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First autochthonous transmission of West Nile virus (WNV) lineage 2 to humans in Spain

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ABSTRACT

West Nile Virus (WNV) lineage 2, primarily endemic to parts of Africa and Europe, has recently emerged as a public health concern in new geographic regions. In 2024, the first autochthonous human case of neuroinvasive disease caused by WNV lineage 2 was identified in Andalusia, Southern Spain. Molecular testing and whole-genome sequencing confirmed WNV lineage 2 as the causative agent. Phylogenetic analysis revealed a close relationship with strains circulating in Central Europe, distinct from previous WNV lineage 2 detections in Spain. Concurrently, WNV lineage 2 RNA was detected in an imperial eagle near the case location, suggesting local viral circulation. This case marks a significant shift in WNV epidemiology in Spain, where lineage 1 has historically been dominant. The findings underscore the expanding range of WNV lineage 2 and the necessity for enhanced vector surveillance, genomic monitoring, and strengthened One Health strategies to mitigate future outbreaks and protect public health.

1. Introduction

West Nile virus (WNV), a member of the Flavivirus genus, is transmitted in an enzootic cycle involving birds as amplifying hosts and mosquitoes as vectors [1]. The virus can also infect mammals, considered dead-end hosts, leading to disease outbreaks in humans and/or horses [2]. Currently, WNV is recognized as a recurrent zoonosis with a broad geographic distribution [3]. Phylogenetic analysis classifies WNV into eight lineages [4], although lineages 1 and 2 are considered the most pathogenic strains [5]. Evidence of WNV circulation in Europe dates back to the 1950s [6], with the first documented human outbreak occurring in 1962 in southern France [7]. Until 2004, lineage 1 was primarily associated with outbreaks in humans and horses in Europe [8]. However, in 2004, lineage 2 was identified for the first time in Hungary [9], coinciding with an independent introduction in southern Russia, from which it subsequently spread [10]. By 2008, lineage 2 had expanded into Central Europe, Russia and the Eastern Mediterranean basin where it has since become endemic [11]. Both lineages now coexist in countries such as Italy, where annual outbreaks involving lineages 1 and 2 have been reported since 2008 [12,13], as well as in Cyprus by 2016 [14] and other countries like Greece and Serbia [15]. These observations demonstrate a clear pattern of expansion of WNV in Europe [16], likely facilitated by global climate change [17], as well as by the recently questioned dead-end host status of the infected mammals [18,19].

However, in Spain only WNV lineage 1 has been responsible for the different outbreaks in humans that occurred in 2008, 2010, 2016 and 2020 [20–23], also detected in mosquitos [24]. Although the presence of lineage 2 was detected for the first time in 2017 in birds in Catalonia (Northeastern Spain) by passive surveillance [25], and an imported case was described in a patient from Romania in 2018 [26], no transmission of WNV lineage 2 to humans has been reported in Spain since then. The

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first human case of neuroinvasive disease caused by WNV lineage 2 through autochthonous transmission has been reported in September 2024. The case was infected in a region (Jaén, Andalucía, Southeastern Spain) where no evidence of WNV circulation had been observed in previous years until this same season. In particular, the first focus of WNV lineage 2 has been declared in an imperial eagle (wild bird) near the location of the case, whose sampling date was at the end of July 2024, according to what was reported by the Animal Health Service Ministry of Agriculture, Fisheries, Water and Rural Development of Andalusia [27].

Here we provide a detailed molecular characterization of WNV lineage 2, isolated from the first locally transmitted human case in Spain.

2. Materials and methods

2.1. Samples, molecular diagnosis and culture isolation

Following the Andalusian Protocol for WNF surveillance and alert [27], West Nile virus tests were requested for a human case reported in September 2024, in the province of Jaen (Andalusia, Southern Spain). Serum, whole blood, urine and CSF samples were sent to the Andalusia Virus Reference Laboratory.

The following diagnostic tests were performed in order to confirm WNV infection [27–29]: detection of virus-specific IgM and IgG antibodies in serum and/or cerebrospinal fluid (CSF) by ELISA testing (Euroimmun,Lübeck, Germany) and detection of specific viral nucleic acids in CSF, serum and/or urine. Nucleic acid extraction from clinical samples was performed by using QIAsymphony DSP virus/pathogen mini kit (Qiagen, Hilden, Germany). Real time reverse transcription polymerase chain reaction (qRT-PCR) targeting a conserved sequence of the 3′-untranslated (3'UTR) region of WNV genome was used to confirm the presence of specific viral RNA [30] in urine, serum and whole blood samples. Molecular diagnosis could not be performed in CSF due to insufficient sample volume. Positive samples were subjected to a one-step qRT-PCR that allows differentiation between lineage 1 and 2 [31]. For virus isolation, all of the procedures were performed within certified biosafety cabinets under biosafety level 3 (BSL3) containment. WNV RNA-positive samples were inoculated onto confluent monolayers of Vero cells. Passage to fresh Vero cell tubes was performed after 10 days of incubation or when cytopathic effect was observed [32,33]. Viral growth was confirmed by qRT-PCR [30] of the cell culture supernatant and viral culture was considered negative after 2 passages without evidence of CPE and negative qRT-PCR of the supernatant.

