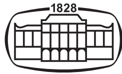

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West Nile virus infections in Hungary: Epidemiological update and phylogenetic analysis of the Hungarian virus strains between 2015 and 2022

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RESEARCH ARTICLE

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ABSTRACT

Following the introduction of the *West Nile virus* (WNV) into Hungary in 2004, it has shortly become one of the most important human arbovirus infections, with a gradually increasing number of cases. The study aimed to summarize the current epidemiological situation in Hungary and sequence the WNV PCR-positive clinical specimens and virus isolates by next-generation whole genome sequencing (NGS) to obtain a detailed phylogenetic analysis of the circulating virus strains. Whole blood and urine samples from confirmed WNV-infected patients and WNV isolates were investigated by reverse transcription PCR assays. Genome sequencing was carried out by Sanger-method, followed by NGS on the Illumina MiSeq platform. Altogether 499 human infections were diagnosed between 2004 and 2022. A particularly remarkable increase in human WNV infections was observed in 2018, while the number of reported cases significantly decreased during the COVID-19 pandemic. Between 2015 and 2022, 15 WNV isolates, and 10 PCR-positive clinical specimens were investigated by NGS. Phylogenetic analysis revealed that the major European WNV lineage 2 clades, namely the Eastern European (or Russian) and the Central European (or Hungarian) clades, are presented in Hungary. Strains of the Balkan and other European clusters within the Central European clade are co-circulating in the country, following a characteristic geographical distribution. In Hungary, the presence and co-circulation of multiple lineage 2 WNV strains could be identified in the last few years. Therefore, in light of the 2018 WNV outbreak, sequence-based typing of the currently circulating strains could highly support outbreak investigations.

KEYWORDS

West Nile virus, phylogeny, arboviruses, lineage 2, emerging pathogens, zoonotic infections, next-generation sequencing

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INTRODUCTION

West Nile virus (WNV), a member of the genus *Flavivirus*, family *Flaviviridae*, is considered one of the most important endemic arboviruses in Hungary. WNV has a single-stranded positive-sense RNA genome of approximately 11 kilobases in length and encodes a single polyprotein [1]. Since the first documented human neuroinvasive infections in 2004,

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West Nile fever (WNF) and the more severe manifestation of the infection, West Nile neuroinvasive disease (WNND) have been regularly diagnosed in Hungary with the highest peak of reported cases in 2018 [2]. The National Reference Laboratory (NRL) for Viral Zoonoses of the National Center for Public Health (NCPH), Hungary is exclusively responsible for the laboratory diagnosis of human infections, across the entire territory of the country. The Department of Epidemiological and Vaccination Surveillance of the NCPH collects, evaluates, and reports epidemiological and microbiological data directly to The European Surveillance System (TESSy) operated by the European Centre for Disease Prevention and Control (ECDC) [2]. For laboratory case confirmation the European Union's case definition criteria are applied: isolation of WNV from blood or cerebrospinal fluid (CSF), detection of WNV nucleic acid in blood or CSF, detection of WNV specific IgM antibodies in CSF, presence of WNV IgM antibodies in high titer and detection of WNV IgG and confirmation by neutralization [3]. In addition, nucleic acid detection in urine samples is also part of the routine laboratory confirmatory protocol [2]. Molecular typing of the WNV RNA by Sanger-method or by next-generation whole genome sequencing (NGS) is also an important element of the surveillance activities, as data regarding the circulating lineages are regularly reported to the ECDC. To date, at least eight genetic variants of WNV have been identified, with huge differences in pathogenicity potential. Among the genetic variants (*lineages*), lineage 1 and 2 pose the largest health risk to humans and animals worldwide [4]. Until the first emergence of WNV lineage 2 in Hungary in 2004, only lineage 1 and 3 WNV strains had been reported across Europe [5]. Shortly after its first emergence, lineage 2 strains began to spread rapidly in the region, characterized by a high degree of genetic diversification. During the last decade, WNV lineage 2 strains were responsible for several outbreaks in European countries (e.g. Austria, Italy, Greece, Romania, etc.) in both human and animal populations [6–7]. Furthermore, the largest European WNV epidemic in 2018 was also caused by lineage 2 strains [8–9]. The different clades and clusters of lineage 2 in Europe show geographic clustering due to the independent introduction from Africa via migratory birds and the local genetic evolution of viral strains [6]. The wide diversity of WNV strains requires continuous monitoring and accurate molecular typing of genomic sequences. This study aimed to sequence or re-sequence WNV strains between 2015 and 2022 to obtain a detailed phylogenetic analysis of currently circulating variants in Hungary. The geographical clustering of the sequences and the cumulative WNV incidence data were also compared.

METHODS

A detailed description of the epidemiological surveillance and data collection, as well as the laboratory diagnostic algorithm, was previously published elsewhere [2].

