

Whole genome sequencing and phylogenetic analysis of West Nile virus lineage 1 and lineage 2 from human cases of infection, Italy, August 2013

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A human outbreak of West Nile virus (WNV) infection caused by WNV lineage 2 is ongoing in northern Italy. Analysis of six WNV genome sequences obtained from clinical specimens demonstrated similarities with strains circulating in central Europe and Greece and the presence of unique amino acid changes that identify a new viral strain. In addition, WNV lineage 1 Livenza, responsible for a large outbreak in north-eastern Italy in 2012, was fully sequenced from a blood donor during this 2013 outbreak.

A human outbreak of West Nile virus (WNV) infection is ongoing in northern Italy. We report here the clinical presentation, laboratory test results, and WNV full genome sequences from human cases of WNV infection which were diagnosed in August 2013 in the Veneto region. This region is one of the most affected by the outbreak. WNV genome sequences obtained from clinical specimens of infected individuals were subjected to phylogenetic analysis, in order to gain information on the origin and evolutionary history of the responsible viral strains.

Cases of West Nile virus infection from the Veneto region

Cases included five patients with laboratory-confirmed West Nile neuroinvasive disease (WNND) and four with West Nile fever (WNF), aged 51 to 88 years-old, who were resident in Rovigo, Padova, and Verona provinces (Figure 1). In addition, three WNV RNA-positive blood and organ donors were identified by screening in Padova, Verona, and Venice provinces and, besides the 12 confirmed cases, further possible cases are currently under investigation. Confirmed human cases of WNND have also been notified in regions neighbouring Veneto, namely Emilia Romagna and Lombardy [1].

Of the 12 confirmed cases reported in Veneto, WNV lineage 2 (lin2) RNA was identified in plasma and/or urine

of seven patients with WNND or WNF, and in a blood donor, while WNV lin1 was respectively detected in an organ donor and in a blood donor. The sites where different WNV lineages were identified are indicated in Figure 1.

Clinical and laboratory findings

A summary of clinical and laboratory findings from confirmed cases is reported in Table 1. Clinical presentation of patients with WNND and WNF included arthralgia, fatigue, fever ($\geq 38^{\circ}\text{C}$), headache, myalgia, while patients with WNND had neurological manifestations, such as encephalitis, meningitis and paralysis. Mild symptoms (i.e. arthralgia, headache, myalgia, but not fever) occurred also in a WNV-positive blood donor a few days before donation. No deaths due to WNV infection were reported.