2.2. Viral sequencing

Purified RNA from WNV isolate was used for sequencing. LunaScript® RT SuperMix Kit (New England Biolab, Ipswich, MA, USA) was used for cDNA synthesis with 15 µl of RNA following the instructions of the manufacturer. To perform multiplex PCR, Q5® Hot Start High-Fidelity (New England Biolabs) protocol was followed by adding primers previously described [34].

After the PCR performed with a set of 18 primer pairs, the amplified regions were purified with Agencourt AMPure XP beads (Beckman Coulter). Library preparation was performed through Illumina DNA Prep kit following manufacturer's recommendation. Samples were pooled in equal concentrations after quantification by Qubit 4 fluorometer (Invitrogen). Sequencing was carried out on a iSeq 100 system using a iSeq 100 i1 Reagent v2 (300-cycle) (Illumina).

2.3. Viral genomic data processing

Sequencing data (150 bp × 2) were analyzed using custom in-house scripts and the nf-core/viralrecon pipeline software (v.2.6.0) [35] (see also [22]). Briefly, after quality filtering of the reads, these were aligned to the lineage 2 WNV genome (OM037673.1), which was recently isolated from birds in northeastern Spain [25], using the *Bowtie 2* (v2.4.4) algorithm [36]. This was followed by the removal of primer sequences and the labeling of duplicate reads. Genomic variants were identified with the *iVar* tool, using a minimum allele frequency threshold of 0.25 for variant calling and applying a filtering step to retain variants with a minimum allele frequency of 0.75. Finally, using the set of high confidence variants and the reference genome, a consensus genome was constructed with *bcftools* (v1.16) [37].

2.4. Phylogenetic analysis

Phylogenetic analysis was performed using the Augur toolkit v.21.0.0 [38]. Multiple alignment was carried out using the strain OM037673 as reference. The phylogenetic tree is recovered by maximum likelihood, using a general time reversible model with unequal rates and unequal base frequencies [39]. Branching date estimation was carried out with the least square dating method using TreeTime v.0.9.4. Branching point reliabilities were estimated by UFBoot [40].

The results can be viewed in the Andalusian Genomic Epidemiology System (SIEGA) [41] local server [42].

3. Results

3.1. Diagnostic tests and culture isolation

IgM antibodies to WNV were detected in CSF and serum samples, with IgG in the latter being indeterminate. The WNV genome was detected in serum, whole blood and urine samples with cycle threshold (ct) values of 36.6, 25.15 and 22.47 respectively. qRT-PCR for differentiation of WNV lineages 1 and 2 was performed on the urine sample with positive result for lineage 2. Urine and whole blood samples were inoculated for viral culture in Vero cells, although WNV was isolated only from the urine sample after one passage.

3.2. Sample sequencing

The sample was processed, sequenced and the resulting sequencing raw data, processed as described in Materials and Methods. The resulting sample exhibited very high quality, with a median depth of 12,757 X and genome coverage of 99.69 %. A total of 82 high-confidence variants were identified by the variant-calling algorithm implemented in the nf-core/viralrecon pipeline, relative to the reference genome (OM037673.1).

The new lineage 2 WNV sequence reported here is available in the European Nucleotide Archive (ENA) database under the project identifier PRJEB43037, sample accession ERS22985467, as well as in the GenBank database under the accession GCA_965119255.1.

A partial sequence of 170 nucleotides of the region NS5 of the WNV isolated from an imperial eagle in the same region and date (PV089519) showed a 100 % similarity with the corresponding sequence of the virus studied here.

3.3. Phylogenetic analysis

A set of 187 whole genomes of WNV lineage 2 and representative sequences from other lineages were found in the GenBank repository (listed in Additional Table 1). All these genomes were downloaded and aligned, together with the Spanish WNV sequence, using the MAFFT program (see Materials and Methods). Fig. 1 shows a detail of the phylogenetic tree of a clade containing the current and previous recent Spanish samples.

A phylogenetic tree including the WNV sequence reported in this study and the 187 WNV sequences listed in Additional Table 1 was reconstructed as described in Materials and Methods (Additional Fig. 1).