Laboratory procedure

Virus isolation from human WNV PCR-positive clinical specimens (serum, plasma, or urine) was performed on Vero E6 cell lines and/or with intracranial inoculation of susceptible suckling mice, following our protocol, available at protocols.io website; doi: dx.doi.org/10.17504/protocols.io.14egn2e7pg5d/v1. The infective titer of virus isolates was also calculated using Vero E6 cell culture. Human clinical samples with insufficient viral load (real-time RT-PCR *Cycle threshold* (Ct) value >35.00) were excluded from the study. Altogether, 15 WNV isolates (14 cell culture supernatant and 1 mouse brain homogenate) and 15 PCR-positive clinical specimens (5 whole blood and 10 urine samples) were used for NGS. Amplicon-based whole genome sequencing of WNV PCR-positive clinical specimens and virus isolates was carried out to characterize the WNV genetic variants. For nucleic acid extraction, Zymo Quick-RNA Miniprep Kit (Zymo Research Corp, Irvine, CA) was used, with on-column DNAse I digestion, according to the manufacturer's instructions. The concentration of residual cellular genomic DNA was measured by Qubit™ 1x dsDNA High Sensitivity (HS) Kit on the Qubit Flex Fluorometer (Invitrogen™, Waltham, MA). The whole WNV genome was amplified in twelve overlapping amplicons by one-step RT-PCR. A detailed description of our *in-house* developed one-step RT-PCR protocol is available at the protocols.io; doi: dx.doi.org/10.17504/protocols.io.e6nvwjopdlmk/v1. For post-PCR size selection clean-up, Agencourt AMPure XP (Beckman Coulter Inc, Brea, CA) reagent was used. DNA amplicons of each sample were pooled in equimolar concentration and amplicon pools were further used for DNA library preparation using the Nextera XT DNA Library Preparation Kit (Reference Guide 15031942v06; Illumina, Inc. San Diego, CA). Sequencing was carried out on the Illumina MiSeq system with MiSeq Reagent Micro kit, v2, 300 cycles (MS-103-1002) chemistry (Illumina, Inc. San Diego, CA).

Sequencing data analysis

For quality control of the Illumina short reads, Genome Detective Virus Tool version 2.41 was used. Genome assembly was done by *mapping to the reference* method (GenBank accession number of the reference genome: KC496015.1), using UNIPRO UGENE software version 38.1 [10], and for pairwise alignment of the genomic sequences we used the Basic Local Alignment Search Tool (BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi). All the sequences were submitted to the NCBI GenBank database (National Center for Biotechnology Information; https://www.ncbi.nlm.nih.gov/genbank/). For phylogenetic analysis, multiple sequence alignment was performed by MAFFT version 7 (Multiple Alignment using Fast Fourier Transform: https://mafft.cbrc.jp/alignment/server/). The aligned sequences were edited by GeneDoc software version 2.7. We constructed a maximum likelihood phylogenetic tree using the Tamura-Nei model with 1000 bootstrap replicates. For phylogeny, we used MEGA11 (Molecular Evolutionary

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Genetics Analysis) software. The phylogenetic tree was visualized using FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

To visualize the geographic distribution of the cumulative incidence data and the geographic location of WNV strains ArcGIS® software by Esri (ArcGIS desktop 10.8. version 10.8.0.12790, ESRI: Environmental Systems Research Institute, Redlands, CA.) was used.

RESULTS

Summary of the epidemiological data

Between 2004 and 2022, altogether 499 human WNF and WNND cases were diagnosed and reported by the NCPH, Hungary (Fig. 1), including 483 autochthonous and 16 imported infections.

To date, the highest peak in case numbers was detected in 2018 (225), during the largest WNV epidemic in Europe, while in 2020 (3) and 2021 (8), a significant decrease could be observed in the number of reported WNF and WNND cases (Fig. 1). This trend coincides with the COVID-19 pandemic, which posed a significant burden on the healthcare system, resulting in a reduced number of samples sent to the reference laboratory of the NCPH for diagnosis of zoonotic infections. The clinical and laboratory diagnostics of infectious agents other than SARS-CoV-2, including WNV remained underrepresented during this period, probably as an indirect effect of the pandemic.

Overall, the cumulative incidence (laboratory-diagnosed WNF and WNND cases per 100,000 inhabitants) was 4.88 in the average population between 2004 and 2022. Considering the geographical distribution of the cumulative incidence rates at NUTS 3 (Nomenclature of Territorial Units for Statistics level 3) Hajdú-Bihar county (12.70) proved to be the most affected area, followed by Békés (11.06), Csongrád-Csanád (10.40), Jász-Nagykun-Szolnok (9.01) and Fejér (8.04) counties. The lowest incidence rate was measured in Zala county (0.35). Overall, the Eastern and Southeastern parts of the country are highly endemic for WNV, while in the Western counties, moderate or low incidence rates were observed. This tendency is also supported by seroprevalence data, obtained from a nationwide WNV serosurvey of blood donors in Hungary, in 2019 [11]. Table 1 summarizes the geographic distribution of the total number of cases and the cumulative incidence rates at the NUTS 3 level in Hungary.

