One Health 19 (2024) 100937

Contents lists available at ScienceDirect

One Health

journal homepage: www.elsevier.com/locate/onehlt

Advancing West Nile virus monitoring through whole genome sequencing: Insights from a One Health genomic surveillance study in Romagna (Italy)

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ARTICLE INFO

Keywords:
West Nile virus
Arbovirus
Genomic surveillance
Whole genome sequencing
One Health approach

ABSTRACT

In the last 6 years, Italy accounted for 36 % of the total autochthonous European West Nile virus (WNV) cases reported to ECDC. Since 2001, the country put in place a multi-species national surveillance plan. The plan was enhanced in 2020 by adopting a fully integrated "One Health" approach, including human, wild bird, equine, and mosquito surveillance for the early detection of WNV. In this context, the systematic acquisition of whole viral genetic information from human patients and animals is fundamental to obtain an in-depth knowledge on the patterns of virus evolution and transmission and to gain insights on the role virus genetics in morbidity and mortality, The purpose of this pilot study was thus to design a One-Health surveillance framework based on the genomic surveillance of WNV circulating at the vector-human-animal interface, in the endemic territory of Romagna (North-Eastern Italy) during the 2023 transmission season. Whole genome sequencing (WGS) analyses confirmed the circulation of WNV lineage 2 showing high nucleotide and amino acid identity of 99.82 % and 99.92 % respectively among viral sequences from human patients, vectors and birds. All the sequences clustered with other Italian strains in the Central and Southern European clade with robust bootstrap support and BLASTn identity exceeding 99.7 %. The highest nucleotide identity was observed with sequences from Emilia-Romagna and Veneto regions (Italy), confirming a local virus circulation and overwintering of WNV lineage 2 with a confined virus spread and no (or limited) external introduction of viral strains. Our results, support the adoption of a One Health approach to WNV surveillance, based on WGS and integrating the clinical diagnosis, epidemiology, and genomic characterisation, to create a suitable operational process for the characterisation of autochthonous and imported Arboviruses circulating in Romagna to effectively integrate the already established surveillance plan.

1. Introduction

West Nile virus (WNV) is a mosquito-borne zoonotic virus belonging to the *Orthoflavivirus* genus, *Flaviviridae* family [1,2]. The two most prevalent and clinically significant WNV lineages are lineage 1 (WNV L1) and lineage 2 (WNV L2). Over the last two decades, WNV lineage 2 established local virus reservoirs that could lead to outbreaks independently from seasonal reintroduction of viral strains from southern Europe or Africa [3]. Since then, WNV lineage 2 has evolved into an endemic virus, causing seasonal outbreaks of viral encephalitis and

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https://doi.org/10.1016/j.onehlt.2024.100937
Received 9 September 2024; Received in revised form 12 November 2024; Accepted 13 November 2024
Available online 19 November 2024

aseptic meningitis in several European countries [4]. While the circulation of WNV lineage 1 in Europe has historically been linked to continuous reintroductions from North Africa [5], recent studies have proven its endemic circulation in southern Spain, where WNV lineage 1 overwinters [6,7]. In Italy, both WNV lineages have been identified with lineage 2 becoming predominant after its introduction in 2011 [8]. Despite all the efforts to control its spread, WNV continues to circulate, posing a significant threat globally. According to the annual ECDC published data, autochthonous West Nile cases account for 95 % of the total recorded in Europe over the period 2018–2023, with Italy being the largest contributor, accounting for an average of 36 % of these infections [9].

Italy's epidemiological WNV surveillance system is now regulated by the "National prevention, surveillance and response plan for arboviral diseases 2020-2025". This plan integrates human and veterinary surveillance for WNV and is managed by the Italian National Institute of Health and the Ministry of Health in coordination with the Research Centre for Exotic Diseases of the Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "Giuseppe Caporale" [10]. Surveillance of imported and local human infections is carried out year-round throughout the country, between early May and late November in Regions where endemic areas have been identified [11]. In Emilia-Romagna region, North-Eastern Italy, an integrated and multidisciplinary WNV surveillance system targeting humans, wild birds, horses, and mosquitoes has been implemented to allow early detection of virus circulation in the environment and mitigation of viral transmission risks via blood and solid organ donations since 2009 [11–13]. Integrating genomic data from infected humans and animals, has the potential to provide new insights on the patterns of virus transmission and the role virus genetics and evolution in causing morbidity and mortality [13] for targeted interventions in high-risk areas. Furthermore, embedding precise metadata to viral genomes (e.g collection date and sample location), which are often unavailable or unreported, is key for precisely calibrating molecular clock for phylogeographic models [5].

The purpose of this study is to describe a One-Health surveillance framework based on WNV integrated whole genomic surveillance, and associated metadata, to complement the existing multi-species genomic surveillance while incorporating WNV genetic analysis into the patients diagnostics workflow.