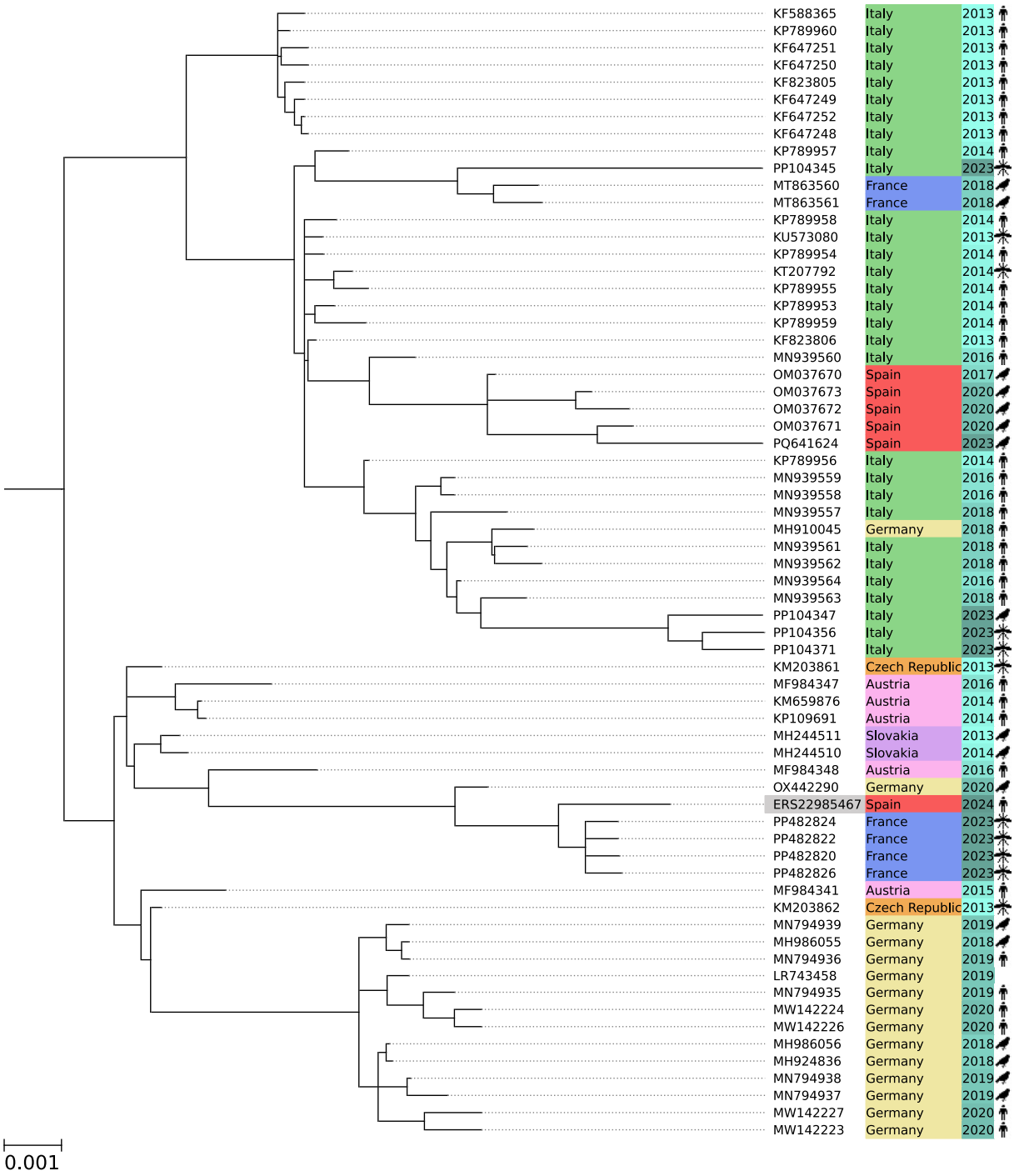

Fig. 1. Detailed phylogenetic tree with the most closely related sequences available.

3.4. The SIEGA Nextstrain server

The Andalusian Genomic Epidemiology System, SIEGA [41], includes a Nextstrain server [42] that offers an interactive view of the complete WNV phylogeny with the WNV sequences reported in this study in the context of some viral sequences of reference (see Additional Table 1). Additional Fig. 1 shows a summarized view of the whole phylogenetic tree as displayed by the SIEGA Nextstrain server [42].

4. Discussion

This study describes the first reported case of neuroinvasive disease caused by autochthonous transmission of WNV lineage 2 in Spain, signaling a concerning milestone in the geographic expansion of this lineage and underscoring the importance of enhanced surveillance and vector control strategies in Spain [11,25]. Most WNV infections in humans are asymptomatic or mild, with neuroinvasive disease being only the tip of the iceberg [43], suggesting intense viral circulation in the region and the existence of many more undetected cases.

However, the phylogenetic analysis (Fig. 1) clearly documents that the sequence obtained in this study was not a direct descendent from previous WNV lineage 2 isolated in Spain from northern goshawk between 2017 and 2023 (sequences OM037670-OM037673 and PQ641624) [44,45]. Actually, these previous isolates were related to the

most recent outbreaks of WNV lineage 2 sequences reported in Europe, which occurred in 2023 in Italy, and had two different phylogenetic origins: one of them, PP104345, is closely related to previous French (2018) and Italian (2014) outbreaks [46], and on the other one, PP104347, PP104356 and PP104371, closely related to previous Italian outbreaks occurring since 2014. Both cases are of Italian origin.

Interestingly, the phylogenetic origin of the genome of this study is closely related (less than 20 nucleotide changes) to a recent French outbreak reported in 2023 (sequences PP482824, PP482822, PP482820 and PP482826) [47] related to another one reported in Germany in 2020 (sequence OX442290). This phylogenetic lineage has origin in previous isolations reported in center Europe, including Austria in 2016 (MF984348) [48], Slovakia (MH244510 and MH244511) [49] and Czech Republic (KM203861) [48] in 2013.