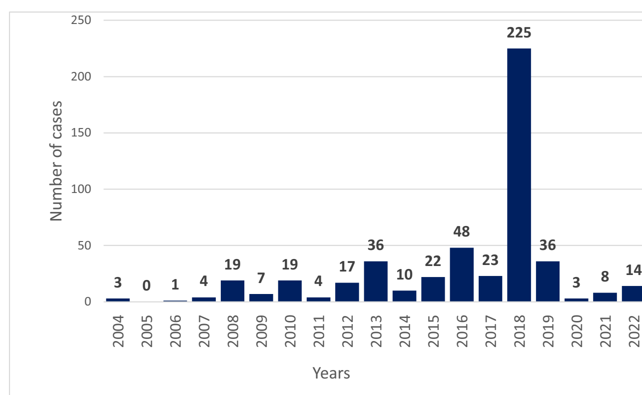

Fig. 1. The annual number of laboratory-diagnosed WNF and WNND cases in Hungary, between 2004 and 2022

Phylogenetic analysis

Between 2015 and 2018 altogether 15 virus isolates were available for sequencing. The number of WNV PCR-positive clinical specimens was more limited, as between 2018 and 2022 only 10 samples were suitable for direct whole genome sequencing. Considering the high potential of the adaptation of the viruses to the cell cultures and the permanent change in the nucleotide sequence of the viral RNA resulting from virus replication, only virus isolates with *passage 0* ($n = 13$ isolates) or *passage 1* ($n = 2$ isolates) were used for the NGS. Horizontal coverage of the consensus whole genomes compared to the reference genome was proved to be 99.2–99.8% in 24 samples, and 95.1% in one urine sample (GenBank accession number: OK239667). The depth of genome coverage varied between 2,782 and 13,597 (mean: 7,660). All relevant data are summarized in the supplementary document. Lineage 2 WNV was identified in all samples, according to our previous results based on the Sanger sequencing method, although whole genome sequencing enabled a more detailed analysis of the circulating virus strains of the country [8]. According to the maximum likelihood phylogenetic tree (Fig. 2), lineage 2 WNV strains form two main clades, the *Central European (*or *Hungarian) clade* and the *Eastern European* (or *Russian) clade*. Only one Hungarian sample (isolated in the Eastern part of the country in 2017) belongs to the Eastern European clade (OK239669; Fig. 2: highlighted in blue). The nucleotide sequence of this strain differs in only 35 nucleotides (99% nucleotide sequence identity) from a Romanian sequence (KJ934710). Most of the samples ($n = 24$) belong to the Central European clade, however, this clade seems to be substantially diverse (Fig. 2). Within the Central European clade, three main clusters can be further separated into the Central European, the Southwest European, and the Balkan clusters (Fig. 2). Hungarian samples collected between 2015 and 2018 form a separate monophyletic group in the phylogenetic tree (highlighted in yellowish green in Fig. 2). This monophyletic group is further defined as the Hungarian cluster within the Central European (Hungarian) clade. Furthermore, other sequences of the Central European cluster can be divided into smaller groups. A WNV PCR-positive urine sample from 2014 (KT359349; Fig. 2: highlighted in dark green) shows high (>99%) nucleotide sequence similarity with a Serbian WNV strain (KC496016 Novi Sad, Serbia; 2010). KT359349 was obtained from an imported WNV case, as the patient traveled to Serbia during the incubation period. Sequence analysis of this WNV strain has already been described in our previously published study [12].

When considering the geographical distribution of the WNV strains, the location of WNV exposure has been taken

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Table 1. Geographic distribution of the cumulative number of WNV cases and the cumulative incidence rates between 2004 and 2022, at the NUTS 3 level of Hungary. (CI: Confidence interval, WNV: West Nile virus) Incidence rates are marked with the same color scale as in Fig. 3

No.	Statistical Regions (NUTS 3)	Average population number between 2004 and 2021[a]	Cumulative number of autochthonous WNV cases between 2004 and 2022	Cumulative incidence with 95% CI
1	Budapest	1 729 204	50	2.89 (2.15 – 3.81)
2	Pest	1 216 235	62	5.10 (3.91 – 6.53)
3	Fejér	423 100	34	8.04 (5.57 – 11.23)
4	Komárom-Esztergom	306 661	10	3.26 (1.56 – 6.00)
5	Veszprém	353 676	10	2.83 (1.35 – 5.21)
6	Győr-Moson-Sopron	450 866	8	1.77 (0.76 – 3.50)
7	Vas	258 055	5	1.94 (0.62 – 4.54)
8	Zala	283 132	1	0.35 (0.00 – 2.00)
9	Baranya	382 143	4	1.05 (0.27 – 2.69)
10	Somogy	316 448	10	3.16 (1.51 – 5.82)
11	Tolna	231 106	5	2.16 (0.69 – 5.06)
12	Borsod-Abaúj-Zemplén	685 860	15	2.19 (1.22 – 3.61)
13	Heves	308 112	16	5.19 (2.96 – 8.44)
14	Nógrád	202 341	2	0.99 (0.10 – 3.60)
15	Hajdú-Bihar	519 763	66	12.70 (9.82 – 16.15)
16	Jász-Nagykun-Szolnok	388 511	35	9.01 (6.28 – 12.53)
17	Szabolcs-Szatmár-Bereg	565 310	33	5.84 (4.02 – 8.20)
18	Bács-Kiskun	522 178	34	6.51 (4.51 – 9.10)
19	Békés	361 606	40	11.06 (7.90 – 15.06)
20	Csongrád-Csanád	413 497	43	10.40 (7.53 – 14.01)
TOTAL:		9 899 191	483	4.88 (4.45 – 5.33)