2. Materials and methods

2.1. Study area

The study area is represented by Romagna (Fig. 1), corresponding to the south-eastern portion of Emilia-Romagna region in northern Italy. Romagna presents a unique environment limited by the Apennines mountains to the southwest, the Reno and Sillaro rivers to the north and west and the Adriatic Sea to the east. Its northern part, corresponding to Ravenna province, borders the delta of the Po River, Europe's second-largest wetlands and is protected by UNESCO as a Biosphere Reserve. For its climate and environmental features, Romagna allowed WNV and other arboviruses endemization. The study was carried out from May to October 2023.

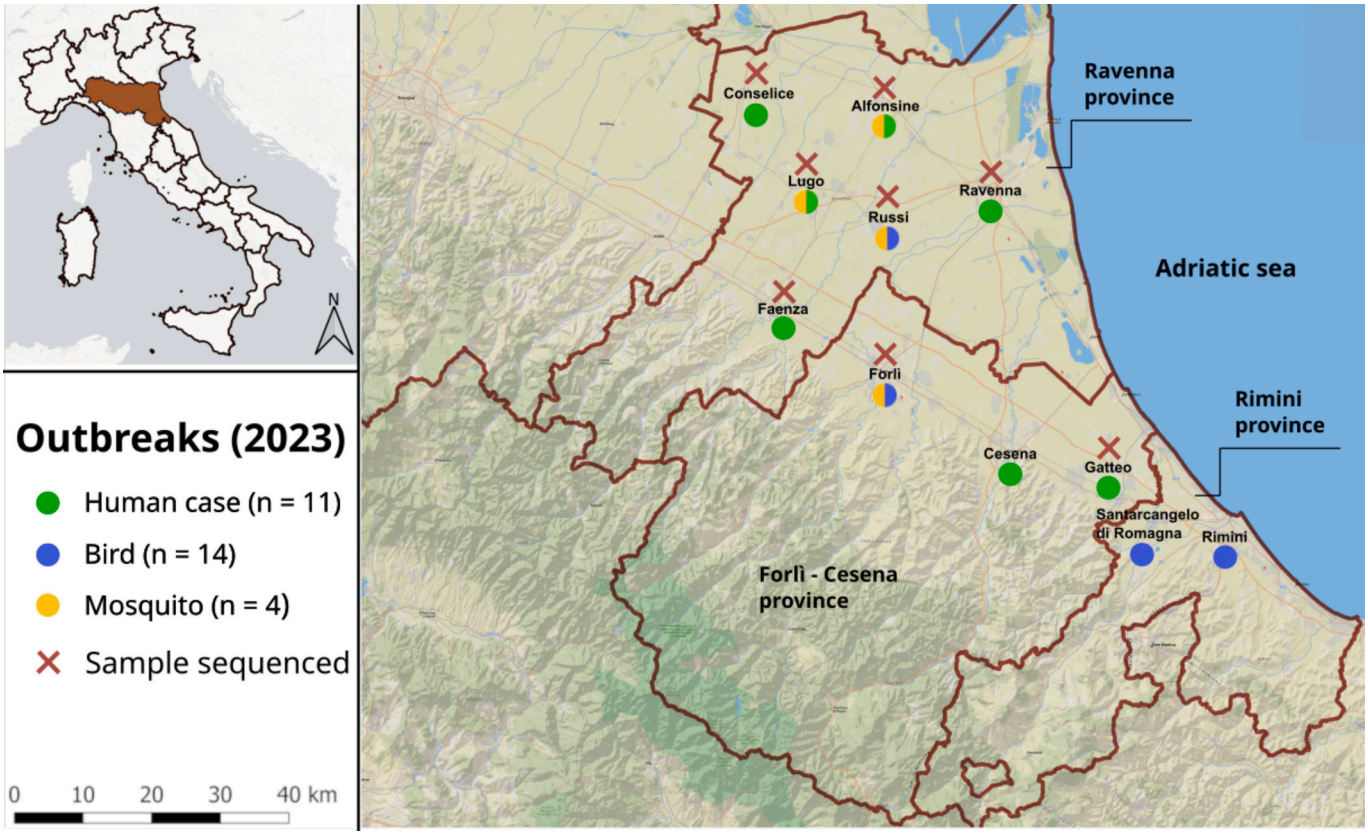

Fig. 1. Map of geo-localization sites of recorded WNV infections. In 2023, a total of 29 cases of WNV were detected in the Romagna region. These cases comprised 11 human cases (green dots on the map), 14 bird cases (blue dots), and 4 sites with WNV-positive mosquitoes (yellow dots). The primary hotspot for WNV was Ravenna province, where 9 human cases, 3 bird cases, and 3 mosquito cases were reported. Additionally, WNV circulation was observed in Forlì-Cesena province (2 human cases, 7 bird cases, 1 site with WNV-positive mosquitoes) and Rimini province (4 bird cases). Samples sequenced in this study are marked on the map with a red cross. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.2. Human infection cases inclusion criteria, samples collection and analysis

The following groups of patients were enrolled: i) Patients from hospitals in Ravenna, Forlì-Cesena, and Rimini provinces with symptoms potentially related to arboviral encephalitis or meningitis, and cerebrospinal fluid (CSF) tests indicative of viral meningitis; healthy blood donors from Association of Voluntary Italian Blood Donors centres of Romagna who tested positive for WNV during systematic screening implemented during transmission season by the local Unit of Transfusion Medicine (Cobas® WNV, Roche Diagnostics International AS, Rotkreuz, Switzerland) [14].