The current and previous Spanish isolates share a common ancestor estimated in 2006–2008, according to the LSD2 method. The Spanish genomes share a common ancestor estimated in 2006–2008 [50], which would correspond to the ancestor of the clade in Fig. 1.

This observation suggests a transcontinental introduction, potentially facilitated by migratory birds, which are recognized as key amplifying hosts for WNV [45,48]. Interestingly, the identified strain exhibited nearly 50 genetic mutations compared to its closest relative from Austria in 2016, reflecting the genetic evolution of WNV during its spread across Europe. These findings are consistent with prior reports of WNV lineage 2 expanding westwards from its initial emergence in Hungary and Russia in the early 2000s [9,10].

Prior WNV outbreaks in Spain have predominantly involved lineage 1 in 2020 and 2021 [22,24], with sporadic detections of lineage 2 in avian populations, between 2017 and 2020 [25,44,45]. The detection of human transmission of lineage 2 in Andalusia suggests a shift in WNV epidemiology in the region. To date, all locally-acquired human cases of the disease in Spain have been caused by WNV lineage 1, mainly in Southwestern Andalusia, with the highest amount of cases reported in the last 2024 season. Notably, Jaén province (Northeastern Andalusia) has reported the first locally acquired human cases, as well as the first documented human case of lineage 2, described in this study.

Factors such as climate change, altered migratory patterns, and increased vector populations may have facilitated this expansion. Similar patterns of co-circulation of WNV lineages 1 and 2 have been observed in Italy and Greece, raising concerns about the potential for larger outbreaks in southern Europe [11,12,17].

The implications of these findings for public health are significant. Firstly, WNV lineage 2, unlike lineage 1, has demonstrated the ability to persist in raptors during winter months when mosquito vectors (e.g., *Culex pipiens*) are typically inactive. A northern goshawk infected with WNV lineage 2 in January 2022 in Umbria, Italy, showed neurological symptoms and tested positive for the virus in multiple organs. This case, along with a similar 2019 infection in a little grebe, confirms year-round circulation of WNV L2 in birds despite low-risk classification of the region [51]. This persistence independent of mosquitoes increases their endemic potential and requires year-round vector control. Also relevant are the known immunological differences between the two WNV lineages. The E protein (DIII domain) contains type- and subtype-specific epitopes critical for neutralizing antibody responses [52]. Thus, the existence of different WNV lineages increases the potentially risk of false negatives or cross-reactivity in diagnostic tests targeting lineage-specific epitopes [53]. Moreover, mutations in the E protein (e.g., DIII lateral ridge) can alter antigenicity, potentially reducing vaccine efficacy if lineage-specific variants are not included [54].

Current surveillance programs in Spain should be expanded to monitor both WNV lineages in avian, mosquito, and human populations [55]. Integrated vector management strategies, including insecticide use, habitat modification, and public awareness campaigns, are urgently needed to reduce the risk of further transmission. Additionally, incorporating real-time genomic epidemiology, such as the SIEGA Nextstrain server, will allow for more rapid detection of emerging strains and their potential origins [41].

This case also highlights the value of a One Health approach, which integrates human, animal, and environmental health to combat zoonotic diseases like WNV. Collaboration between clinicians, epidemiologists, veterinarians, and public health officials will be essential to mitigate the threat posed by WNV lineage 2 in Spain and beyond [11,56].

In conclusion, this study provides critical insights into the emergence and genetic characterization of WNV lineage 2 in Spain. Our findings emphasize the importance of proactive surveillance, vector control, and genomic monitoring to prevent further outbreaks, like the Comprehensive Vector Surveillance and Control Program for West Nile Fever in Andalusia [51]. Continued research into the environmental and genetic factors driving WNV spread is essential to anticipate and respond to future public health challenges. This underscores the importance of, genomics-informed, real-time, global pathogen surveillance tools for the control of zoonosis [56], such as the SIEGA initiative [41].

CRediT authorship contribution statement

Maria Lara: Visualization, Investigation, Formal analysis, Data curation. **Carlos S. Casimiro-Soriguer:** Writing – original draft, Methodology, Investigation, Formal analysis. **Irene Pedrosa-Corral:** Investigation. **Cristina Gómez-Camarasa:** Investigation. **Nicola Lorusso:** Resources. **Jose M. Navarro-Marí:** Investigation. **Joaquin Dopazo:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization. **Javier Perez-Florido:** Writing – original draft, Formal analysis, Conceptualization. **Sara Sanbonmatsu-Gámez:** Writing – review & editing, Writing – original draft, Investigation, Conceptualization.

Ethical issues

Viral samples were provided and sequenced in the context of the Andalusian Protocol for WNF surveillance and alert [27]. Only epidemiological data disconnected from the affected individual are used in the study, and no ethical approval was required.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Joaquin Dopazo reports administrative support, article publishing charges, and statistical analysis were provided by Horizon Europe. Joaquin Dopazo reports administrative support and statistical analysis were provided by Carlos III Health Institute. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.