[a]According to the Hungarian Central Statistical Office, regional population data are available until 2021.

into account. In one case (OK129334) the location of the WNV exposure could not be precisely determined (only at NUTS 3 level), therefore this sample was omitted from the map. Figure 3 represents the geographical distribution of the WNV sequences, as well as the geographical distribution of the cumulative incidence between 2004 and 2022. Our sequence data represent mainly the Central, Eastern, and Southeast parts of Hungary, as lower incidence rates were measured in the Western part of the country, resulting in the lack of cases and PCR-positive samples from this region (Fig. 3). Based on our data, a characteristic geographical distribution of WNV strains, that belong to different clusters can be established. Virus strains of the Balkan cluster (Fig. 3, symbolized by green asterisks) are located in the Eastern and Southeast parts of Hungary, while Central European WNV strains are distributed in the Central and Southern counties (Fig. 3). The virus strain that belongs to the Eastern European (Russian) clade originated from an East Central county of Hungary, namely Jász-Nagykun-Szolnok (Fig. 3, marked with a blue asterisk). This strain was isolated in 2017 from a human urine sample of an autochthonous case of WNV infection.

DISCUSSION

The main limitation of our retrospective study was the available number of suitable PCR-positive human samples and virus isolates. Besides environmental and ecological factors, the number of diagnosed WNV cases also depends on the number of samples received by the NRL for Viral Zoonoses for analysis. During the COVID-19 pandemic, the number of tested samples for WNV decreased, thereby limiting the number of PCR-positive samples and sequencing between 2020 and 2021. The time period between sampling and symptom onset, sample storage, and shipment conditions also affect the integrity of viral RNA and the detection of viral nucleic acid [13]. As human WNV infections can be characterized by a low level of *viremia*, in many cases WNV load is insufficient for virus isolation on cell cultures or for the amplification of the whole viral genome. Anticoagulant-treated whole blood and urine samples are the most suitable sample types for WNV nucleic acid detection [14–15]. In our study, whole genome amplification was successful from nine urine samples, and one whole blood (real-time PCR Ct values for each sample are available in the supplementary document). To date, according to our results, urine proved to be the most suitable sample type for virus isolation, and it may be more suitable for whole genome amplification and sequencing as well. However, a further comparative examination of more clinical specimens is needed.

Prior to 2004 only lineage 1 WNV strains were detected in Europe, while in 2004 WNV lineage 2 was first identified outside of Africa [7]. The virus was isolated from the brain

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Central European cluster
Southwest European cluster
Hungarian cluster
Smaller clusters of Central European lineage 2
Balkan cluster
Smaller group of Serbian/Balkan cluster
Eastern European clade