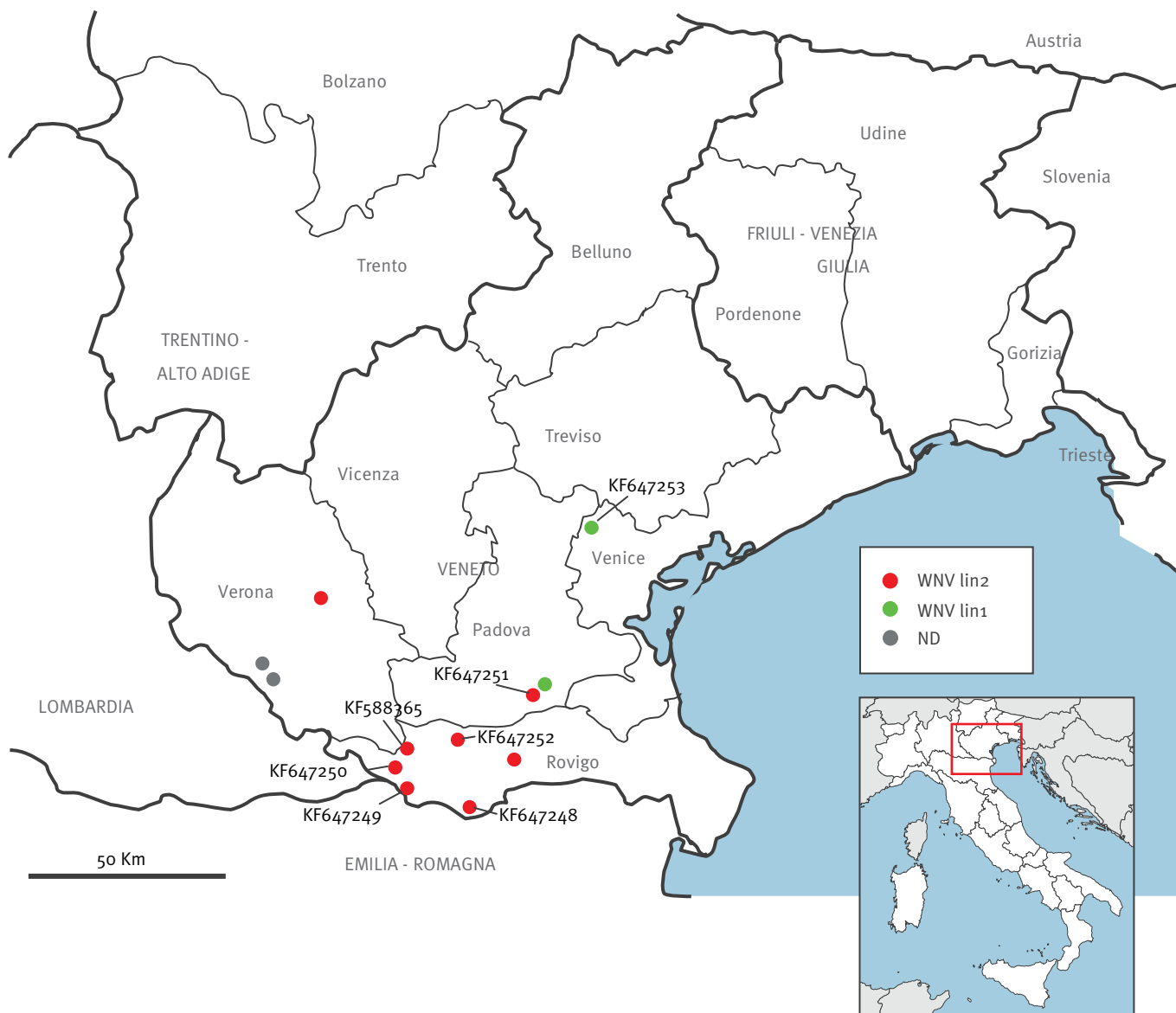
Isolation of the virus in cell cultures was obtained from urine samples collected from three patients with WNND or WNF and from two blood donors. Laboratory methods were performed as previously described [2].

Epidemiological situation of West Nile virus infection in Europe and Italy

Since 2010, WNV, a mosquito-borne flavivirus, has become a public health concern in Europe, as it has been responsible for an increasing number of epidemic outbreaks in European countries and in neighbouring countries in the Mediterranean basin as well as in the Russian Federation [3]. In fact, after large human outbreaks with hundreds of cases of neuroinvasive disease (WNND) occurred in Romania in 1996 and in Russia in 1999, only small outbreaks were reported in European and Mediterranean countries, generally caused by WNV lin1 strains [4]. The epidemiological situation in Europe changed in 2010, when two large human outbreaks occurred in Greece [5] and in the Volgograd region, Russia [6,7]. During these two outbreaks, two

FIGURE 1

Map showing the places of residence of human cases of West Nile virus (WNV) infection confirmed in the Veneto region, Italy, August 2013 (n=12)



ND: WNV case whereby WNV lineage was not determined; WNV lin1: WNV case with WNV lineage 1 infection; WNV lin2: WNV case with WNV lineage 2 infection.

GenBank accession numbers of WNV genome sequences are indicated near the corresponding case symbols.

unrelated WNV lin2 genotypes were respectively characterised. The genotype in the Greek outbreak (Greece-Nea Santa-2010) [8] was similar to that first found in a goshawk in Hungary in 2004 (Hungary04 strain) [9] while in the Volgograd outbreak the genotype was similar to that prior detected in human brain and blood samples in 2007 in Volgograd [6]. Recent epidemiological data indicate that WNV lin2 of the Greek/Hungarian cluster is spreading to other central and southern European countries [10,11], such as Serbia, where a large human outbreak occurred in 2012 [7] and is ongoing in 2013 [1].