Prior to the enrolment, all participants provided their written informed consent for the collection and use of biological samples and access to clinical data. The study was conducted in accordance with the Declaration of Helsinki and approved by the Romagna Local Ethical Board (Comitato Etico della Romagna, CEROM) under protocol code C. ARBO.SEQ of 13 May 2023.

CSF, plasma and urine samples were collected from patients hospitalized for suspected meningitis within two weeks from symptoms onset. Plasma and urine were collected from blood donors at the time of donation. All samples were conferred to the Unit of Microbiology of The Greater Romagna Area Hub Laboratory, Cesena, Italy.

After RNA extraction with TANBead Maelstrom 9600 automated extractor (Taiwan Advanced Nanotech Inc., Taiwan), samples were subjected to a first-line TaqMan one-step RT-PCR (Reverse-Transcription Polymerase Chain Reaction) for the detection of WNV using primers pair and probe targeting the 3' UTR region (UnTranslated Region) designed by Tang et al. [15]. Positive samples were further tested for lineage-discrimination according to Del Amo et al. [16].

2.3. Animal samples collection and analysis

Mosquitoes and birds were collected in the frame of the Regional Plan for Arbovirus surveillance [17] by the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna "Bruno Ubertini" (IZSLER). Mosquitoes were sampled in 13 georeferenced traps sited in the Romagna area, every two weeks from May to October 2023. Collected mosquitoes were identified at the species level using morphological keys [18] and males were discarded. Monospecific pools were obtained with a maximum of 200 specimens per pool and homogenized. Birds, collected as part of active (specifically targeting Eurasian magpies, Hooded crows and Eurasian jays) and passive (carried out on wild birds died in wildlife rehabilitation centres) surveillance, were necropsied and brain, spleen, heart, and kidney samples were pooled and homogenized. Viral RNA was extracted with KingFisher (ThermoFisher, Waltham, Massachusetts, USA). All samples were tested by a WNV real-time RT-PCR, according to the method described by Eiden et al. [19] (Assay2, target: WNVNS2A) followed by the protocol of Del Amo et al. [16] and by a universal PCR protocol [20] for lineage confirmation.

2.4. Viral propagation

For positive human (Ct $<$ 40) and animal samples (Ct $<$ 30) viral propagation was attempted on Vero E6 cells (ATCC CRL-1587). Infected monolayers were incubated for a minimum of three days and a maximum of five days. For each sample, three blind passages were carried out, after which a TaqMan RT-PCR was performed to identify effective viral isolation and propagation. All activities involving virus manipulation and propagation were conducted at a Biological Safety Level 3 facility, adhering to biocontainment rules.

2.5. NGS whole genome viral sequencing

Following viral RNA extraction, library preparation was carried out by tagmentation with Illumina RNA Prep with Enrichment Tagmentation (Illumina, San Diego, California, USA) and hybrid-capture-mediated target enrichment with Viral Surveillance Panel oligos (Illumina). Protocol steps (RNA denaturation, cDNA synthesis, tagmentation with enrichment and indexing, library normalization, three-plex enrichment and oligo-mediated capture) were performed according to the manufacturer instructions [21,22]. Libraries were normalized to 10 nM, pooled in equimolar ratios and diluted to a final concentration of 650 pM (onboard denaturation). 2 % PhiX was used as a spike-in control for low-diversity libraries. Paired-end sequencing was carried out on a NextSeq 2000 instrument (Illumina), with a 300-cycles P1 Reagents kit.

2.6. Sequence assembly, variant and phylogenetic analysis

Index sequences were automatically removed from fastq by the instrument-integrated Real Time Analysis software (RTA3). After preprocessing, the trimmed reads were aligned to the reference genome (GenBank accession no. **NC_001563.2**) by using Bowtie2 v.2.4.1 [23], and the consensus genome sequences were called using BCFtools v.1.13 [24]. In short, a Bowtie2 index was created to facilitate the incorporation of viral segment sequences via the bowtie2-build program. Subsequent short-read analysis was conducted using Bowtie2 in paired-end mode with the —S option, resulting in SAM format output. The resulting SAM format alignment files underwent sequential processing using SAMtools v.1.13 commands, including view and sort. A consensus sequence was generated through the "bcftools consensus" command.