LR743426-1_Germany_2019
LR743435-1_Germany_2019
LR743433-1_Germany_2019
LR743455-1_Germany_2019
LR743429-1_Germany_2019
LR743436-1_Germany_2019
MW142227-1_Leipzig_Germany_2020
LR743458-1_Germany_2019
OK239660-1_Budapest_Hungary_2018
OK239667-1_Kiskunmajsa_Hungary_2018
OK239671-1_Újtelek_Hungary_2018
OK129334-1_Hajdú-Bihar county_Hungary_2017
OK239664-1_Dunaújváros_Hungary_2018
OK129336-1_Tahitótfalu_Hungary_2017
OK239668-1_Jászberény_Hungary_2018
OK129335-1_Martfű_Hungary_2017
KM203862-1_Lednice_Czech Republic_2013
KM203861-1_Mikulov_Czech Republic_2013
KP780837-1_Austria_2008
MF984344-1_Austria_2015
MH244512-1_Velky Biel_Slovakia_2013
KP789960-1_Mantova_Italy_2013
KF647249-1_Rovigo_Italy_2013
MT863560-1_France_2018
MT863561-1_France_2018
MN939564-1_Rovigo_Italy_2016
MN939563-1_Verona_Italy_2018
KP789956-1_Verona_Italy_2014
OM037670-1_Lerida_Spain_2017
OM037671-1_Lerida_Spain_2020
OM037672-1_Tarragona_Spain_2020
OM037673-1_Tarragona_Spain_2020
OK129330-1_Kisújszállás_Hungary_2016
OK129329-1_Enying_Hungary_2016
OK129332-1_Dunaújváros_Hungary_2015
OK129331-1_Mátyásdomb_Hungary_2015
OK239659-1_Kecskemét_Hungary_2018
OK239662-1_Kiskunhalas_Hungary_2018
OK129333-1_Szentmártonkáta_Hungary_2017
MH244513-1_Secovce_Slovakia_2013
KM203860-1_Mikulov_Czech Republic_2013
KM203863-1_Lednice_Czech Republic_2013
KF179640-1_Austria_2008
OP179288-1_Budapest_Hungary_2021
LR743454-1_Germany_2019
MN481597-1_Imathia_Greece_2012
MN481595-1_Thessaloniki_Greece_2010
KY594040-1_Greece_2010
HQ537483-1_Nea Santa_Greece_2010
KJ883349-1_Xanthi_Greece_2013
KC496015-1_Hungary_2010
KT757323-1_Vojvodina_Serbia_2013
KT757318-1_Vojvodina_Serbia_2013
KU206781-1_Sofia_Bulgaria_2015
OK239661-1_Orosháza_Hungary_2018
OK239663-1_Nyíregyháza_Hungary_2019
OK239672-1_Tiszalúc_Hungary_2018
MT341472-1_Pleven_Bulgaria_2018
MN480793-1_Pella_Greece_2018
OP179287-1_Szarvas_Hungary_2021
MN480794-1_Thessaloniki_Greece_2018
OK239665-1_Debrecen_Hungary_2018
MH021189-1_Hungary (Antwerpen, Belgium)_2017
OK239666-1_Békéscsaba_Hungary_2018
MK947397-1_Slovenia_2018
MW561633-1_Kavecany_Slovakia_2018
OK239658-1_Hajdúszoboszló_Hungary_2018
OK239670-1_Hajdúszoboszló_Hungary_2018
KT359349-1_Hungary_2014
KC496016-1_Novi Sad_Serbia_2010
MW383508-1_Zambezi region_Namibia_2020
KY523178-1_Queen Elizabeth National Park_Uganda_2009
JN393308-1_Tiegerpoort_Pretoria_South Africa_2008
EF429197-1_South Africa_1989
EF429198-1_South Africa_2001
DQ318019-1_Senegal
DQ318020-1_Central African Republic
EF429200-1_South Africa_1958
EF429199-1_South Africa_2000
KM052152-1_South Africa_1977
MH939154-1_Danube Delta_Romania_2014
OK239669-1_Jászkisér_Hungary_2017
KJ934710-1_Enisala_Romania_2013
MH939153-1_Danube Delta_Romania_2014
HM147824-1_Republic of the Congo_1958
HM147823-1_Madagascar_1988
AY688948-1_Israel
GQ903680-1_Cyprus_1968
HM147822-1_South Africa_1958
MW383507-1_Zambezi region_Namibia_2020
MZ605382-3_Hungary_2004
MF797870-1_Cyprus_2016
MT863559-1_France_2015
EF206350-1_Hungary_2005

Central European/Hungarian Clade
Eastern European/Russian Clade

3.0

Fig. 2. Maximum likelihood phylogenetic tree of the lineage 2 WNV sequences. (Accession numbers and names of the Hungarian samples are written in red.) Sequence data are defined as follows: GenBank accession number, the geographical location where the virus was isolated or detected, and the year of isolation or detection

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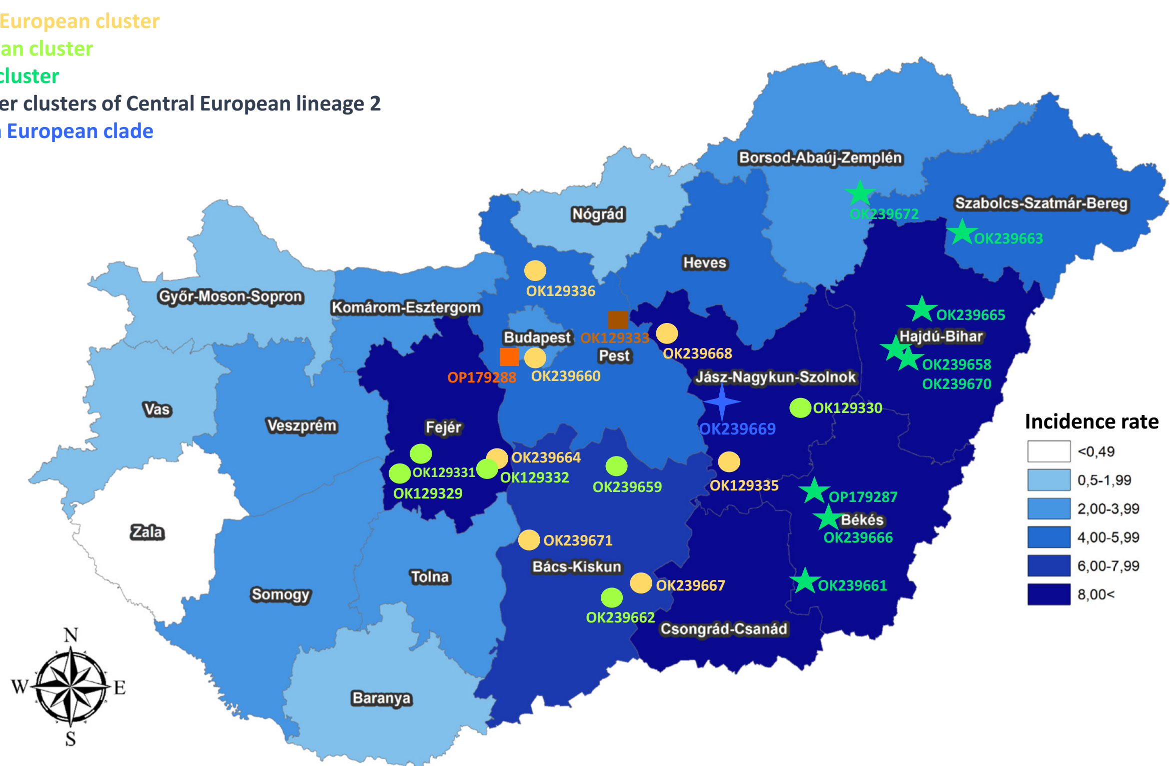

