 outbreaks were also reported in the Volgograd region of Russia (Platonov *et al.* 2008), in Romania (Sirbu *et al.* 2011), in Greece (Chaskopoulou *et al.* 2011), and in Italy (Savini *et al.* 2012). Although WNV strains of both lineages are capable to infect a wide variety of bird, reptile, and mammalian species, severe nervous symptoms including ataxia, recumbency, and muscle fasciculation have been described only in a small percentage (< 1%) of birds, horses, and/or humans (Carson *et al.* 2012, Angenvoort *et al.* 2013). This paper reports evidence of WNV lineage 2 circulation in Karacabey, Bursa region, in the Western Turkey.

In the beginning of August, clinical symptoms including fever, lack of appetite, depression and incoordination of hind limbs were observed in a 9-year old mare housed in Karacabey Boarding Stud Farm, Bursa region, Turkey (Figure 1). Symptomatic and supportive treatment provided to the animal did not evoke any relief of symptoms and the animal became nervous and aggressive starting to kick out with its hind legs. The clinical conditions then worsened. Due to the inability to maintain the standing posture, and in order to stop its suffering, the animal was eventually euthanized. Brain, lung, liver, heart, spleen, kidney, gut tissues, defibrinated blood, nasal and tracheal swabs were collected and tested for the presence of WNV RNA. The tissue samples were diluted 1/5 with sterile Minimum Essential Medium (MEM) containing antibiotics, homogenized and then centrifuged to remove the cell debris. The supernatants were stored at -80°C until use. Total RNA was purified by using the Exiprep Plus Viral DNA/RNA kit (Bioneer, Daejeon, Korea) according to the manufacturer's instructions. The nucleic acids were eluted in 50 µl of distilled sterile water and used for the subsequent molecular activities. The assay for the viral RNA detection was essentially the method described by Eiden and colleagues (Eiden *et al.* 2010) with some minor modifications.

In order to define the region with viral circulation and possible vectors, serum samples from 27 horses living in the outbreak surrounding area were collected and 7 CDC light traps baited with CO_2 were placed in the infected premises (4,996,000 m^2), either in the paddocks with housed animals or in the surrounding grazing areas. Traps were placed in the evening and mosquitos collected in the morning for 5 consecutive days in October 2014. Sera were tested by WNV IgM and IgG competitive ELISAs (IDVet, Grabels, France).

Upon collection, mosquitoes were identified to species level (Becker *et al.* 2010) and sorted by pools of 50 individuals according to species. Two pools of *Culex pipiens*, 2 pools of *Ochlerotatus caspius*, and 1 pool of *Culex theileri* were tested for WNV with real time polymerase chain reaction (RT-PCR) as described above.

To better characterise the detected strain, positive samples were further tested by nested RT-PCR, as described by Chaskopoulou and colleagues (Chaskopoulou *et al.* 2011) targeting 423 bp of the NS3 coding region. The amplicon was purified with the Qiaquick PCR Purification kit (Qiagen, Hilden, Germany) and used for direct sequencing in both directions using the internal primers. The sequencing reaction was set up using the Big Dye Terminator kit (Applied Biosystems, Carlsbad, USA), the excess

Figure 1. *Geographic localization of 2014 West Nile virus lineage 2 outbreak in Turkey.* GPS coordinates: 40,099213° latitude and 28,358451° longitude.

Table I. *Details of the West Nile virus strains included in the phylogenetic analysis.*

Strain Name	Genbank ID	Collection Date	Host	Country
KC496016/Serbia_2010	KC496016	2010	Mosquito (*Culex pipiens*)	Serbia
DQ116961/Hungary_2004	DQ116961	2004	Goshawk (*Accipiter gentilis*)	Hungary
KF179640/Austria_2008	KF179640	2008	Goshawk (*Accipiter gentilis*)	Austria
HQ537483/Greece_2010	HQ537483	2010	Mosquito (*Culex pipiens*)	Greece
KF179639/Greece_2012	KF179639	2012	Human (*Homo sapiens*)	Greece
KC407673/Serbia_2012	KC407673	2012	Goshawk (*Accipiter gentilis*)	Serbia
KF588365/Italy_2013	KF588365	2013	Human (*Homo sapiens*)	Italy
KF647251/Italy_2013	KF647251	2013	Human (*Homo sapiens*)	Italy
AY532665/Uganda_B956	AY532665	N/A	Unknown	Uganda
FJ425721/Russia_2007	FJ425721	2007	Human (*Homo sapiens*)	Russia
AY688948/Israel	AY688948	N/A	Unknown	Israel
15556/Italy_2012	KP407863	2012	Mosquito (*Culex pipiens*)	Italy
16103/Italy_2012	KP407864	2012	Mosquito (*Culex pipiens*)	Italy
23743 NS3/Italy_2011	KP407865	2011	Collared dove (*Streptopelia decaocto*)	Italy
21612 NS3/Italy_2011	KP407866	2011	Mosquito (*Culex pipiens*)	Italy
20168/Italy_2012	KP407867	2012	Goshawk (*Accipiter gentilis*)	Italy
JN858070.1/Italy_2011	JN858070.1	2011	Human (*Homo sapiens*)	Italy
Turkey 2014/1-291	KP407868	2014	Horse (*Equus caballus*)	Turkey