For maximum likelihood (ML) phylogeny, a dataset consisting of 106 West Nile lineage 2 sequences was downloaded from NCBI GenBank database [25]. The sequences were selected following spatial and temporal criteria, including sequences deposited in the last twenty years, with focus on data from Central and Southern European countries. WNV sequences used for phylogeny reconstruction and their metadata (sampling date, geographical origin, host, genome length) are reported in Table S1 (see web-only Supplementary Table S1). Among selected sequences, 93 were complete genomes, while 13 were nearly complete. Multiple sequence alignments (MSA) were executed using MAFFT v7.505 [26] with the –auto option. The MSA was visualized and inspected by the Molecular Evolutionary Genetics Analysis (MEGA) software v.11.0.13 [27]. The resulting alignment was subsequently trimmed utilizing trimAl v1.4.rev15 [28] with the gappyout setting. Maximum likelihood (ML) trees for viral phylogenetic inference were constructed employing IQ-TREE 2.0.3 [29] with automatic selection of the best nucleotide substitution model and 1000 ultrafast bootstrap replicates. Finally, the phylogenetic trees were visualized using FigTree v.1.4.4 and midpoint rooted. Sequences showing a sister relationship with those sequenced in this study were compared to the NCBI database using the BLASTn tool [25] to determine their percentage identity.

Pairwise comparisons (using Needleman-Wunsch algorithm for global alignment)) of complete coding sequences and translated polyproteins were performed to assess sequence identity among sequenced isolates and WNV lineage 2 reference sequence (GenBank accession nos. **NC_001563.2** and **NP_041724.2** for reference strain nucleotide and amino acid sequence, respectively).

3. Results

3.1. Human infection cases clinical presentation

A total of 28 patients with suspected arboviral meningoencephalitis were enrolled from May to September 2023, aged between 19 and 90, with a mean age of 54 years old (SD (standard deviation) = 20 years, median age = 56 years, IQR (interquartile range) = 71–39). Of these, 8 patients (32 %) aged between 19 and 90, with a mean age of 70 years old (SD = 21 years, median age = 77.5 years, IQR = 84.75–63.5), comprising 2 females and 6 males, tested positive for WNV by molecular methods. All patients reported headache and fever, with three patients

experiencing severe prostration, debilitating myalgias, photophobia, and nausea. The mean hospitalization period of WNV-positive patients was 32 days. Three of WNV-positive patients presented with a severe form of meningitis and required hospitalization in Intensive Care Unit (ICU).

Three further subjects, 2 men and a woman, included in the study, were blood donors aged between 44 and 57 years old (mean age = 49, standard deviation = 6 years, median age = 48 years, IQR (interquartile range) = 57–47) who tested WNV positive in screening tests; they all tested positive on plasma at the time of donation and urine at the 7-days follow-up.

A total of 9 infections were reported in Ravenna province and 2 in Forlì-Cesena province, with none of the patients reporting travels abroad or to other Italian WNV-endemic areas. All infections were hence classified as autochthonous, with the place of infection approximated to the municipality of residence. Regarding lineage identification, carried out by multiplex lineage-discriminating RT-PCR (see method section), 9 samples were identified as lineage 2, while 2 were identified as lineage 1.

3.2. Animal samples

During the surveillance period in Romagna, 79,939 *Culex* mosquitos were trapped and grouped in 489 pools, 198 birds were screened during active surveillance and 998 during passive surveillance. Of these, 4 mosquitoes (*Culex pipiens*) pools collected between end of June and end of August 2023, and 14 birds of different target species (1 seagull [*Larus ridibundus*], 7 magpies [*Pica pica*], 3 jays [*Garrulus glandarius*] and 3 blackbirds [*Turdus merula*]) collected between beginning of June and end of August through active ($n = 10$) and passive ($n = 4$) surveillance, tested positive for WNV lineage 2. Overall, 7 WNV-positive birds were reported from Forlì-Cesena province, 4 from Rimini province, and 3 from Ravenna province. Interestingly, despite the detection of WNV lineage 1 in two meningitis cases, this lineage was not detected in any mosquito or bird. Recorded WNV-positive mosquito pools and birds are reported in Fig. 1.

3.3. Viral propagation

WNV was successfully isolated on Vero E6 cells from 4 human clinical specimens, including 1 CSF from a severe meningitis case and 3 plasma samples from healthy blood donors; all isolates belonged to WNV lineage 2. Viruses from animal sample were not successfully propagated, potentially due to a very low viable viral load. Virus-induced cytopathic effect was detectable on the monolayer after approximately three days of infection, with evident cell rounding and replication plaques formation. Prolonged incubation (3–5 days) resulted in detachment. All propagates had a Ct of about 8–10, compared with Ct of the respective parent samples above 24.