Fig. 3. Geographical distribution of the Hungarian WNV strains and the geographic distribution of the cumulative incidence rate of WNF and WNND cases between 2004 and 2022. (Geographical location of strains belonging to different clusters is marked with the same colors as clusters in Fig. 2.)

of a goshawk *(Accipiter gentilis)* in Hungary [7]. After its first detection, an explosive spread of lineage 2 strains could be observed across Europe, resulting in local epidemics in Greece, Italy, and Austria. Furthermore, lineage 2 WNV strains have already been detected in France, Spain, and the northern parts of Europe, such as the Czech Republic, Germany, and the Netherlands [6, 16]. Following its geographical clustering, Southwest, Balkan, and Central European clusters could be distinguished within the Central European (Hungarian) clade (Fig. 2).

Among the sequenced strains between 2015 and 2022, only one Hungarian WNV strain belongs to the Eastern European (Russian) clade (OK239669; Fig. 2). Regardless of the appearance of Hungarian WNV lineage 2 in 2004, another lineage 2 strain with African origin (GenBank accession number: FJ425721.1) was recorded in Volgograd; Southern Russia, in 2007 [17]. Subsequently, in 2010, similar lineage 2 strains caused a large WNND epidemic in Romania and since then, these strains became endemic in Romania and Russia, forming the Eastern European (Russian) clade of WNV lineage 2 [17]. OK239669 has 99.68% nucleotide sequence homology with a WNV strain detected in a *Hyalomma marginatum marginatum* tick collected from a song thrush *(Turdus philomelos)*, in the Danube delta; Eastern Romania in 2013 (GenBank Accession number: KJ934710; Fig. 2) [17]. Phylogenetically closely related WNV strains were recorded in Israel, Iran, and Italy [18–19]. In addition, during the 2018 epidemic, Eastern European lineage 2 WNV was detected in a 72-year-old female WNND patient in Greece, however, the other WNV sequences from 2018 were similar to strains found in previous years [18]. Overall, based on our current knowledge, the detection of Eastern European lineage 2 WNV sequences is uncommon in Central Europe.

Phylogenetic analysis revealed that virus isolates from the 2018 WNV epidemic belonged to different clusters within the Central European clade (Fig. 2). The fact that these samples do not form a single monophyletic group suggests that the 2018 European outbreak was rather associated with the impact of certain environmental factors, than with the emergence of a novel genetic variant. These results are consistent with our previously published study, based on partial genomic sequence analysis [8]. Since 2003, no lineage 1 WNV has been detected in Hungary, neither in humans nor in animals, although, at the same time, a high degree of genetic heterogeneity of lineage 2 strains has been observed. In conclusion, our phylogenetic analysis demonstrates the co-circulation of different WNV lineage 2 clusters in Hungary with characteristic geographical distribution. However, any possible differences in the virulence of these strains are currently not clear and require further investigations, such as plaque size assays or *in vivo* studies of pathogenicity in vertebrate hosts. The geographical distribution of different genetic clusters of WNV lineage 2 might be related to the bird migration routes and breeding sites. In Hungary, there are many important bird areas (IBA), that

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provide favorable habitats for one or more bird species, including sites for breeding, wintering, or routes of migration. According to the Hungarian Bird Watching Association's website (https://www.hungarianbirdwatching.com/), there are 54 IBAs, covering 1.4 million hectares (representing 15.1% of the territory of Hungary). Some of the most important birding hotspots are localized in the Eastern and Southeastern parts of the country, such as Jász-Nagykun-Szolnok and Hajdú-Bihar counties, which are characterized by high WNV incidence rates (Table 1 and Fig. 3). It can be hypothesized, that besides the genetic evolution of the local strains in a given geographic area, the diversity of WNV in Central Europe is associated with the independent introduction and continuous fluctuation of viral variants between Africa and Europe, triggered by migratory species.

