Putative New West Nile Virus Lineage in *Uranotaenia unguiculata* Mosquitoes, Austria, 2013

Karin Pachler,1 Karin Lebl, Dominik Berer, Ivo Rudolf, Zdenek Hubalek, and Norbert Nowotny

West Nile virus (WNV), the most widespread flavivirus, is distributed throughout Africa, Asia, Europe, and Australia, and since 1999, WNV has also been present in the Americas (1). Within the last 2 decades, WNV infection has caused an increasing number of cases of neuroinvasive disease in humans and become a substantial public health problem (1).

Up to 8 lineages of WNV, based on genetic differences, have been proposed (1,2) (Table 1). Lineage 1 is widely distributed and further divided into lineage 1a, which includes the American strains; lineage 1b, which is also referred to as Kunjin virus and mainly described in Australia; and lineage 1c, which is also referred to as lineage 5 and comprises isolates from India. Lineage 2 has been detected in Africa and several parts of Europe, lineage 3 (Rabensburg virus) has been isolated only in the Czech Republic, and lineage 4 has been reported from Russia (3). A putative sixth lineage, based on a small genome fragment, has been described from Spain (4), and putative lineages 7 (Koutango virus) and 8 have been reported from Senegal (2).

WNV is maintained in an enzootic cycle between mosquitoes and wild birds (1). In 2013, ≈100 *Uranotaenia unguiculata* Edwards, 1913, mosquitoes were trapped during mosquito-monitoring projects at Lake Neusiedl-Seewinkel National Park in Austria and near Sedlec in the Czech Republic. In Russia, *Ur. unguiculata* mosquitoes have been described as hosting lineage 4 WNV strains (A. Platonov, unpub. data) (GenBank accession nos. FJ154906–49 and FJ159129–31). To determine whether *Ur. unguiculata* mosquitoes in Austria and the Czech Republic also host WNV, we investigated the mosquitoes collected in 2013 for the presence of WNV, focusing on lineage 4 viruses.

The Study

During May–October 2013, ≈11,300 female mosquitoes belonging to 13 species were trapped at 4 sites in Lake Neusiedl-Seewinkel National Park in Burgenland State, Austria. Mosquito species were determined according to morphologic criteria (5). Individual mosquitoes were pooled by species and collection site and date. A total of 47 *Ur. unguiculata* mosquitoes were collected in Austria (12 pools, 1–12 mosquitoes/pool). The relative abundance of *Ur. unguiculata* mosquitoes among the total collected in Austria was 0.42%. During August 2013, ≈39,000 mosquitoes were trapped at 2 fish ponds (Nesyt and Novy) in Sedlec, Czech Republic, near the border with northeastern Austria. A total of 47 female *Ur. unguiculata* mosquitoes were grouped into 4 pools (2 with 1 mosquito each, 1 with 4 mosquitoes, and 1 with 41 mosquitoes). The relative abundance of *Ur. unguiculata* mosquitoes among the total collected in the Czech Republic was 0.12%.

The mosquito pools were homogenized in RNase-free water, and RNA was extracted by using the QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA). The samples were screened for the presence of flavivirus nucleic acid by reverse transcription PCR, using universal flavivirus primers MAMD (6) and CFD2 (6,7) for amplification of a partial nonstructural protein (NS) 5 sequence. Results were negative for the samples from Czech Republic. One pooled sample from Austria was positive; the pool contained 9 mosquitoes that had been captured in late August in Illmitz, a village east of Lake Neusiedl (47.76997°N, 16.752887°E). We obtained the complete polyprotein coding sequence and partial 5' and 3' noncoding ends of this novel WNV strain (GenBank accession no. KJ831223), which was designated West Nile virus-*Uranotaenia unguiculata*-Lake Neusiedl-Austria-2013 (WNV-Uu-LN-AT-2013). Primer sequences and amplification protocols are available upon request.

The complete polyprotein gene sequence of WNV-Uu-LN-AT-2013 shares a maximum identity of ≈83% with lineage 4 WNV strains isolated from *Ur. unguiculata* mosquitoes and *Dermacentor marginatus* ticks in Russia (3). At the amino acid level, the entire polyproteins of WNV-
showed a close grouping of WNV-Uu-LN-AT-2013 viruses from Austria, the virus from Spain, and the lineage 4 viruses from Russia; similarity was slightly higher between the viruses from Austria and Spain (Figure, panel B).

WNV-Uu-LN-AT-2013 encodes a polyprotein of 3,432 aa. The envelope protein carries 1 putative N-linked glycosylation site at asparagine residue N-154, which has been associated with increased WNV pathogenicity and neuroinvasiveness (9). The 3 highly conserved N-linked glycosylation sites at NS1 positions N-130, N-175, and N-207 in WNV strains were also calculated for WNV-Uu-LN-AT-2013 by using NetNGlyc 1.0 software (http://www.cbs.dtu.dk/services/NetNGlyc/). Glycosylation of NS1 at these 3 positions has been implicated in neuroinvasiveness (10), as has proline at NS1 aa position 250 (11), which is also present in WNV-Uu-LN-AT-2013. The NS2A-encoding nucleotide region contains a foo motif, which can mediate production of NS1′, a variant of NS1 that plays a role in neuroinvasiveness (12). A ffo motif, which has been described for the nonpathogenic mosquito-specific flaviviruses (13), could not be determined for WNV-Uu-LN-AT-2013.