3.4. Sequence and phylogenetic analysis

Five sequences derived from human clinical samples (4 meningitis cases and 1 blood donor), 4 from cell culture isolates (1 meningitis case and 3 blood donors) and 4 from animal samples (3 *Cx. pipiens* and 1 *P. pica*) were obtained. Overall, genome sequencing of human and animal samples, and cell isolates yielded 98.8–100 % coverage and consensus reconstructed genome sequences ranging from 10.841 to 10.995 nucleotides length (Table 1). All sequences obtained belonged to WNV lineage 2b; neither of the two samples identified as lineage 1 were successfully sequenced because of the very low viral load and were not efficiently propagated on cell culture.

When sequences from clinical samples and their respective isolate were available, only the sequence from the clinical samples were considered for variant and phylogenetic analysis. In 2 patients, the viral load of the clinical sample was too low for sequencing, for this their

Table 1
Whole genome sequencing results. Results of WNV-positive human and animal samples and cell culture isolates sequencing by tagmentation and hybrid-capture target enrichment using Illumina RNA Prep with Enrichment Tagmentation protocol with Viral Surveillance Panel oligos.

Sample ID	Municipality of origin (province)	Collection date	Sample matrix	RT-PCR Ct value	WNV lineage	Total number of reads	Number of WNV reads	% of WNV mapped reads	Coverage %	Mean depth	Sequence length
LU_300723	Lugo (RA)	30 Jul 2023	urine	26	2	11.117.638	90.207	0.8	99.6	420	10.920
LU_130823	Conselice (RA)	31 Aug 2023	CSF	36	2	1.840.980	166.470	9.0	98.8	77	10.841
			Vero E6 isolate	8.53	2	19.827.442	19.214.209	96.9	99.9	32.869	10.956
RA_180823	Ravenna (RA)	18 Aug 2023	urine	25.04	2	15.278.922	12.343.939	80.8	100	13.906	10.963
FA_010923	Faenza (RA)	1 Sep 2023	urine	31.41	2	1.987.836	21.385	1.1	99.8	126	10.936
FA_240823	Faenza (RA)	24 Aug 2023	Vero E6 isolate	9.08	2	11.651.273	10.937.191	93.9	100	24.971	10.995
CE_040923	Gatteo (FC)	4 Sep 2023	Vero E6 isolate	6.79	2	8.117.842	7.545.500	92.9	100	19.404	10.959
RA_040923	Russi (RA)	4 Sep 2023	urine	30.45	2	5.807.664	342.927	5.9	99	91	10.849
			Vero E6 isolate	7.07	2	12.176.029	11.601.017	95.3	100	25.823	10.957
Cx. pipiens_241,729	Forlì (FC)	3 Aug 2023	homogenate	24.58	2	11.666.237	4.938.263	42.3	99.9	5.129	10.955
Cx. pipiens_246,218	Russi (RA)	8 Aug 2023	homogenate	24.27	2	6.144.291	637.647	10.4	99.8	459	10.937
Cx. pipiens_261,861	Alfonsine (RA)	24 Aug 2023	homogenate	28.41	2	10.921.464	285.682	1.1	99.6	348	10.914
P.pica_248,896–6	Russi (RA)	10 Aug 2023	homogenate	29.35	2	7.385.939	80.153	2.6	99	136	10.853

CSF = cerebrospinal fluid.

respective cell culture isolate sequences were considered for further analyses, highlighting how cell culture is key to amplify the original viral load, which is often very low in clinical samples, hence facilitating a comprehensive genomic analysis. Overall, a total of 11 sequences (5 human clinical samples, 2 cell culture isolates from human samples, and 4 animal samples) were included in phylogenetic analysis. Non-coding regions (3′-5' UTR) were excluded from analysis as they are affected by very large variations often attributable to sequencing artefacts. Nucleotide identity attested at above 99 % for all pairwise comparisons (average 99.82 %). Deduced amino acid sequences were highly conserved, with an average identity of 99.92 %. Compared to WNV lineage 2 reference sequence, mean nucleotide and amino acid identity are 97.28 % and 98.99 %, respectively (Table 2).

The phylogenetic analysis revealed that the WNV lineage 2 sequences obtained in this study clustered within the Central-Southern European clade with robust bootstrap support (Fig. 2). Specifically, these sequences formed a distinct cluster with viruses isolated in the Emilia-Romagna and Veneto regions of Italy from 2021 to 2023, supported by high bootstrap values and a BLASTn identity exceeding 99.7 %, indicating close genetic relatedness. Sequences CE_040923 and LU_300723 are closely related to a virus isolated from *Culex pipiens* mosquitoes in Emilia-Romagna on July 26th, 2023 (GenBank accession no. **PP104338.1**), with 99.86 % BLASTn identity, suggesting they belong to the same quasispecies. Additionally, sequence *Cx. pipiens*_241,729 shows a sister relationship with a virus detected from *Culex pipiens* mosquitoes in Emilia-Romagna on August 11th, 2023 (GenBank accession no. **PP104356.1**), supported by a significant bootstrap value and 99.72 % nucleotide identity. The fact that the Italian sequences clustered together suggests a local virus spread with no external introductions [30]. Our findings are consistent with previous ones, highlighting WNV lineage 2 eco-epidemiological and genetic evolutionary features that are characterised by the evolution of permanent and stable local cycles with rare incursions into new area, leading to high genetic similarity of WNV lineage 2 strains clustering within the same geographical areas [5].