Our study is limited to the sequencing of WNV PCR-positive human samples or virus isolates derived from human clinical specimens, as the NRL for Viral Zoonoses of the NCPH, Hungary is responsible for the microbiological diagnosis of human infections. In Hungary, a passive equine and avian surveillance system exists and animal cases (e.g. equine encephalomyelitis due to WNV) are notified by the Veterinary Diagnostic Directorate of the National Food Chain Safety Office and regularly reported to the European Animal Disease Notification System (ADNS) of the European Commission. A country-wide mosquito surveillance system is still not in operation in Hungary, however real-time monitoring of native and invasive mosquito species, based on citizen science and local trapping is being developed within the One Health concept. In light of the 2018 WNV outbreak in Europe, the European Centre for Disease Prevention and Control anticipates the need for multi-country outbreak investigations through sequence-based typing. Further findings in different European countries also strengthen the need for such an approach: In 2019 a death of a little grebe *(Tachybaptus ruficollis subsp. ruficollis)* in Italy was associated with WNV lineage 2 infection [20]. Furthermore, in January 2022, WNV lineage 2 was also detected in a female goshawk rescued in the region of Umbria [20]. WNV circulation has never been reported in this territory before 2019 [20]. The westward spread of lineage 2 WNV was also evidenced, as WNV lineage 2 was molecularly detected in 2017, for the first time in Catalonia, Spain, away from the lineage 1-affected areas of the country [21]. In 2020, WNV lineage 2, belonging to the Southwest European cluster within the Central European (Hungarian) clade was detected again in northern goshawks with clinical signs compatible with WNV infection, highlighting the spread of WNV lineage 2 from the central regions of Europe to the west [21]. Due to the emerging number of human and animal cases and the increasing geographical spread of lineage 2 strains, WNV should be kept under extensive surveillance, including whole genome sequencing of viral variants, following the One-Health approach. Based on previous findings, it seems that Hungary has an essential role in the dissemination of WNV strains in Europe, thus it is important to enhance the collection of whole genome sequencing data from this region of Europe. An increase in the number of tested human clinical samples and diagnosed cases may help to obtain even more data regarding the circulating virus variants. Besides whole blood, the collection of urine samples can highly support virus isolation, viral RNA detection, and molecular typing.

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Ethical statement: Ethical approval and special permission was not needed for this study as it was performed as part of the routine diagnostic and surveillance activities.

Data availability: All relevant data are within the manuscript and its Supporting Information files.

Conflict of interest: None declared.

Authors' contributions: AN, MT conceptualized the study, AH, AN, NM, and CSN performed laboratory experiments, AH, AN interpreted the laboratory results and performed bioinformatics analysis, JH helped in NGS data analysis and reviewed the results, EM provided epidemiological data and prepared Fig. 3, ON, AK, MT reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

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REFERENCES

1. Hayes EB, Sejvar JJ, Zaki SR, Lanciotti RS, Bode AV, Campbell GL. Virology, pathology, and clinical manifestations of West Nile virus disease. Emerg Infect Dis 2005; 11(8): 1174–9. http://doi.org/10.3201/eid1108.050289b.
2. Nagy A, Mezei E, Nagy O, Bakonyi T, Csonka N, Kaposi M, et al. Extraordinary increase in West Nile virus cases and first confirmed human Usutu virus infection in Hungary, 2018. Euros Surveill 2019; 24(28): 1–9. http://doi.org/10.2807/1560-7917.ES.2019.24.28.1900038.
3. European Commission. Commission Implementing Decision 2012/506/EU of 8 August 2012 amending Decision 2002/253/EC laying down case definitions for reporting communicable diseases to the Community network under Decision No 2119/98/EC of the European Parliament and of the Council (notified under document C(2012) 5538). Luxembourg: Publication Office of the European Union; 2012. Available from: https://op.europa.eu/en/publication-

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detail/-/publication/10ed460f-0711-11e2-8e28-01aa75ed71a1/language-en (Accessed: 27 March 2023).