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Data availability

Data is available in the European Nucleotide Archive (ENA) database under the project identifier PRJEB43037, sample accession ERS22985467

References

[1] J.S. Mackenzie, D.J. Gubler, L.R. Petersen, Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses, Nat. Med. 10 (2004) S98–S109, https://doi.org/10.1038/nm1144.

[2] L.D. Kramer, L.M. Styer, G.D. Ebel, A global perspective on the epidemiology of West Nile virus, Annu. Rev. Entomol. 53 (2008) 61–81, https://doi.org/10.1146/annurev.ento.53.103106.093258.

[3] Z. Hubálek, J. Halouzka, West Nile fever–a reemerging mosquito-borne viral disease in Europe, Emerg. Infect. Dis. 5 (1999) 643, https://doi.org/10.3201/eid0505.990505.

[4] G. Fall, N. Di Paola, M. Faye, M. Dia, C.C. de Melo Freire, C. Loucoubar, P.M. de Andrade Zanotto, O. Faye, Biological and phylogenetic characteristics of west African lineages of West Nile virus, PLoS Negl. Trop. Dis. 11 (2017) e0006078, https://doi.org/10.1371/journal.pntd.0006078.

[5] E. Pérez-Ramírez, F. Llorente, J. Del Amo, G. Fall, A. Lubisi, S. Lecollinet, A. Vázquez, M.Á. Jiménez-Clavero, Pathogenicity evaluation of twelve West Nile virus strains belonging to four lineages from five continents in a mouse model: discrimination between three pathogenicity categories, J. Gen. Virol. 98 (2017) 662–670, https://doi.org/10.1099/jgv.0.000743.

[6] V. Bardos, J. Adamcová, S. Dedei, N. Gjini, B. Rosický, A. Simkova, Neutralizing antibodies against some neurotropic viruses determined in human sera in Albania, J. Hyg. Epidemiol. Microbiol. Immunol. 3 (1959) 277–282.

[7] L. Joubert, J. Oudar, C. Hannoun, D. Beytout, B. Corniou, J.C. Guillon, R. Panthier, Epidemiology of the West Nile virus: study of a focus in Camargue. IV. Meningo-encephalomyelitis of the horse, Ann Inst Pasteur. 118 (1970) 239–247.

[8] H. Zeller, I. Schuffenecker, West Nile virus: an overview of its spread in Europe and the Mediterranean basin in contrast to its spread in the Americas, Eur. J. Clin. Microbiol. Infect. Dis. 23 (2004) 147–156, https://doi.org/10.1007/s10096-003-1085-1.

[9] T. Bakonyi, É. Ivanics, K. Erdélyi, K. Ursu, E. Ferenczi, H. Weissenböck, N. Nowotny, Lineage 1 and 2 strains of encephalitic West Nile virus, Central Europe, Emerg. Infect. Dis. 12 (2006) 618, https://doi.org/10.3201/eid1204.051379.

[10] A.E. Platonov, L.S. Karan, T.A. Shopenskaia, M.V. Fedorova, N.M. Koliasnikova, N. M. Rusakova, L.V. Shishkina, T.E. Arshba, V.I. Zhuravlev, M.V. Govorukhina, A. A. Valentseva, G.A. Shipulin, Genotyping of West Nile fever virus strains circulating in southern Russia as an epidemiological investigation method: principles and results, Zh. Mikrobiol. Epidemiol. Immunobiol. 29-37 (2011).

[11] L.M. Hernández-Triana, C.L. Jeffries, K.L. Mansfield, G. Carnell, A.R. Fooks, N. Johnson, Emergence of West Nile virus lineage 2 in Europe: a review on the introduction and spread of a mosquito-borne disease, Front. Public Health 2 (2014) 271, https://doi.org/10.3389/fpubh.2014.00271.

[12] C. Rizzo, C. Napoli, G. Venturi, S. Pupella, L. Lombardini, P. Calistri, F. Monaco, R. Cagarelli, P. Angelini, R. Bellini, West Nile virus transmission: results from the integrated surveillance system in Italy, 2008 to 2015, Eurosurveillance 21 (2016) 30340, https://doi.org/10.2807/1560-7917.ES.2016.21.37.30340.

[13] L. Barzon, M. Pacenti, E. Franchin, E. Lavezzo, G. Masi, L. Squarzon, S. Pagni, S. Toppo, F. Russo, M. Cattai, R. Cusinato, G. Palù, Whole genome sequencing and phylogenetic analysis of West Nile virus lineage 1 and lineage 2 from human cases of infection, Italy, august 2013, Eurosurveillance 18 (2013) 20591, https://doi.org/10.2807/1560-7917.ES2013.18.38.20591.

[14] J. Richter, C. Tryfonos, A. Tourvas, D. Floridou, N.I. Paphitou, C. Christodoulou, Complete genome sequence of West Nile virus (WNV) from the first human case of neuroinvasive WNV infection in Cyprus, Genome Announc. 5 (2017), https://doi.org/10.1128/genomea.01110-17.