The outbreak described in this report represents the first human outbreak of WNV lin2 infection reported in Italy. In fact, in Italy, most cases of human infection reported before 2013 were caused by WNV lin1. In particular, two unrelated WNV lin1 strains, both classified within the Mediterranean cluster by phylogenetic analysis, had been responsible for two different outbreaks in northern Italy. The first outbreak between 2008 and 2009 was caused by the WNV lin1 Italy 2008–2009 strain [12–14] and occurred in the same areas that are currently affected by WNV lin2 circulation, namely those surrounding the Po river in the Veneto, Emilia-Romagna, and Lombardy regions [12,15]; the second

TABLE 1

Clinical and laboratory data of laboratory-confirmed cases of West Nile virus infection, Veneto region, Italy, August 2013 (n=12)

Case	Time to diagnosis (days) ^a	Symptoms	Laboratory findings ^b	WNV RNA load in plasma (GE/mL)	WNV RNA load in urine (GE/mL)	Type of sample where WNV was isolated	WNV genome sequencing (GenBank name, accession number, type of sample)	WNV lineage
WNND	3	Fever, meningitis, upper limbs' paralysis	WNV RNA in plasma and urine, WNV IgM+/IgG- in serum and CSF, viral isolation in culture	1,300	8,300,000	Urine	Italy/2013/Rovigo/32.1, KF588365; urine	2
WNND	12	Arthralgia, encephalitis, fever, headache	WNV RNA in plasma, WNV IgM+/IgG- in serum and CSF	190,000	NA	WNV not isolated	Italy/2013/Rovigo/34.1, KF647248; plasma	2
WNND	2	Fever, meningitis,	WNV RNA in urine, WNV IgM+/IgG+ in serum and CSF, viral isolation in culture	Undetectable	15,000,000	Urine	Italy/2013/Padova/34.1, KF647251; urine	2
WNND	4	Fever, encephalitis, headache	WNV RNA in plasma and urine, WNV IgM+/IgG+ in serum	100	1,300,000	WNV not isolated	Italy/2013/Rovigo/35.1, KF647252; urine	2
WNND	2	Fatigue, fever, encephalitis, vomiting, rash	WNV RNA in plasma and urine, WNV IgM+/IgG- in serum and CSF	400	350,000	WNV not isolated	ND	2
WNF	21	Arthralgia, fatigue, fever, myalgia	WNV RNA in urine, WNV IgM+/IgG+ in serum	Undetectable	350	WNV not isolated	ND	ND
WNF	10	Arthralgia, fatigue, fever, headache, myalgia	WNV RNA in urine, WNV IgM+/IgG- in serum	Undetectable	1,200,000	WNV not isolated	Italy/2013/Rovigo/33.1, KF647250; urine	2
WNF	8	Arthralgia, fatigue, fever, headache, rash	WNV RNA in urine, WNV IgM+/IgG- in serum, viral isolation in culture	Undetectable	180,000	Urine	Italy/2013/Rovigo/33.2, KF647249; urine	2
WNF	14	Arthralgia, fatigue, fever, headache, myalgia	WNV IgM+/IgG+ in serum	Undetectable	Undetectable	WNV not isolated	ND	ND
Organ donor	0	NR	WNV RNA in plasma	3,800	NA	NA	ND	1
Blood donor	5	Asymptomatic	WNV RNA in plasma and urine, WNV IgM-/IgG-, viral isolation in culture	3,900	1,000	Urine	Italy/2013/Livenza/35.1, KF647253; urine	1
Blood donor	4	Arthralgia, headache, myalgia	WNV RNA in plasma and urine, WNV IgM-/IgG-, viral isolation in culture	50,000	3,200	Urine	ND	2

CSF: cerebrospinal fluid; GE: genome equivalents; NA: sample not available; ND: not determined; NR: not reported; WNF: West Nile fever; WNND: West Nile neuroinvasive disease; WNV: West Nile virus.

