**First evidence of West Nile virus lineage 2 circulation in Turkey**

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**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.

**Keywords**

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

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

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.

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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 CO₂ were placed in the infected premises (4,996,000 m²), either in the paddocks with housed animals or in the surrounding grazing areas. Traps were placed in the evening and mosquitoes 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.
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 (KF407868) 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 pippiens s.l.* (58%), 154 *Ochlerotatus caspius* (31%), 50 *Culex theileri* (10%), and 5 *Anopheles hycanus* (1%).

No WNV RNA was detected in pools of *Cx. pippiens* (n = 2), *Os. 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) (Kalayçioğlu 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

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

<table>
<thead>
<tr>
<th>Strain Name</th>
<th>Genbank ID</th>
<th>Collection Date</th>
<th>Host</th>
<th>Country</th>
</tr>
</thead>
<tbody>
<tr>
<td>KC496016/Serbia_2010</td>
<td>KC496016</td>
<td>2010</td>
<td>Mosquito (Culex pippiens)</td>
<td>Serbia</td>
</tr>
<tr>
<td>DQ116961/Hungary_2004</td>
<td>DQ116961</td>
<td>2004</td>
<td>Goshawk (Accipiter gentilis)</td>
<td>Hungary</td>
</tr>
<tr>
<td>KF179640/Austria_2008</td>
<td>KF179640</td>
<td>2008</td>
<td>Goshawk (Accipiter gentilis)</td>
<td>Austria</td>
</tr>
<tr>
<td>HQ537483/Greece_2010</td>
<td>HQ537483</td>
<td>2010</td>
<td>Mosquito (Culex pippiens)</td>
<td>Greece</td>
</tr>
<tr>
<td>KF179639/Greece_2012</td>
<td>KF179639</td>
<td>2012</td>
<td>Human (Homo sapiens)</td>
<td>Greece</td>
</tr>
<tr>
<td>KF407673/Serbia_2012</td>
<td>KF407673</td>
<td>2012</td>
<td>Goshawk (Accipiter gentilis)</td>
<td>Serbia</td>
</tr>
<tr>
<td>KF588365/Italy_2013</td>
<td>KF588365</td>
<td>2013</td>
<td>Human (Homo sapiens)</td>
<td>Italy</td>
</tr>
<tr>
<td>KF647251/Italy_2013</td>
<td>KF647251</td>
<td>2013</td>
<td>Human (Homo sapiens)</td>
<td>Italy</td>
</tr>
<tr>
<td>AYS32665/Italy_2010</td>
<td>AYS32665</td>
<td>N/A</td>
<td>Unknown</td>
<td>Turkey</td>
</tr>
<tr>
<td>FJ425721/Russia_2007</td>
<td>FJ425721</td>
<td>2007</td>
<td>Human (Homo sapiens)</td>
<td>Russia</td>
</tr>
<tr>
<td>AY688948/Israel</td>
<td>AY688948</td>
<td>N/A</td>
<td>Unknown</td>
<td>Israel</td>
</tr>
<tr>
<td>15556/Italy_2012</td>
<td>KP407863</td>
<td>2012</td>
<td>Mosquito (Culex pippiens)</td>
<td>Italy</td>
</tr>
<tr>
<td>16103/Italy_2012</td>
<td>KP407864</td>
<td>2012</td>
<td>Mosquito (Culex pippiens)</td>
<td>Italy</td>
</tr>
<tr>
<td>23743 NS3/Italy_2011</td>
<td>KP407865</td>
<td>2011</td>
<td>Collared dove (Streptopelia decaocto)</td>
<td>Italy</td>
</tr>
<tr>
<td>21612 NS3/Italy_2011</td>
<td>KP407866</td>
<td>2011</td>
<td>Mosquito (Culex pippiens)</td>
<td>Italy</td>
</tr>
<tr>
<td>20168/Italy_2012</td>
<td>KP407867</td>
<td>2012</td>
<td>Goshawk (Accipiter gentilis)</td>
<td>Italy</td>
</tr>
<tr>
<td>JN585070.1/Italy_2011</td>
<td>JN585070.1</td>
<td>2011</td>
<td>Human (Homo sapiens)</td>
<td>Italy</td>
</tr>
<tr>
<td>Turkey 2014/1-291</td>
<td>KP407868</td>
<td>2014</td>
<td>Horse (Equus caballus)</td>
<td>Turkey</td>
</tr>
</tbody>
</table>
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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.

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


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