of dyes was removed using Cleanseq (Beckman Coulter, Brea, USA), and the nucleotide sequences were determined using the DNA sequencer ABI PRISM 3100 (Applied Biosystems, Carlsbad, USA). Raw sequence data were assembled using Contig Express (Vector NTI suite 9.1; Invitrogen, Carlsbad, USA) and consensus sequence (KP407868) aligned with the corresponding sequences deposited in the Genbank database with ClustalW (Table I). A total of 18 WNV strains representative of different geographic regions (Table I) was used to infer the evolutionary history by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei 1993). The tree with the highest log likelihood (-567.5543) is shown in Figure 2.

Evolutionary analyses on 290 bp in the region coding for the NS3 protein were conducted in MEGA6 (Tamura *et al.* 2013). Statistical support at the internodes on the tree was assessed by 1,000 bootstrap replications.

Traces of WNV lineage 2 RNA were detected in the brain tissue of the dead horse. Horse sera collected from the infected area revealed 13 animals with WNV IgG antibodies, in 2 of them levels of IgM were also detected.

The CDC light traps allowed the collection of 499 mosquitoes. The mosquito catches included 290 *Culex pipiens s.l.* (58%), 154 *Ochlerotatus caspius* (31%), 50 *Culex theileri* (10%), and 5 *Anopheles hyrcanus* (1%). No WNV RNA was detected in pools of *Cx. pipiens* (n = 2), *Oc. caspius* (n = 2), and *Cx. theileri* (n = 1).

Once the NS3 coding gene sequence was aligned, the overall difference between the Turkish strain and the sequences included in the alignment varied from 1 nucleotide (Greek strains 2010-2012, position 5428, T instead of C) to 18 bp (Israeli strain AY688948). The phylogenetic analyses of 290 bp (positions 5268-5558 ref. AY532665) of the NS3 coding region clustered the Turkish strain with the 2010 and 2012 Greek isolates (Figure 2).

In the last decade WNV circulation has been extensively documented in humans, birds mosquitoes and horses (Ergunay *et al.* 2014) in different regions of Turkey. As it is the case for many countries facing the Mediterranean Basin, WNV strains belonging to lineage 1 were responsible for these infections, which were rarely associated with clinical symptoms either in humans or in horses. In 2010, however, neurological signs due to WNV infection were reported in 2 horses in the Izmir province (Turkey) (Kalaycioglu *et al.* 2012) and the following year in 2 purebred Arabian foals in the Eskisehir Province in Central Anatolia (Ozkul *et al.* 2013). In 1 of them, WNV lineage 1 was detected in the brain tissue. In this study a horse with severe neurological disorders was observed in Bursa region, and a WNV lineage 2 strain was detected in the brain tissue. To the best of our knowledge, this is the first evidence of WNV lineage 2 circulation in Turkey. Even though the phylogenetic analysis is based on a relatively short sequence (290 bp) of NS3 coding region, it clearly shows a high similarity between the Turkish isolate and those circulating in Greece since