[15] R.T. Koch, D. Erazo, A.J. Folly, N. Johnson, S. Dellicour, N.D. Grubaugh, C. B. Vogels, Genomic epidemiology of West Nile virus in Europe, One Health 18 (2024) 100664, https://doi.org/10.1016/j.onehlt.2023.100664.

[16] European Centre for Disease Prevention Control, Epidemiological Update: West Nile Virus Transmission Season in Europe. www.ecdc.europa.eu/en/news-events/epidemiological-update-west-nile-virus-transmission-season-europe-2019, 2019 accessed 2 april 2025.

[17] S. Paz, Climate change impacts on West Nile virus transmission in a global context, Philos. Trans. R. Soc. B 370 (2015) 20130561, https://doi.org/10.1098/rstb.2013.0561.

[18] S. Higgs, B.S. Schneider, D.L. Vanlandingham, K.A. Klingler, E.A. Gould, Nonviremic transmission of West Nile virus, Proc. Natl. Acad. Sci. 102 (2005) 8871–8874.

[19] C.C. Lord, C.R. Rutledge, W.J. Tabachnick, Relationships between host viremia and vector susceptibility for arboviruses, J. Med. Entomol. 43 (2006) 623–630.

[20] E. Sotelo, J. Fernandez-Pinero, F. Llorente, A. Vazquez, A. Moreno, M. Agüero, P. Cordioli, A. Tenorio, M.A. Jimenez-Clavero, Phylogenetic relationships of Western Mediterranean West Nile virus strains (1996–2010) using full-length genome sequences: single or multiple introductions? J. Gen. Virol. 92 (2011) 2512–2522, https://doi.org/10.1099/vir.0.033829-0.

[21] N. López-Ruiz, M. del Carmen Montaño-Remacha, E. Durán-Pla, M. Pérez-Ruiz, J. M. Navarro-Marí, C. Salamanca-Rivera, B. Miranda, S. Oyonarte-Gómez, J. Ruiz-Fernández, West Nile virus outbreak in humans and epidemiological surveillance, West Andalusia, Spain, 2016, Eurosurveillance 23 (2018) 17–00261, https://doi.org/10.2807/1560-7917.ES.2018.23.14.17-00261.

[22] C.S. Casimiro-Soriguer, J. Perez-Florido, J.L. Fernandez-Rueda, I. Pedrosa-Corral, V. Guillot-Sulay, N. Lorusso, L.J. Martinez-Gonzalez, J.M. Navarro-Marí, J. Dopazo, S. Sanbonmatsu-Gámez, Phylogenetic analysis of the 2020 West Nile virus (WNV) outbreak in Andalusia (Spain), Viruses 13 (2021) 836, https://doi.org/10.3390/v13050836.

[23] L.G.S.M. Rodríguez-Alarcón, B. Fernández-Martínez, M.J.S. Moros, A. Vázquez, P. J. Pachés, E.G. Villacieros, M.B.G. Martín, J.F. Borras, N. Lorusso, J.M.R. Aceitero, Unprecedented increase of West Nile virus neuroinvasive disease, Spain, summer 2020, Eurosurveillance 26 (2021) 2002010, https://doi.org/10.2807/1560-7917.ES.2021.26.19.2002010.

[24] M.J. Ruiz-López, M. Muñoz-Chimeno, J. Figuerola, A.M. Gavilán, S. Varona, I. Cuesta, J. Martínez-de la Puente, Á. Zaballos, F. Molero, R.C. Soriguer, Genomic analysis of West Nile virus lineage 1 detected in mosquitoes during the 2020–2021 outbreaks in Andalusia, Spain, Viruses 15 (2023) 266, https://doi.org/10.3390/v15020266.

[25] N. Busquets, M. Laranjo-González, M. Soler, O. Nicolás, R. Rivas, S. Talavera, R. Villalba, E. San Miguel, N. Torner, C. Aranda, Detection of West Nile virus lineage 2 in North-Eastern Spain (Catalonia), Transbound. Emerg. Dis. 66 (2019) 617–621, https://doi.org/10.1111/tbed.13086.

[26] M. Velasco, M.P. Sánchez-Seco, C. Campelo, F. de Ory, O. Martin, L. Herrero, O. J. Salmerón Béliz, T. Minguito, M. Campos, F. Molero, Imported human West Nile virus lineage 2 infection in Spain: neurological and gastrointestinal complications, Viruses 12 (2020) 156, https://doi.org/10.3390/v12020156.

[27] Regional Government of Andalusia, Epidemiology Andalusian Surveillance System (SVEA). Protocolo de vigilancia y alerta de Fiebre del Nilo Occidental. [Protocol for West Nile Fever Surveillance And Alert]. https://juntadeandalucia.es/export/drupaljda/salud_5af95879cc803_fiebre_nilo250817.pdf, 2017 (accessed 2 April 2025).

[28] A.I.N. Antón, F. de Ory Manchón, M.P.S.-S. Fariñas, L.F. Narváez, M.I.G. Cámara, J. M.N. Mari, A.T. Matanzo, Diagnóstico microbiológico de arbovirosis y robovirosis emergentes, Enferm. Infecc. Microbiol. Clin. 33 (2015) 197–205, https://doi.org/10.1016/j.eimc.2013.07.011.

