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BRIEF REPORT

The complete genome sequence analysis of *West Nile virus* strains isolated in Slovakia (central Europe)

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Abstract

The present study reports the first complete genome sequence analysis of West Nile virus (WNV) strains isolated from brain samples from raptors. The results prove the circulation of closely related WNV lineage II strains in central Europe and genetic analysis revealed seven amino acid substitutions in structural (PrM_3, E_{159} and E_{231}) and in non-structural ($NS1_{109}$, $NS5_{259}$, $NS5_{310}$ and $NS5_{600}$) proteins. Observed amino acid substitutions Phe_3 and Ser_{231} were common only within the lineage VII Koutango strain isolated from *Rhipicephalus guilhoni* tick in Senegal. Further research could reveal whether these substitutions influence the biological properties of WNV, including virulence and neuroinvasiveness.

West Nile virus (WNV, *Flavivirus, Flaviviridae*) is a mosquito-borne flavivirus that belongs to the Japanese encephalitis virus serocomplex. WNV was originally isolated from the blood of a febrile woman in the West Nile district of Uganda in 1937 [1]. WNV is in nature maintained through ornithophilic mosquitoes and birds; whereas mammals, including humans are accidental hosts considered as dead-end hosts. First reports on WNV presence in Europe are from 1958 from Albania, where neutralizing antibodies against WNV were detected in human sera. Since then, WNV was reported in several European countries and detected in vectors, birds, horses and humans causing seasonal outbreaks [2]. Phylogenetic studies revealed the existence of at least eight evolutionary lineages of WNV, however strains from human and animal WNV infections in Europe belong to lineage I and II [3]. WNV lineage I (strain goose-Hungary/03) was detected in the brain of goose in Hungary in 2003. One year later, a lineage II WNV (strain goshawk-Hungary/04) was detected from a goshawk [4]. Since then, in central Europe annual seasonal outbreaks of WNV recorded in humans and animals were caused by lineage II WNV [2]. In Slovakia, first reports of WNV were from the 1960s, when antibodies were detected in human sera [5]. From that time onwards, no clinical cases of WNV infection have been reported in humans or animals. In 2016, WNV RNA was detected in the brains of raptors which died during the clinical course of infection [6]. Several studies identifying molecular markers of virulence in WNV lineage II strains have been published [7, 8]. Phylogenetic study revealed genetic stability in the envelope (E) and membrane (M) proteins among lineage II strains. Nevertheless, absence of the NYS glycosylation motif in the E protein in position $E_{154–156}$, was observed in non-neuroinvasive lineage I and II strains [7]. Flavivirus E glycoproteins are involved in cell-binding and determinate host cell susceptibility [9]. Non-structural (NS) proteins are essential for flavivirus replication. Mutation P250L in NS1 leads to slower viral replication in cell culture and seems to abolish the neuroinvasiveness [8]. WNV strains with

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mutation D73H or M108K in NS2A replicate poorly and fail to cause mortality in mice [10].

This study is based on a previous screening of bird samples for WNV RNA [6]. Virus RNA positive brain samples used for virus isolation in Vero E6 cell line were originated from raptors that died during clinical onset. Monolayers showed cytopathic effect in four samples and cultivation media were used for whole genome analysis. Sequences designed as WNV strain 486.B/14/Velky Biel/SVK, WNV strain 291.B/13/Velky Biel/SVK, WNV strain 286.B/13/Velky Biel/SVK and WNV strain 200.B/2013/Secovce/SVK were submitted to GenBank under accession numbers MH244510–MH244513. Isolates 486.B/14/Velky Biel/SVK, 291.B/13/Velky Biel/SVK, 286.B/13/Velky Biel/SVK were originated from Northern Goshawks (*Accipiter gentilis*) from Veľký Bieľ (Senec County, western Slovakia) and 200.B/2013/Secovce/SVK strain from Eurasian Sparrow Hawk (*A. nisus*) from Sečovce (Trebišov County; eastern Slovakia). Twenty-two overlapping sequences covering the whole WNV genome were submitted for Sanger sequencing in both directions. Description of brain samples and the methods are described in detail in the Supplementary data 1. After assembly and validation, the acquired sequences were aligned (Clustal W) with reference lineage I (n = 7) and lineage II (n = 9) sequences. The phylogenetic tree was constructed in MEGA 7 and Usutu virus as the outgroup had been added to the alignment (Figure 1).