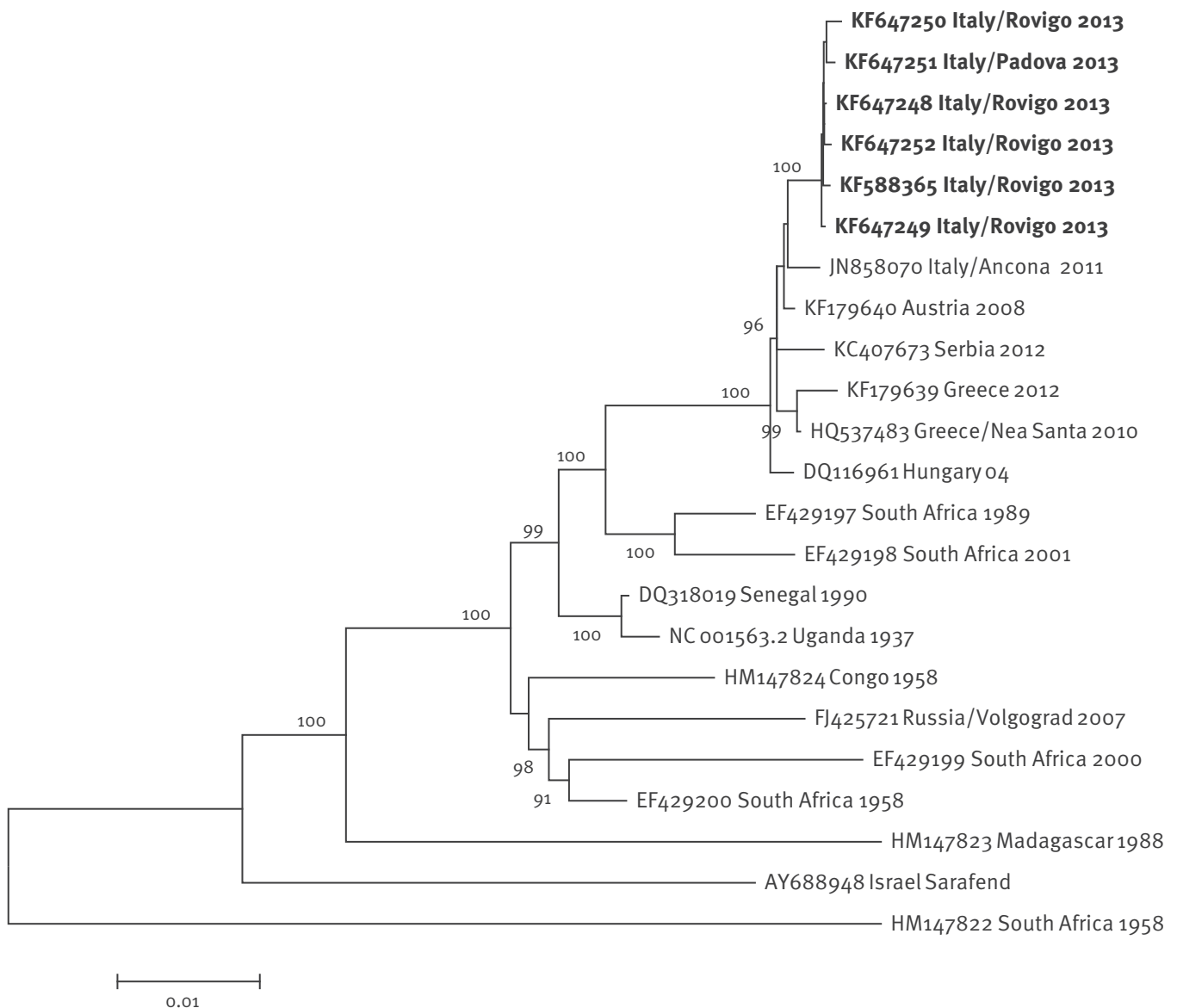
Fever is determined as body temperature $\geq 38^{\circ}\text{C}$.

^a This is the time interval between symptom onset or blood/organ donation (for blood or organ donors) and diagnosis.

^b WNV RNA was detected by real-time reverse-transcription polymerase chain reaction; WNV IgM and IgG antibodies were detected by enzyme-linked immunosorbent assay (ELISA) and confirmed by plaque reduction neutralisation assay.

FIGURE 2

Molecular phylogenetic analysis of six West Nile virus lineage 2 genome sequences detected in individuals from the Veneto region, Italy, August 2013



Sequences detected in individuals from the Veneto region are shown in bold font. The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model [25]. The bootstrap consensus tree inferred from 1,000 replicates [26] is taken to represent the evolutionary history of the taxa analysed [26]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches (values ≥ 80) [26]. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 23 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 8,586 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [27].

outbreak took place between 2011 and 2012 in the Venice and Treviso provinces of the Veneto region and was caused by the WNV lin1 Livenza strain [16,17].