4. Discussion

The value of WGS to improve time-scaled phylogenetic resolution and bridge knowledge gaps on WNV spread dynamics, was recently reported by several authors [13,31,32]. However, the number of publicly accessible whole WNV genomes from Europe is yet considered insufficient to reconstruct the complete phylogeographic spread history, particularly from Central and Southeastern regions, where WNV have been detected in both humans and animals [32]. In fact, more than 60 % of publicly available sequences from Europe are still represented by partial genomes, sufficient for lineage discrimination, but unable to provide the same level of resolution as whole genomes for more complex phylogenetic analysis [13]. Furthermore, due to partial genome sequencing, important regions that may be associated with differences in virus transmissibility or virulence [33] can be missed.

In this pilot study we describe the methodology and report the results of an integrated One-Health surveillance framework based on WGS genomic monitoring of WNV strains circulating in humans, mosquitoes and birds in Romagna. Our results confirmed WGS to be valuable in elucidating the spatiotemporal patterns of viral circulation, identifying potential amplification hosts, and assessing the risk of spillover to humans. The genomic analyses performed on the complete genomic sequences of 11 WNV lineage 2 showed a high sequence identity among viral isolates, with a mean nucleotide identity of 99.82 % calculated on the coding sequence region and a deduced polyprotein amino acid identity of 99.92 % [5]. Phylogenetic maximum likelihood tree, revealed a geographically based distribution of WNV genomic diversity, and clustering with other Italian, Spanish and French isolates sampled between 2013 and 2023. Other isolates from northern regions (Germany, Austria, and the Czech Republic) or Balkan region and Greece were shown to be more genetically divergent. One of the drivers of this regional genomic diversity is linked to the flyways within the European continent and the distribution of European wetlands, which represent nesting areas for migratory birds and hence hotspots for virus evolution and diversification [2,32]. Interestingly, the highest number of human cases were recorded in the Ravenna province, which is highly urbanised and close to the Delta of the Po area, a wetland reserve in northeastern Italy. These results are consistent with previous findings that WNV is likely to spread to areas with a high degree of urbanization where *Culex pipiens* vectors, the most represented vector captured in the study area, prefers to live due to the availability of aquatic habitats, reduced number of predators and presence of warmer temperatures, thus having a preferential impact of WNV on the population of common bird species [32].

Despite including a small cohort of patients, and an even smaller number of animal samples, our pilot study provides an insight into the methodology that can be put in place to study the genomic epidemiology of WNV strains circulating in the study area. Emilia-Romagna is a WNV endemic region, favouring the presence of animals and vectors in wetland areas where WNV lineage 2 is more likely to cluster [32]. Due to its socio-ecological characteristics, Romagna is an ideal study area in Europe for implementing genomic epidemiological surveillance for monitoring the genome diversity of WNV and other arboviruses, such as Toscana virus and Usutu virus. The implementation of a genomic

Table 2
Percentage nucleotide and amino acid identity. Identities were determined among sequenced samples and against WNV lineage 2 reference sequence (nucleotide reference sequence accession no. **NC_001563.2**, amino acid reference sequence accession no. **NP_041724.2**). Percentage nucleotide identity is reported in regular type and highlighted in orange on the left side; amino acid identity is reported in italics and highlighted in green.

	NC_001563.2	LU_300723	LU_130823	RA_180823	FA_010923	FA_240823	CE_040923	RA_040923	Cx.pipiens_241729	Cx.pipiens_246218	Cx.pipiens_261861	P.pica_248896-6
		aminoacid										
NP_041724.2		*99*	*99*	*99*	*99.03*	*99*	*98.97*	*99*	*99*	*99*	*98.94*	*98.97*
LU_300723	97.26		*100*	*100*	*99.97*	*99.94*	*99.97*	*100*	*99.88*	*99.94*	*99.94*	*99.91*
LU_130823	97.26	99.96		*100*	*99.97*	*99.94*	*99.97*	*100*	*99.88*	*99.94*	*99.94*	*99.91*
RA_180823	97.3	99.89	99.89		*99.97*	*99.94*	*99.97*	*100*	*99.88*	*99.94*	*99.94*	*99.91*
FA_010923	97.4	99.83	99.83	99.88		*99.91*	*99.94*	*99.97*	*99.85*	*99.91*	*99.91*	*99.88*
FA_240823	97.42	99.79	99.79	99.84	99.85		*99.91*	*99.94*	*99.82*	*99.88*	*99.88*	*99.85*
CE_040923	97.25	99.93	99.95	99.89	99.82	99.79		*99.97*	*99.85*	*99.91*	*99.91*	*99.88*
RA_040923	97.26	99.96	99.96	99.89	99.83	99.79	99.95		*99.88*	*99.94*	*99.94*	*99.91*
Cx.pipiens_241729	97.18	99.8	99.82	99.76	99.69	99.66	99.81	99.82		*99.88*	*99.82*	*99.85*
Cx.pipiens_246218	97.25	99.82	99.82	99.78	99.71	99.68	99.81	99.82	99.74		*99.88*	*99.97*
Cx.pipiens_261861	97.25	99.93	99.93	99.89	99.82	99.79	99.92	99.93	99.79	99.81		*99.85*
P.pica_248896-6	97.29	99.74	99.74	99.79	99.76	99.72	99.73	99.74	99.66	99.88	99.73	
		nucleotide										