The length of sequenced WNV genomes was 11026 bp (291.B/13/Velky Biel/SVK), 11025 bp (486.B/14/Velky Biel/SVK), 11013 bp (286.B/13/Velky Biel/SVK) and 11012 bp (200.B/2013/Secovce/SVK) and nucleotide pairwise identity among them ranged between 99.6–99.9% (Supplementary data 2a). Phylogenetic analysis showed close clustering with central European WNV lineage II strains detected in Hungary, the Czech Republic, Austria and Serbia (Figure 1). Pairwise identity among these and the Slovak strains ranged from 99.5–99.8%. Each polyprotein was coded by a single ORF (10302 bp) flanked by a 96 bp and 614–627 bp long non-coding regions at the 5′ and 3′ ends, respectively. Translation resulted in a 3434 amino acid long polypeptide. The comparison with the reference WNV lineage I and II strains showed seven unique and uncommon amino acid substitutions in the PrM, E, NS1 and NS5 proteins (Table 1). These substitutions were further studied by comparison with polyprotein sequences of WNV strains isolated all over the world (n = 1903; Supplementary data 2b) and polyprotein sequences of European WNV lineage I and II strains (n = 45; Supplementary data 2c). Observed amino acid substitutions and the possible importance of WNV polyprotein sites are summarized in Supplementary data 3.