Before 2013 only two unrelated human cases of WNV lin2 infection representing the Greek/Hungarian cluster were documented in the country. These had occurred in 2011 and included one case in Ancona (Marche region) and one in Olbia (Sardinia region), respectively [18,19]. In 2011 and 2012, however, WNV lin2 belonging to the

Greek/Hungarian cluster was detected by entomological and veterinary surveillance in the island of Sardinia as well as in the Veneto and Friuli-Venezia Giulia regions in north-eastern Italy in areas where WNV lin1 was also circulating [20-22].

The fact that most human cases from Veneto in August 2013 are affected by WNV lin2 could suggest that this lineage has now become more widespread in north-eastern Italy and that it is playing an important part in

TABLE 2

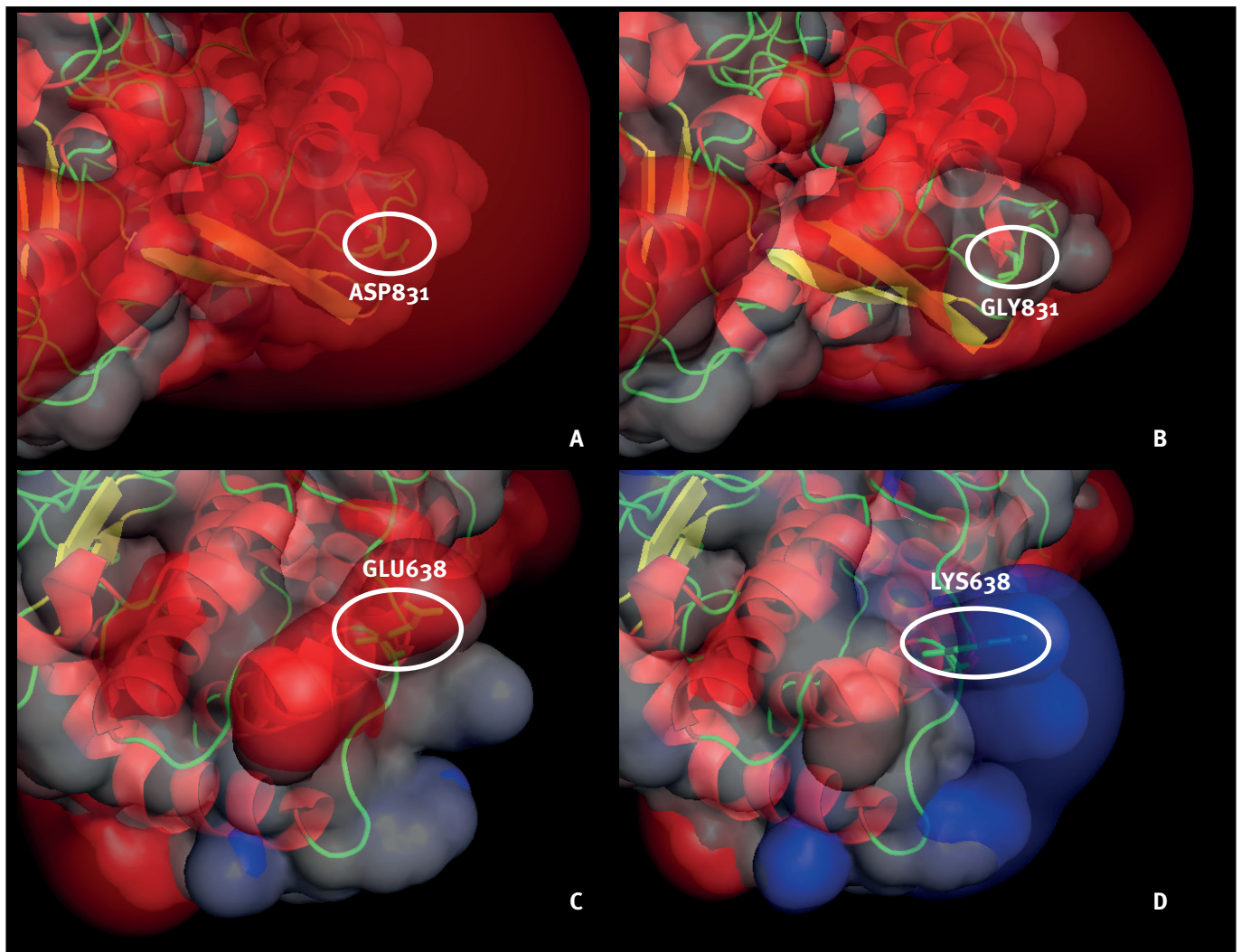
Variant amino acids encoded by West Nile virus (WNV) lineage 2 genome sequences, including the reference Hungary 2004 sequence, other related sequences, and six genome sequences obtained during the WNV outbreak in Italy, August 2013