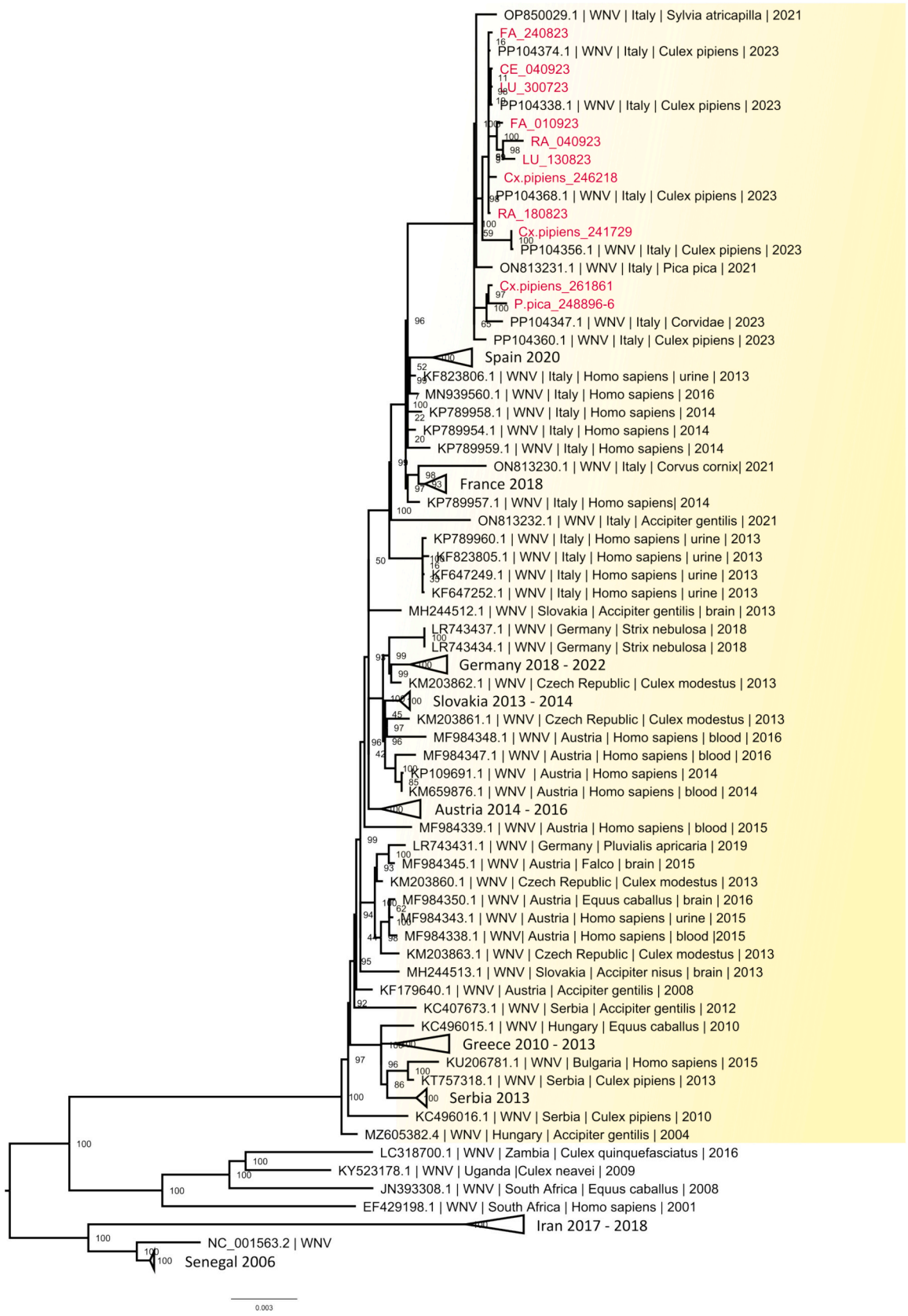

Fig. 2. Phylogenetic analysis of (nearly) complete genome nucleotide sequences of WNV lineage 2. The maximum-likelihood tree was inferred using IQ-TREE v.2.0.3, under automated model selection (GTR + F + G4) and 1000 bootstrap replicates. The viruses sequenced in this study are marked in red (n = 11 samples). GenBank accession numbers are indicated for each strain, with country, host, and year of isolation. Background shading distinguishes the Central-Southern European clade. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

surveillance programme may facilitate the timely tracking of genetic diversity, the evolution, the emergence of new variants and the introduction of genetically distinct strains from other parts of the world. Active surveillance on the territory, could also enable a deeper characterisation of other neglected arboviruses, such as Tahyna virus (*Bunyavirales*, *Peribunyaviridae*, *Orthobunyavirus*) a mosquito-borne virus whose spread in Romagna and ability to cause infections in humans have been demonstrated [34]. On the other hand, a proactive surveillance plan could also be of paramount importance for monitoring imported arboviruses including Chikungunya, Zika, and Dengue.