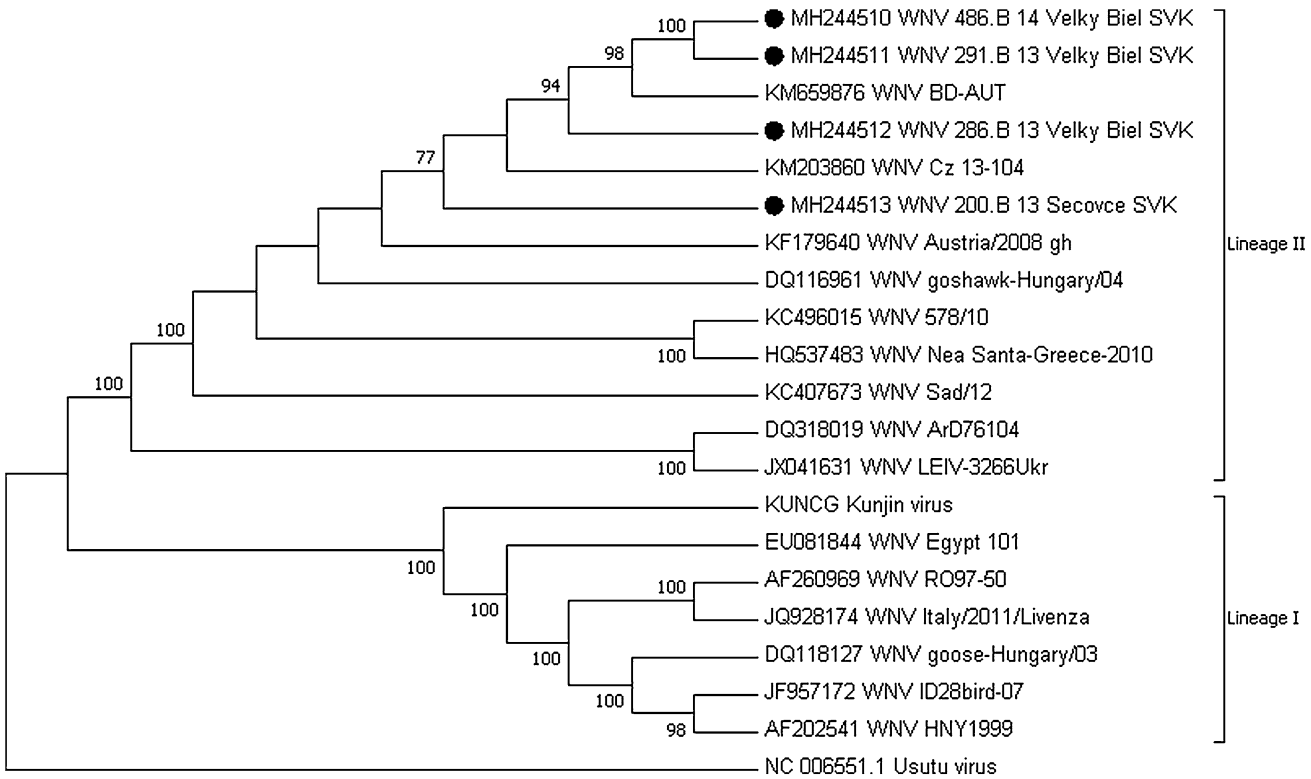

Fig. 1 Molecular phylogenetic analysis of full genome nucleotide sequences of WNV strains isolated from raptor's brains. *Legend: WNV isolated and analysed in this study are marked with black dots*

Table 1 Amino acid changes in the polyprotein sequences of WNV isolates from Slovakia and selected reference WNV lineage I and II strains

WNV strain	Substitution position in polyprotein (in corresponding protein)						
	PrM	E		NS1	NS5		
	126 (3)	449 (159)	521 (231)	900 (109)	2776 (259)	2827 (310)	3117 (600)
486.B/14/Velky Biel/SVK	F	A	T	E	R	T	D
291.B/13/Velky Biel/SVK	F	A	S	E	K	T	D
286.B/13/Velky Biel/SVK	L	T	T	G	K	T	D
200.B/13/Secovce/SVK	L	T	T	E	K	T	G
578/10	L	I	T	E	K	A	D
BD-AUT	L	A	T	E	K	T	D
Austria/2008_gh	L	I	T	E	K	A	D
CZ 13-104	L	T	T	E	K	T	D
Hungary/04	L	I	T	E	K	A	D
Nea Santa Greece 2010	L	I	T	E	K	A	D
SAD/12	L	I	T	E	K	A	D
ArD/76/104	L	I	T	E	K	A	D
LEIV-3266Ukr	L	I	T	E	K	A	D
Hungary/03	L	V	T	E	R	E	D
ID28Bird-07	L	A	T	E	R	E	D
Egypt 101	L	I	T	E	R	E	D
Kunjin	L	T	N	E	K	E	D
RO97-50	L	V	T	E	R	E	D
HNY1999	L	V	T	E	R	E	D
Italy/2011/Livenza	L	I	T	E	R	E	D

Legend: Lineage II strains: 486.B/14/Velky Biel/SVK (*MH244510*)*, 291.B/13/Velky Biel/SVK* (*MH244511*)*, 286.B/13/Velky Biel/SVK* (*MH244512*)*, 200.B/13/Secovce/SVK* (*MH244513*)*, 578/10* (*KC496015*)*, BD-aut* (*KM659876*)*, Austria/2008_gh* (*KF179640*)*, CZ 13-104* (*KM203860*)*, Goshawk Hungary/04* (*DQ116961*)*, Nea Santa Greece 2010* (*HQ537483*)*, SAD/12* (*KC407673*)*, ArD/76/104* (*DQ318019*) *and LEIV-3266Ukr* (*JX041631*)*. Lineage I WNV strains: Goose-Hungary/03* (*DQ118127*)*, ID28Bird-07* (*JF957172*)*, Egypt 101* (*EU081844*)*, Kunjin* (*KUNCG*)*, RO97-50* (*AF260969*)*, HNY1999* (*AF202541*) *and Italy/2011/Livenza* (*JQ928174*)