[29] M.J. Martínez Yoldi, M. Ruiz, M.P. Sánchez-Seco Fariñas, A. Vázquez González, Diagnóstico microbiológico de las principales arbovirosis importadas y autóctonas, in: E. Mansilla Cercenado, R. Moreno Cantón (Eds.), Procedimientos en Microbiología Clínica: Sociedad Española de Enfermedades Infecciosas y Microbiología Clínica (SEIMC), 2020, pp. 1–51.

[30] A. Vázquez, L. Herrero, A. Negredo, L. Hernández, M.P. Sánchez-Seco, A. Tenorio, Real time PCR assay for detection of all known lineages of West Nile virus, J. Virol. Methods 236 (2016) 266–270, https://doi.org/10.1016/j.jviromet.2016.07.026.

[31] J. Del Amo, E. Sotelo, J. Fernández-Pinero, C. Gallardo, F. Llorente, M. Agüero, M. A. Jiménez-Clavero, A novel quantitative multiplex real-time RT-PCR for the simultaneous detection and differentiation of West Nile virus lineages 1 and 2, and of Usutu virus, J. Virol. Methods 189 (2013) 321–327, https://doi.org/10.1016/j.jviromet.2013.02.019.

[32] T.P. Monath, The Arboviruses:: Epidemiology and Ecology, CRC Press, Boca Raton, FL, 2020.

[33] L. Barzon, M. Pacenti, E. Franchin, L. Squarzon, A. Sinigaglia, S. Ulbert, R. Cusinato, G. Palù, Isolation of West Nile virus from urine samples of patients with acute infection, J. Clin. Microbiol. 52 (2014) 3411–3413, https://doi.org/10.1128/jcm.01328-14.

[34] B. Tešović, J. Nišavić, B.B. Đeri, T. Petrović, A. Radalj, M. Šekler, K. Matović, Z. Debeljak, N. Vasković, M. Dmitrić, Development of multiplex PCR based NGS protocol for whole genome sequencing of West Nile virus lineage 2 directly from biological samples using Oxford Nanopore platform, Diagn. Microbiol. Infect. Dis. 105 (2023) 115852, https://doi.org/10.1016/j.diagmicrobio.2022.115852.

[35] H. Patel, S. Monzón, S. Varona, J. Espinosa-Carrasco, M. García, M. Heuer, A. Underwood, G. Gabernet, P. Ewels, M. Julia, S. Kelly, H. Tavares, F. Hanssen, S. Wilson, K. Sameith, M. Wells, K. Menden, nf-core/viralrecon: nf-core/viralrecon v2.6.0 - Rhodium Raccoon (2.6.0). https://zenodo.org/records/7764938, 2023.

[36] B. Langmead, S.L. Salzberg, Fast gapped-read alignment with bowtie 2, Nat. Methods 9 (2012) 357–359, https://doi.org/10.1038/nmeth.1923.

[37] P. Danecek, J.K. Bonfield, J. Liddle, J. Marshall, V. Ohan, M.O. Pollard, A. Whitwham, T. Keane, S.A. McCarthy, R.M. Davies, Twelve years of SAMtools and BCFtools, GigaScience 10 (2021) giab008, https://doi.org/10.1093/gigascience/giab008.

[38] J. Huddleston, J. Hadfield, T.R. Sibley, J. Lee, K. Fay, M. Ilcisin, E. Harkins, T. Bedford, R.A. Neher, E.B. Hodcroft, Augur: a bioinformatics toolkit for phylogenetic analyses of human pathogens, J. Open Source Softw. 6 (2021) 2906, https://doi.org/10.21105/joss.02906.

[39] S. Tavaré, Some probabilistic and statistical problems in the analysis of DNA sequences, Lect. Math. Life Sci. 17 (1986) 57–86.

[40] D.T. Hoang, O. Chernomor, A. Von Haeseler, B.Q. Minh, L.S. Vinh, UFBoot2: improving the ultrafast bootstrap approximation, Mol. Biol. Evol. 35 (2018) 518–522, https://doi.org/10.1093/molbev/msx281.

[41] C.S. Casimiro-Soriguer, J. Pérez-Florido, E.A. Robles, M. Lara, A. Aguado, M. A. Rodríguez Iglesias, J.A. Lepe, F. García, M. Pérez-Alegre, E. Andújar, V. E. Jiménez, L.P. Camino, N. Loruso, U. Ameyugo, I.M. Vazquez, C.M. Lozano, J. A. Chaves, J. Dopazo, The integrated genomic surveillance system of Andalusia