4. Barzon L, Pacenti M, Ulbert S, Palù G. Latest developments and challenges in the diagnosis of human West Nile virus infection. Expert Rev Anti Infect Ther 2015; 13(3): 327–42. https://doi.org/10.1586/14787210.2015.1007044.
5. Bakonyi T, Ivanics É, Erdélyi K, Ursu K, Ferenczi E, Weissenböck H, et al. Lineage 1 and 2 strains of encephalitic West Nile virus, Central Europe. Emerg Infect Dis 2006; 12(4): 618–23. https://doi.org/10.3201/eid1204.051379.
6. Chaintoutis SC, Papa A, Pervanidou D, Dovas CI. Evolutionary dynamics of lineage 2 West Nile virus in Europe, 2004–2018: phylogeny, selection pressure and phylogeography. Mol Phylogenet Evol 2019; 141: 106617. https://doi.org/10.1016/j.ympev.2019.106617.
7. Bakonyi T, Ferenczi E, Erdélyi K, Kutasi O, Csörgo T, Seidel B, et al. Explosive spread of a neuroinvasive lineage 2 West Nile virus in Central Europe, 2008/2009. Vet Microbiol 2013; 165(1–2): 61–70. https://doi.org/10.1016/j.vetmic.2013.03.005.
8. Zana B, Erdélyi K, Nagy A, Mezei E, Nagy O, Takács M, et al. Multi-approach investigation regarding the West Nile virus situation in Hungary, 2018. Viruses 2020; 12(1): 1–12. https://doi.org/10.3390/v12010123.
9. Barzon L, Montarsi F, Quaranta E, Monne I, Pacenti M, Michelutti A, et al. Early start of seasonal transmission and co-circulation of West Nile virus lineage 2 and a newly introduced lineage 1 strain, northern Italy, June 2022. Euro Surveill 2022; 27(29): 1–6. https://doi.org/10.2807/1560-7917.ES.2022.27.29.2200548.
10. Okonechnikov K, Golosova O, Fursov M, UGENE team. Unipro UGENE: a unified bioinformatics toolkit. Bioinformatics 2012; 15; 28(8): 1166–7. https://doi.org/10.1093/bioinformatics/bts091.
11. Nagy A, Csonka N, Takács M, Mezei E, Barabás É. West Nile and Usutu virus seroprevalence in Hungary: a nationwide serosurvey among blood donors in 2019. PLoS One 2022; 17(4): 1–12. https://doi.org/10.1371/journal.pone.0266840.
12. Nagy A, Bán E, Nagy O, Ferenczi E, Farkas Á, Bányai K, et al. Detection and sequencing of West Nile virus RNA from human urine and serum samples during the 2014 seasonal period. Arch Virol 2016; 161(7): 1797–806. https://doi.org/10.1007/s00705-016-2844-5.
13. Papa A, Bakonyi T, Xanthopoulou K, Vázquez A, Tenorio A, Nowotny N. Genetic characterization of west nile virus lineage 2, Greece, 2010. Emerg Infect Dis 2011; 17(5): 920–2. https://doi.org/10.3201/eid1705.101759.
14. Lustig Y, Mannasse B, Koren R, Katz-Likvornik S, Hindiyeh M, Mandelboim M, et al. Superiority of west nile virus RNA detection in whole blood for diagnosis of acute infection. J Clin Microbiol 2016; 54(9): 2294–7. https://doi.org/10.1128/JCM.01283-16.
15. Pacenti M, Sinigaglia A, Franchin E, Pagni S, Lavezzo E, Montarsi F, et al. Human West Nile virus lineage 2 infection: epidemiological, clinical, and virological findings. Viruses 2020; 12(4): 458. https://doi.org/10.3390/v12040458.
16. Bergmann F, Trachsel DS, Stoeckle SD, Bernis Sierra J, Lübke S, Groschup MH, et al. Seroepidemiological survey of West Nile virus infections in horses from Berlin/Brandenburg and North Rhine-Westphalia, Germany. Viruses 2022; 14(2): 243. https://doi.org/10.3390/v14020243.
17. Kolodziejek J, Marinov M, Kiss BJ, Alexe V, Nowotny N. The complete sequence of a West Nile virus lineage 2 strain detected in a *Hyalomma marginatum marginatum* tick collected from a song thrush (*Turdus philomelos*) in Eastern Romania in 2013 revealed closest genetic relationship to strain Volgograd 2007. PLoS One 2014; 9(10), e109905. https://doi.org/10.1371/journal.pone.0109905.
18. Papa A, Papadopoulou E, Chatzixanthouliou C, Glouftsios P, Pappa S, Pervanidou D, et al. Emergence of West Nile virus lineage 2 belonging to the Eastern European subclade, Greece. Arch Virol 2019; 164(6): 1673–5. https://doi.org/10.1007/s00705-019-04243-8.
19. Ravagnan S, Montarsi F, Cazzin S, Porcellato E, Russo F, Palei M, et al. First report outside Eastern Europe of West Nile virus lineage 2 related to the Volgograd 2007 strain, northeastern Italy, 2014. Parasites and Vectors 2015; 8(1): 1–5. https://doi.org/10.1186/s13071-015-1031-y.
20. Mencattelli G, Iapaolo F, Polci A, Marcacci M, Di Gennaro A, Teodori L, et al. West Nile virus lineage 2 overwintering in Italy. Trop Med Infect Dis 2022; 7(8): 160. https://doi.org/10.3390/tropicalmed7080160.
21. Aguilera-Sepúlveda P, Napp S, Llorente F, Solano-Manrique C, Molina-López R, Obón E, et al. West Nile virus lineage 2 spreads westwards in Europe and overwinters in North-Eastern Spain (2017–2020). Viruses 2022; 14(3): 569. https://doi.org/10.3390/v14030569.

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