In PrM protein, a point mutation (C→T) at the position 475 nt of WNV isolates 291.B/13/Velky Biel/SVK and 486.B/14/Velky Biel/SVK, resulted in PrM_3 in substitution to Phe_3. From the total number of aligned WNV sequences (n = 1903), Phe_3 was observed only in WNV lineage VII Koutango strain ArD96655/1993/SN (KY703855) isolated from *Rhipicephalus guilhoni* tick in Senegal [11]. The other WNV strains isolated from ticks (KJ934710, JX041633 and AY277251) *Hyalomma marginatum*, *H. plumbeum* and *Dermacentor marginatus*, respectively has Leu (n = 1895) or Met (n = 5) (Supplementary data 2b). Koutango and the 291.B/13/Velky Biel/SVK strains share another substitution. This one is located at position 1657 nt in the E protein, where a substitution A→T at residue E_{231} resulted in Ser (Ser_{231}). In the rest of the compared sequences was Thr (Supplementary data 2b). The PrM and E protein amino acid sequence identity between Koutango and 291.B/13/Velky Biel/SVK strains is 81.3% and 75.9%, respectively. Both amino acid substitutions are located in structural proteins, which might have influence on their antigenicity. PrM and E protein are the aim of several studies not only for antibody-dependent enhancement (ADE) mechanism [12, 13] but also as a target in serodiagnostics [14] or vaccine development [10, 12]. PrM antibodies restored the infectivity of immature WNV NY99 particles, which after infection of mice caused viremia, have been found in the brain and have caused disease and death of the animals [12]. These results indicate that during natural WNV infection, PrM antibodies and immature virions produced during the replication may mediate ADE of WNV infection. There is limited information on the ADE mechanism mediated by WNV proteins. However, the amino acid sequence of DENV PrM was analysed for its ADE capacity. All analysed monoclonal PrM antibodies recognized a single antigenic site with overlapping epitopes. Seven different residues on the PrM are necessary for antibody binding and among those, Leu_3 has been found to be critical for binding of nearly half of the antibodies [13]. The residue E_{231} is part of domain III, which maintains an immunoglobulin-like fold, appears to be involved in receptor binding and is a major target of neutralizing antibodies [15]. In a study of neutralizing antibody response, mice were infected with sublethal dose of

the Koutango strain. There was no detectable neutralizing antibody response up to 2 weeks after infection, but mice infected with WNV NY99 had average titer 67 and 533 at days 7 and 14, respectively. At 21 days after infection, an average titer of 40 was detected in mice infected by the Koutango strain and 2560 after NY99 strain [16]. The 291.B/13/Velky Biel/SVK and Koutango strains have low PrM and E protein identity, but studies on replication kinetic, in either *ex vivo* and/or *in vivo* models would show a deeper insight into the consequences of the shared Phe_3 and E_{231} substitutions, which are unique among the compared polyprotein sequences.

Substitutions in the E protein, present in the nucleotides position 1441–1443 resulted in two codons, GCA and ACA. In the 486.B/14/Velky Biel/SVK and 291.B/13/Velky Biel/SVK strains, GCA codes in residue 159 (E_{159}) Ala (Ala_{159}). In the 286.B/13/Velky Biel/SVK and 200.B/2013/Secovce/SVK strains ACA codes Thr (Thr_{159}). The N-linked glycosylation motif NYS (E_{154}–E_{156}) is implicated in the virulence of WNV and changes in its sequence results in altered neuroinvasiveness [17]. In the total number of aligned WNV genome sequences (n = 1903) Ala_{159} substitution was observed in 1469 sequences and Thr_{159} in 39 sequences. Presence of Ala_{159} was first described in North America, where the original NY99 (lineage I) genotype was displaced by a new WN02 genotype [18]. As these two genotypes differ only in residue E_{159}, some suggest that Ala_{159} could be a cause of higher infection rates in birds as a result of shorter extrinsic incubation periods in mosquitoes for strains of the WN02 genotype [18, 19]. Among the European WNV strains (Supplementary data 2c), Ala_{159} was present also in lineage II strains from Austria (KM659876) and the Czech Republic (KM203861 and KM203862). KM659876 has been detected in Austria from an asymptomatic blood donor, which later developed myalgia and a generalized maculopapular rash. Strains KM203861 and KM203862 have been detected in the Czech Republic and originated from *Culex modestus* mosquitoes. Thr_{159} has been observed in Slovak strains 286.B/13/Velky Biel/SVK and 200.B/2013/Secovce/SVK but also in other European lineage I and lineage II strains from Austria, Italy and the Czech Republic (Supplementary data 2c). Austrian WNV lineage II strains (KP780837, KP780838, KP780839 and KP7808740) were detected from keas (*Nestor notabilis*) showing clinical signs of WNV infection. Three of them recovered, but within six years all of them died. Autopsies showed different grades of non-suppurative encephalitis and viral RNA had been detected in the brains. Another Austrian strain (KP109692) sharing Thr_{159} was detected from *Culex pipiens* collected in 2014. Strains from Italy sharing Thr_{159} (KP789954, KP789956, KP789959, KP789960, KF647251, KF588365 and JN858070) were all isolated from human patients with neuroinvasive WNV infection, mostly from northern Italy in years 2013–2014. Czech strains with Thr_{159} (KM203860 and KM203863) were detected from *Culex modestus* mosquitoes collected in South Moravia.