(SIEGA) provides a one health regional resource connected with the clinic, Sci. Rep. 14 (2024) 19200, https://doi.org/10.1038/s41598-024-70107-0.
[42] SIEGA, West Nile Virus Epidemiological Local Server - SIEGA. http://nextstrain.clinbioinfosspa.es/wnv, 2020 (accessed 2 April 2025).
[43] L.D. Kramer, J. Li, P.-Y. Shi, West nile virus, Lancet Neurol. 6 (2007) 171–181, https://doi.org/10.1016/S1474-4422(07)70030-3.
[44] J. Figuerola, M.Á. Jiménez-Clavero, M.J. Ruíz-López, F. Llorente, S. Ruiz, A. Hoefer, P. Aguilera-Sepúlveda, J. Jiménez-Peñuela, O. García-Ruiz, L. Herrero, A one health view of the West Nile virus outbreak in Andalusia (Spain) in 2020, Emerg. Microb. Infect. 11 (2022) 2570–2578, https://doi.org/10.1080/22221751.2022.2134055.
[45] P. Aguilera-Sepúlveda, S. Napp, F. Llorente, C. Solano-Manrique, R. Molina-López, E. Obón, A. Solé, M. Jiménez-Clavero, J. Fernández-Pinero, N. Busquets, West Nile virus lineage 2 spreads westwards in Europe and overwinters in north-eastern Spain (2017-2020), Viruses 14 (2022), https://doi.org/10.3390/v14030569.
[46] C. Beck, I. Leparc Goffart, F. Franke, G. Gonzalez, M. Dumarest, S. Lowenski, Y. Blanchard, P. Lucas, X.D. Lamballerie, G. Grard, Contrasted epidemiological patterns of West Nile virus lineages 1 and 2 infections in France from 2015 to 2019, Pathogens 9 (2020) 908, https://doi.org/10.3390/pathogens9110908.
[47] C. Bigeard, L. Pezzi, R. Klitting, N. Ayhan, G. L'Ambert, N. Gomez, G. Piorkowski, R. Amaral, G.A. Durand, A.M.G. Colmant, C. Giraud, K. Ramiara, C. Migné, G. Grard, T. Touzet, S. Zientara, R. Charrel, G. Gonzalez, A. Duvignaud, D. Malvy, X. de Lamballerie, A. Fontaine, Molecular Xenomonitoring (MX) allows real-time surveillance of West Nile and Usutu virus in mosquito populations, PLoS Negl. Trop. Dis. 18 (2024) e0012754, https://doi.org/10.1371/journal.pntd.0012754.
[48] J. Kolodziejek, C. Jungbauer, S.W. Aberle, F. Allerberger, Z. Bagó, J.V. Camp, K. Dimmel, P. De Heus, M. Kolodziejek, P. Schiefer, Integrated analysis of human-animal-vector surveillance: West Nile virus infections in Austria, 2015–2016, Emerg. Microb. Infect. 7 (2018) 1–15, https://doi.org/10.1038/s41426-018-0021-5.
[49] P. Drzewnioková, L. Barzon, E. Franchin, E. Lavezzo, T. Bakonyi, J. Pistl, T. Csank, The complete genome sequence analysis of West Nile virus strains isolated in Slovakia (Central Europe), Arch. Virol. 164 (2019) 273–277, https://doi.org/10.1007/s00705-018-4056-7.
[50] T.-H. Top, M. Jung, S. Lycett, O. Gascuel, Fast dating using least-squares criteria and algorithms, Syst. Biol. 65 (2016) 82–97, https://doi.org/10.1093/sysbio/syv068.
[51] G. Mencattelli, F. Iapaolo, A. Polci, M. Marcacci, A. Di Gennaro, L. Teodori, V. Curini, V. Di Lollo, B. Secondini, S. Scialabba, M. Gobbi, E. Manuali, C. Cammà, R. Rosà, A. Rizzoli, F. Monaco, G. Savini, West Nile virus lineage 2 overwintering in Italy, Trop. Med. Infect. Dis. 7 (2022), https://doi.org/10.3390/tropicalmed7080160.
[52] K. Maezono, S. Kobayashi, K. Tabata, K. Yoshii, H. Kariwa, Development of a highly specific serodiagnostic ELISA for West Nile virus infection using subviral particles, Sci. Rep. 11 (2021) 9213.
[53] T. Vilibic-Cavlek, M. Bogdanic, V. Savic, Z. Hruskar, L. Barbic, V. Stevanovic, L. Antolasic, L. Milasincic, D. Sabadi, G. Miletic, I. Coric, A. Mrzljak, E. Listes, G. Savini, Diagnosis of West Nile virus infections: evaluation of different laboratory methods, World J. Virol. 13 (2024) 95986, https://doi.org/10.5501/wjv.v13.i4.95986.
[54] S. Ulbert, West Nile virus vaccines - current situation and future directions, Hum. Vaccin. Immunother. 15 (2019) 2337–2342, https://doi.org/10.1080/21645515.2019.1621149.
[55] Boletín Oficial de la Junta de Andalucía, Acuerdo de 9 de marzo de 2021, del Consejo de Gobierno, por el que se toma conocimiento del Programa de Vigilancia y Control Integral de Vectores de la Fiebre del Nilo Occidental. https://www.juntadeandalucia.es/boja/2021/48/4, Boletín Oficial de la Junta de Andalucía, (accessed 2 April 2025).
[56] J.L. Gardy, N.J. Loman, Towards a genomics-informed, real-time, global pathogen surveillance system, Nat. Rev. Genet. 19 (2018) 9, https://doi.org/10.1038/nrg.2017.88.