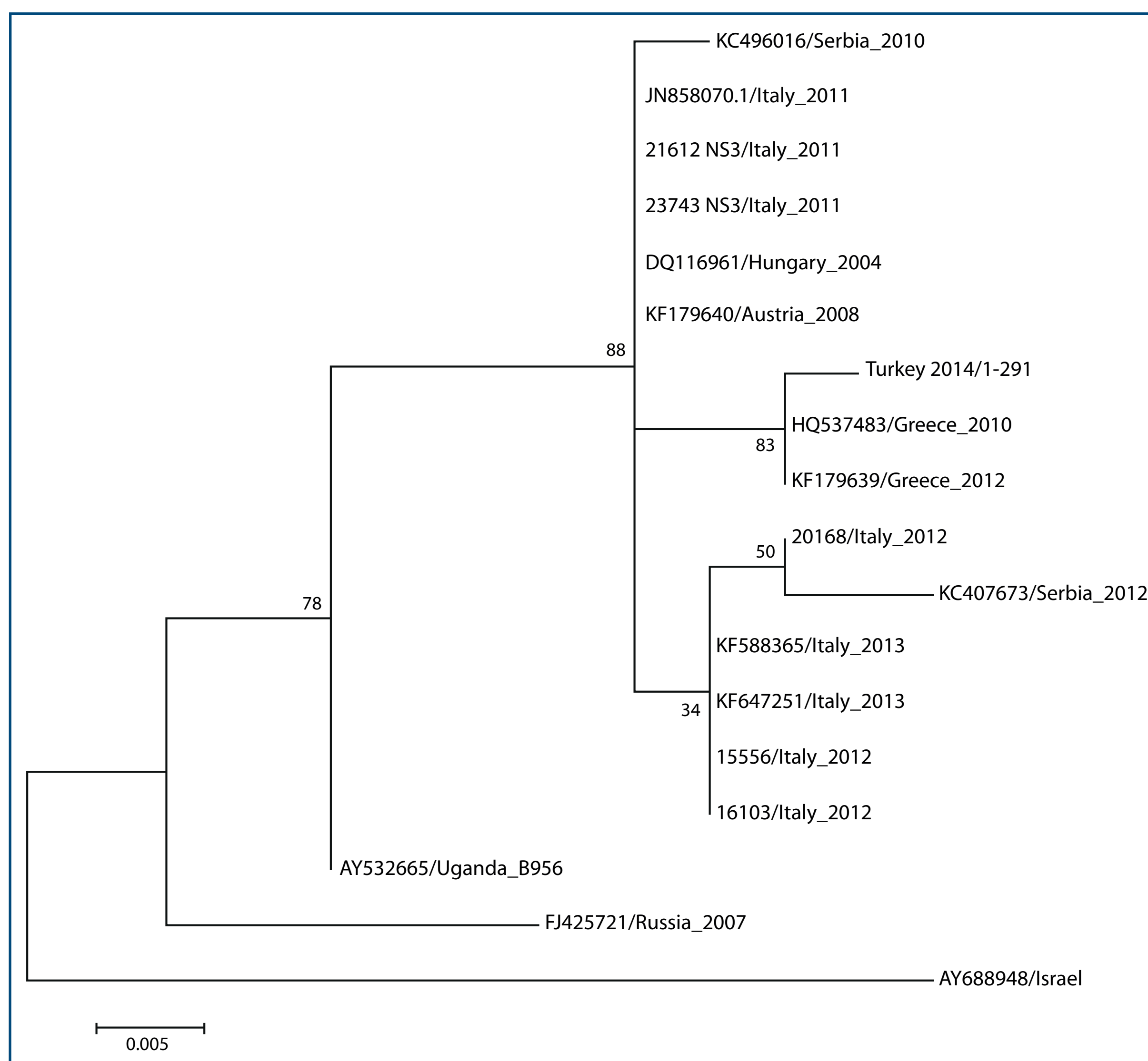

Figure 2. *Phylogenetic analysis by Maximum Likelihood method of the 2014 Turkish strain.* The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model . The tree with the highest log likelihood (-567.5543) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 18 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 290 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

2010, suggesting a common origin of these isolates. It is worth to remind that the Greek isolates were highly pathogenic. According to these results and in view of the close proximity of the region involved, it is likely that short route migratory birds have played an important role in the introduction of the infection, which was able to spread in the area – as suggested by the relative high number of positive horses – establishing a local cycle involving native host and vector species. Unfortunately the lack of WNV detection from the collected mosquitoes did not allow for identifying any potential vector. However, all the tested species are able to transmit the virus, thus strengthening the hypothesis of their involvement as WNV vectors in the outbreak (Hubálek and Halouzka 1999, Monaco *et al.* 2010, Munoz *et al.* 2012). Further studies are required to assess whether the infection has endemized in the area.

References

Angenvoort J., Brault A.C., Bowen R.A. & Groschup M.H.. 2013. West Nile viral infection of equids. *Vet Microbiol*, **167**, 168-180.

Bakonyi T., Ivanics É., Erdélyi K., Ursu K., Ferenczi E., Weissenböck H. & Nowotny N. 2006. Lineage 1 and 2 strains of encephalitic West Nile virus, Central Europe. *Emerg Infect Dis*, **12**, 618-623.

Becker N., Petric D., Zgomba M., Boase C., Madon M. & Kaiser A 2010. Mosquitoes and their Control, 2nd Edition. Springer, Heidelberg Dordrecht London New York.

Carson P.J., Borchardt S.M., Custer B., Prince H.E., Dunn-Williams J., Winkelman V., Tobler L., Biggerstaff B.J., Lanciotti R., Petersen L.R. & Busch M.P. 2012. Neuroinvasive disease and West Nile virus infection, North Dakota, USA, 1999–2008. *Emerg Infect Dis*, **18**, 684-686.