Genomic epidemiology, advanced virus sequencing and phylogenetics are crucial for the characterisation of outbreak and transmission chain tracking [35]. Genomic surveillance holds great potential for improving our ability to detect, monitor, and control emerging arboviruses. Expanding genomic surveillance of WNV, by including human patients along reservoirs and vectors, has the potential to provide important insights in its global patterns of emergence and spread [13].

However, realizing this potential requires a paradigm shift towards integrated approaches to bridge human and animal health, and cross traditional disciplinary boundaries to ensure an active engagement of all the relevant stakeholders. Through the collaboration of biologists, geneticists, entomologists, human physicians, veterinarians, and local health authorities we aimed to provide evidence that it will be possible to integrate the multi-species "One Health" approach for WNV surveillance plan with genomics surveillance to support transition from partial to whole genome sequencing [13,31].

In this context, sequencing infrastructures and data-sharing platforms developed during the COVID-19 pandemic would serve as a foundation for proactive surveillance of other pathogens by sentinel territorial surveillance networks [31]. Consistent with the One Health Joint Plan of Action 2022–2026, promoted by the Quadripartite [36], this framework is aimed at strengthening One Health surveillance targeting animal–human–environment interfaces to mitigate future health challenges. Our future perspectives include the development of an institutionalised framework, integrating the clinical diagnostic workflows, entomological and ornithological monitoring, epidemiological and genomic surveillance, to take a new step forward in the collaborative arboviruses epidemic intelligence in the Romagna hotspot.

Ethical approval statement

The study was conducted in accordance with the Declaration of Helsinki, and approved by the Romagna Local Ethical Board (Comitato Etico della Romagna, CEROM) under protocol code C.ARBO.SEQ of 13 May 2023.Informed Consent Statement: Prior to the enrolment in the present study all participants gave their written informed consent for the collection and use of biological samples and access to clinical data. Information useful for reconstructing the clinical history of the subjects included in the study was retrieved from medical records.

Data sharing statement

All sequences have been deposited with links to the BioProject accession no. **PRJNA1096139** in the NCBI BioProject database.

Funding

This research was supported by EU funding within the NextGenerationEU-MUR PNRR Extended Partnership initiative on Emerging Infectious Diseases (Project no. PE00000007, INF-ACT).

CRediT authorship contribution statement

Martina Brandolini: Conceptualization, Methodology, Formal analysis, Writing – original draft. **Alessandra Mistral De Pascali:** Conceptualization, Methodology, Writing – original draft. **Irene Zaghi:** Conceptualization, Methodology, Investigation. **Giorgio Dirani:** Conceptualization, Methodology, Investigation, Data curation, Supervision. **Silvia Zannoli:** Conceptualization, Methodology, Investigation, Data curation, Supervision. **Ludovica Ingletto:** Formal analysis, Data curation, Writing – original draft. **Antonio Lavazza:** Methodology, Investigation, Data curation. **Davide Lelli:** Methodology, Investigation, Data curation. **Michele Dottori:** Methodology, Investigation, Data curation. **Mattia Calzolari:** Methodology, Investigation, Data curation. **Massimiliano Guerra:** Investigation, Data curation, Writing – review & editing. **Carlo Biagetti:** Conceptualization, Methodology, Investigation, Supervision. **Francesco Cristini:** Conceptualization, Methodology, Investigation, Supervision. **Paolo Bassi:** Conceptualization, Methodology, Investigation, Supervision. **Rino Biguzzi:** Conceptualization, Methodology, Investigation, Supervision. **Monica Cricca:** Project administration, Writing – review & editing. **Alessandra Scagliarini:** Project administration, Writing – review & editing. **Vittorio Sambri:** Project administration, Funding acquisition.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

accession code for public repository has been shared on the manuscript

Acknowledgments

We would like to express our gratitude to all the collaborators who took part in the project, from the Units on Infectious Diseases and Transfusion Medicine who managed patients enrolment, to the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna for their collaborative spirit and support. Their expertise, commitment and dedication were paramount for the successful completion of an innovative study in the field of genomic surveillance of arboviruses, highlighting how a multi-stakeholder One-Health-oriented approach may represent a turning point for arbovirosis management.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.onehlt.2024.100937.

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