Genome sequence name GenBank accession number	Mature peptide Amino acid position in mature peptide (in polyprotein)																													
	PrM	M	E			NS1			NS2A	NS2B	NS3	NS4A	NS4B			NS5														
Hungary 04 DQ116961	A	T	I	S	I	V	L	G	A	A	H	S	V	Q	H	I	V	S	T	V	A	Y	G	A	N	R	E	T	D	885 (3414)
Italy/2013/ Rovigo/32.1 KF588365	A	A	I	P	T	V	L	E	V	A	Y	C	V	Q	H	I	V	S	T	V	T	H	G	T	S	R	K	T	D	A
Italy/2013/ Rovigo/33.1 KF647250	A	A	I	P	T	V	L	E	V	A	Y	C	V	Q	H	I	V	S	T	V	T	H	G	T	S	R	E	T	G	A
Italy/2013/ Padova/34.1 KF647251	A	A	M	P	T	G	S	E	V	A	Y	C	V	Q	H	I	V	S	T	V	T	H	G	T	S	R	E	T	D	A
Italy/2013/ Rovigo/34.1 KF647248	A	A	I	P	T	V	L	E	V	A	Y	C	-	Q	H	I	M	S	T	V	T	H	G	T	S	R	E	T	D	A
Italy/2013/ Rovigo/33.2 KF647249	A	A	I	P	T	V	L	E	V	A	Y	C	I	Q	H	I	V	S	T	V	T	H	G	T	S	R	E	T	D	A
Italy/2013/ Rovigo/35.1 KF647252	A	A	I	P	T	V	L	E	V	A	Y	C	V	Q	H	I	M	S	T	V	T	H	G	T	S	R	E	T	D	A
Austria 2008 KF179640	A	T	I	P	I	V	L	E	A	A	Y	S	V	Q	H	I	V	S	T	V	T	Y	G	A	N	R	E	T	D	V
Greece-Nea Santa-2010 HQ537483	A	T	I	P	I	V	L	E	A	A	Y	S	I	Q	P	I	V	G	A	M	T	Y	G	A	N	R	E	T	D	V
Italy-AN-2 2011 JN858070	V	T	I	P	T	V	L	E	A	V	Y	S	V	Q	H	T	V	S	T	V	T	Y	S	T	N	H	E	A	D	V
Serbia 2012 KC407673	A	T	I	P	I	V	L	E	A	A	Y	S	V	H	H	I	V	S	T	V	T	Y	G	A	N	R	E	T	D	V

E: envelope protein; M: membrane protein; NS: non-structural protein; PrM: pre-membrane protein. Amino acids that are unique in WNV lineage 2 genomes from Italy 2013 are highlighted in red. (-) indicates missing sequence information in partially sequenced genome.

FIGURE 3

3D images of mutations in the RNA-directed RNA polymerase domain of West Nile virus non-structural protein 5 (NS5)



Panels A and B show a mutation from ASP831 to GLY831 and panels C and D show a mutation from GLU638 to LYS638.

The 3 D images are predicted by *in silico* site-directed mutagenesis based on the Protein Data Bank (PDB) 2HCN template [28] Best rotamer of side chain of LYS638 has been placed in place of corresponding GLU638 in 2HCN template. GLY831 has been obtained by removing the side chain of corresponding ASP831 in 2HCN template. Structures rendering has been obtained by Pymol 1.5.0.3 (<http://www.pymol.org>). Per-atom charge and radius have been calculated by CHARMM force field using pdb2pqr tool (<http://www.poissonboltzmann.org/pdb2pqr> [29,30]). Molecular surface is coloured according to the potential on solvent accessible surface and has been calculated using APBS tool (<http://www.poissonboltzmann.org/apbs> [31]) for Poisson-Boltzmann electrostatics and shown in the range of -5/+5 kT/e.