Chaskopoulou A., Dovas C.I., Chaintoutis S.C., Bouzalas I., Ara G. & Papanastassopoulou M. 2011. Evidence of enzootic circulation of West Nile virus (Nea Santa-Greece-2010, lineage 2), Greece, May to July 2011. *Euro Surveill*,**16** (31), pii:19933.

Eiden M., Vina-Rodriguez A., Hoffmann B., Ziegler U. & Groschup M.H. 2010. Two new real-time quantitative reverse transcription polymerase chain reaction assays with unique target sites for the specific and sensitive detection of lineages 1 and 2 West Nile virus strains. *J Vet Diagn Invest*, **22**, 748-753.

Ergunay K., Gunay F., Erisoz Kasap O., Oter K., Gargari S., Karaoglu T., Tezcan S., Cabalar M., Yildirim Y., Emekdas G., Alten B. & Ozkul A. 2014. Serological, molecular and entomological surveillance demonstrates widespread circulation of West Nile virus in Turkey. *PLoS Negl Trop Dis*, **8** (7), e3028.

Kalaycioglu H., Korukluoglu G., Ozkul A., Oncul O., Tosun S., Karabay O., Gozalan A., Uyar Y., Caglayık D.Y., Atasoylu G., Altas A.B., Yolbakan S., Ozden T.N., Bayrakdar F., Sezak N., Pelıtlı T.S., Kurtcebe Z.O., Aydın E. & Ertek M. 2012. Emergence of West Nile virus infections in humans in Turkey, 2010 to 2011. *Euro Surveill*, **17** (21), pii: 20182.

Heinz F.X. & Stiasny K. 2012. Flaviviruses and their antigenic structure. *J Clin Virol*, **55**, 289-295.

Hubálek Z. & Halouzka J. 1999. West Nile fever - a reemerging mosquito-borne viral disease in Europe. *Emerg Infect Dis*, **5**, 643-650.

Monaco F., Lelli R., Teodori L., Pinoni C., Di Gennaro A.P., Polci A., Calistri P. & Savini G. 2010. Re-emergence of West Nile virus in Italy. *Zoonoses Public Health*, **57**, 476-486.

Munoz J., Ruiz S., Soriguer R., Alcaide M., Viana D.S., Roiz D., Vazquez A. & Figuerola J. 2012. Feeding patterns of potential West Nile virus vectors in South-West Spain. *PLoS ONE*, **7** (6), e39549.

Ozkul A., Ergunay K., Koysuren A., Alkan F., Arsava E.M., Tezcan S., Emekdas G., Hacioglu S., Turan M. & Us D. 2013. Concurrent occurrence of human and equine West Nile virus infections in Central Anatolia, Turkey: the first evidence for circulation of lineage 1 viruses. *Int J Infect Dis*, **17**, 546-551.

Pachler K., Lebl K., Berer D., Rudolf I., Hubalek Z. & Nowotny N. 2014. Putative new West Nile virus lineage in Uranotaenia unguiculata mosquitoes, Austria, 2013. *Emerg Infect Dis*, **20**, 2119-2122.

Platonov A.E., Fedorova M.V., Karan L.S., Shopenskaya T.A., Platonova O.V. & Zhuravlev V.I. 2008. Epidemiology of West Nile infection in Volgograd, Russia, in relation to climate change and mosquito (Diptera: Culicidae) bionomics. *Parasitol Res*, **103**, 45-53.

Savini G., Capelli G., Monaco F., Polci A., Russo F., Di Gennaro A., Marini V., Teodori L., Montarsi F., Pinoni C., Pisciella M., Terregino C., Marangon S., Capua I. & Lelli R. 2012. Evidence of West Nile virus lineage 2 circulation in Northern Italy. *Vet Microbiol*, **158**, 267-273.

Sirbu A., Ceianu C.S., Panculescu-Gatej R.I., Vazquez A., Tenorio A., Rebreanu R., Niedrig M., Nicolescu G. & Pistol A. 2011. Outbreak of West Nile virus infection in humans, Romania, July to October 2010. *Euro Surveill*, **16** (2), pii:19762.

Tamura K. & Nei M. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol Biol Evol*, **10**, 512-526.

Tamura K., Stecher G., Peterson D., Filipski A. & Kumar S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol Biol Evol*, **30**, 2725-2729.

Wodak E., Richter S., Bagó Z., Revilla-Fernández S., Weissenböck H., Nowotny N. & Winter P. 2011. Detection and molecular analysis of West Nile virus infections in birds of prey in the eastern part of Austria in 2008 and 2009. *Vet Microbiol*, **149**, 358-366.