Table 2. Sequence identities between the newly identified WNV strain from Austria, WNV-Uu-LN-AT-2013, and other strains representing different WNV lineages

| Strain/lineage† | WNV-Uu-LN-AT-2013 | 1a | 1b | 1c/5 | 2 | 3 | 4 | 6 (Spain)§ | 7 (Koutango virus) | Usutu virus legal accessions
|-----------------|-------------------|-----|-----|------|---|---|---|-------------|-------------------|----------------------|
| WNV-Uu-LN-AT-2013 |                  |     |     |      |   |   |   |             |                   |                      |
| 1a              |                  | 88.3| 87.9| 87.0 | 88.8| 86.7| 96.2| 95.9        | 85.3              | 81.2              | 75.5
| 1b              |                  | 75.4| 88.2| 92.7 | 93.5| 89.8| 88.3| 91.2        | 88.9              | 92.0              | 76.1
| 1c/5            |                  | 76.3| 80.5| 79.7 | 92.1| 88.8| 87.4| 89.1        | 87.7              | 92.1              | 76.1
| 2               |                  | 77.0| 79.8| 79.6 | 79.1| 90.9| 89.2| 92.6        | 89.3              | 92.0              | 76.0
| 3               |                  | 75.9| 78.3| 77.3 | 77.3| 78.7| 87.0| 91.4        | 86.6              | 89.2              | 75.5
| 4               |                  | 82.8| 76.0| 76.2 | 76.2| 76.7| 76.5| 95.0        | 80.5              | 81.0              | 74.7
| 6 (Spain)§      |                  | 83.2| 78.1| 78.1 | 77.7| 78.6| 79.5| 81.7        | 88.6              | --                | 80.8
| 7 (Koutango virus) |                  | 75.1| 77.7| 77.4 | 77.0| 77.6| 73.6| 75.6        | 78.0              | 86.8              | 75.3
| Usutu virus     |                  | 71.2| 72.4| 72.6 | 72.4| 71.3| 71.0| 73.6        | 72.4              | 72.5              | 72.5

*Alignment was performed by using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). WNV, West Nile virus; WNV-Uu-LN-AT-2013, West Nile virus strain Uranotaenia ungueiculata-Lake Neusiedl-Austria 2013; --, comparison between lineages 6 and 8 was not possible because the available partial sequences do not cover the same nucleotide regions.
†GenBank accession nos. are as follows for the polyprotein genes/polyproteins: WNV-Uu-LN-AT-2013 (KJ831223), lineage 1a (AF404756/AAM81752), lineage 1b (D00246/BAA00176), lineage 1c (DQ256376/ABZ5712), lineage 2 (DQ116981/AAZ91684), lineage 3 (AY752684/AW887711), lineage 4 (FJ159129/AHC995030), lineage 6 (Spain) (GU047875/ADD9956), lineage 7 (Koutango virus) (EU082200/ABW76844), lineage 8 (KJ131502/AVH84343), Usutu virus (AY453411/AA59402).
‡Comparison was based only on partial NS5 gene sequences.
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Conclusions

WNV lineages 1–4 and putative lineage 6 have been detected in Europe, but only WNV lineage 1a has spread across the American continents. Circulation of such a genetically diverse group of WNV strains in Europe may partly explain the epidemiologic differences observed between WNV disease in Europe and the Americas. In Europe, the presence of less pathogenic WNV strains may inhibit the spread of more pathogenic strains.

We propose that the WNV-Uu-LN-AT-2013 strain from Austria either constitutes a new lineage (lineage 9) or can be grouped into lineage 4 as sublineage 4c, with the strains from Russia and Spain as sublineages 4a and 4b, respectively. However, the short sequence available for the strain from Spain does not allow a clear-cut conclusion to be drawn with regard to lineage 4. We suggest that future designation of new WNV lineages should be restricted to viruses for which at least the complete polyprotein gene sequences have been determined. In addition, rules for defining virus lineages should be established by the International Committee on Taxonomy of Viruses.

Strain WNV-Uu-LN-AT-2013 has been detected only in *U. unguiculata* mosquitoes. These mosquitoes are mainly distributed in the southern half of Europe (5); in eastern Europe, they have spread from southern Ukraine and the Volga Delta through middle and southwestern Asia to Iran and Pakistan (5). In the Lake Neusiedl area of Austria, *U. unguiculata* mosquitoes seem to be an indigenous species, which was first reported in 1970 (14). In the Czech Republic, *U. unguiculata* mosquitoes seem to be an indigenous species, which was first reported in 1970 (14). In the Czech Republic, *U. unguiculata* mosquitoes seem to be an indigenous species, which was first reported in 1970 (14).
The pathogenicity of strain WNV-Uu-LN-AT-2013 in humans and animals has not been elucidated. Genetic data show that the strain carries typical WNV pathogenicity markers and suggest that WNV-Uu-LN-AT-2013 is not restricted to mosquitoes. Additional monitoring studies involving cell culture and animal isolation experiments are necessary to evaluate the pathogenic potential of this virus for humans and animals.

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Dr Pachler is a postdoctoral researcher at the Institute of Virology, University of Veterinary Medicine, Vienna, Austria. Her research interests include the molecular biology of emerging and vectorborne viruses.

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Address for correspondence: Norbert Nowotny, Viral Zoonoses, Emerging and Vector-Borne Infections Group, Institute of Virology, University of Veterinary Medicine Vienna, Veterinaerplatz 1, 1210 Vienna, Austria; email: norbert.nowotny@vetmeduni.ac.at and nowotny@ssu.edu.om