In NS1, a point mutation (A→G) at position 2795 nt of the 286.B/13/Velky Biel/SVK strain resulted at residue $NS1_{109}$ in substitution to Gly (Gly_{109}). In most other strains Glu_{109} was present, but Gly_{109} was observed in American WNV lineage I strains collected from mosquitoes (HM488228, KX547374, KX547592, KX547342, KX547495) and corvids (KJ501318, KJ501351) in years 2002–2011, in Serbian lineage II strains detected from mosquitoes (KT757320, KT757321, KT757322, KT757323) and in a human (KX375812) in 2013 (Supplementary data 2b). When expressed in the insect cell line, NS1 has been N-glycosylated at three positions $NS1_{130}$, $NS1_{175}$ and $NS1_{207}$. Although the Gly_{109} is not part of these sites, it lacks a side chain, therefore may facilitate conformational changes in the protein. Chung and colleagues mapped 21 monoclonal antibodies specific against three NS1 portions. Four of these antibodies (10NS1, 14NS1, 16NS1 and 17NS1) showed prophylactic properties as 75–95% of mice were protected against lethal WNV infections. Efficient post-exposure therapeutic effect was shown by combined administration of the 14NS1 and 16NS1 antibodies, which recognized distinct epitope binding sites on the NS1 protein. Eighty percent of infected mice survived WNV infection. Moreover, based on WNV quantification, a single dose of combined 14NS1 and 16NS1 antibodies provided a significantly higher (60%) survival rate of mice in comparison after single dose of either 14NS1 (40%), or 16NS1 (44%) [20].

In NS5, a nucleotide change A→G (AGA) at position 8423 nt of the 486.B/14/Velky Biel/SVK strain resulted at residue $NS5_{259}$ to Arg (Arg_{259}). Among the selected European WNV strains (Supplementary data 2c), Arg_{259} is present in all WNV lineage I (n = 5) strains, however in all other lineage II strains including the rest of Slovak isolates (n = 39) Lys_{259} is present. All Slovak isolates shared a point mutation at position 8575 nt (G→A) that resulted at residue $NS5_{310}$ to Thr (Thr_{310}), however in most other WNV sequences Glu was present. In the total number of compared WNV sequences, Thr_{310} was also observed in WNV lineage II strains (JN858070, KF588365, KF823805, KF823806, KF647248, KF647249, KF647250, KF647251, KF647252, KP789953, KP789954, KP789955, KP789956, KP789957, KP789958, KP789959, KP789960 and KT207792) from humans and mosquitoes from Italy; and from humans, birds and mosquitoes in Austria (KM659876, KP109692, KP780837 and KP780838). Thr_{310} was also observed in sequences from mosquitoes collected in the Czech Republic (KM203860, KM203861, KM203862 and KM203863; Supplementary data 2b).

A point mutation (A→G) was identified at position 9446 nt of strain 200.B/2013/Secovce/SVK resulted at residue

$NS5_{600}$ to Gly (Gly_{600}), where in other strains Asp_{600} was present. Gly_{600} was unique among selected European WNV strains (Supplementary data 2c), but also present in lineage IX or sublineage IV strains (KJ831223, FJ159129, FJ159130 and FJ159131) detected from *Uranotaenia unguiculata* in Austria, Russia and Africa. Gly_{600} is also present in the AY277251 strain of the lineage IV originating from *Dermacentor marginatus* tick in the Caucasus.

In conclusion, comparison of nucleotide and polyprotein sequences of new central European WNV isolates showed rare amino acid substitutions in structural and non-structural proteins. In three Slovak isolates (200.B/13/Secovce/SVK, 291.B/13/Velky Biel/SVK a 486.B/14/Velky Biel/SVK) unique amino acid substitutions have been observed in the PrM, E and the NS5 proteins, which were observed only in tick and mosquito isolated strains among the other compared strains. Further research should be focused on the influence of amino acid substitutions Phe_3, Ser_{231} a Gly_{600} on biological properties of WNV in model infections.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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