Negative isosurface and positive isosurface are shown with a gradient that goes from red (-2 kT/e) to blue (+2 kT/e) respectively.

GLY831 weakens the negative surface (see panel B compared with panel A) whereas LYS638 contributes to change the sign of the surface charge from negative to positive (see panel C compared with panel D).

the current human outbreak. To gain more insight into the origin of the WNV lin2 and WNV lin1 involved in this ongoing outbreak, respective genome sequences were sought.

Genome sequences derived from the 2013 West Nile virus outbreak cases in Veneto

A total of seven WNV lin2 and one WNV lin1 genome sequences were derived from blood or urine samples of cases, including two full genome sequences of WNV

lin2 sequenced from samples collected at three days-interval from the same patient. Six WNV complete genome sequences and one almost complete were submitted to GenBank with accession numbers KF588365 and KF647248–KF647253.

Analysis of the West Nile virus lineage 1 sequence
Sequencing of the full genome of WNV lin1 detected in a blood donor from Venice province (i.e. Italy/2013/Livenza/35.1, GenBank accession number: KF647253)

demonstrated over 99.9% nucleotide sequence identity with the Livenza strains fully sequenced in 2011 and 2012 and responsible for the large human outbreak that occurred in Venice and Treviso provinces in 2012 [16,17]. This finding demonstrates that the Livenza strain is still circulating in the affected area.

Phylogenetic and amino acid analyses of West Nile virus lineage 2 sequences

Sequence alignment demonstrated that all the genome sequences derived from the cases infected with WNV lin2 (WNV lin2 Italy/2013) shared over 99.9% nucleotide sequence identity, and the two WNV lin2 genome sequences derived from the same patient had 100% identity. At variance, the identity with other WNV lin2 genomes was lower, e.g. 99.5% vs the WNV lin2 Hungary04 strain [9] and 99.4% vs a WNV lin2 Greece-Nea Santa-2010 strain [8]. Likewise 99.7% and 99.1% nucleotide sequence identities were also respectively observed with the NS3 and NS5 regions of a WNV lin2 detected in a mosquito pool collected in 2012 in Rovigo province, in the same area of the current outbreak [21]. Finally the present outbreak sequences presented 99.5% nucleotide sequence identity with the full genome of the WNV lin2 isolated from the patient in Ancona in 2011 [19]. Phylogenetic analysis showed that the WNV lin2 Italy/2013 genomes were included in the Greek/Hungarian cluster which contains the Hungarian (Hungary04) and Greek (Greece-Nea Santa-2010) strains, but generated a distinct branch in the phylogenetic tree, indicating that they represent a new strain (Figure 2). This finding suggests that a single monophyletic group of WNV lin2 is arising from the Greek/Hungarian cluster, which corresponds to a group of viruses that are evolving as they reach new territories in their spread from central Europe and areas in the Balkans.

At protein level, the WNV lin2 Italy/2013 genomes encoded a set of unique amino acids compared to other fully sequenced WNV lin2 genomes of the Greek/Hungarian cluster (Table 2). Most of the substitutions compared to the Hungary 04 reference apparently seem not to change dramatically the properties of referring proteins. Nonetheless, mutations observed on the surface of the non-structural protein 5 (NS5) protein (i.e. E638K* and D831G) in two individual genome sequences were predicted by *in silico* site-directed mutagenesis to cause a local altered electrostatic potential in the RNA-directed RNA polymerase domain (Figure 3). The relevance of these mutations will be assessed by further sequencing of WNV genome sequences and by experimental studies with viral isolates and site-directed mutagenesis of infectious clones. Of note, none of the WNV lin2 Italy/2013 genomes had the H249P substitution in non-structural protein 3 (NS3) that characterises the Greece-Nea Santa-2010 strain.

Conclusion

Overall, the results of this molecular epidemiology study shows that genetically different lineages of WNV

are capable of establishing in Europe, remain circulating for several years in the same territory, and spread slowly to neighbouring areas, in agreement with other reports from Europe [13,23]. In this local transmission and spread dynamics in Europe, WNV overwintering in mosquitoes and amplification in local susceptible bird populations are key factors, while WNV re-introduction by migrating birds from long-distance Euro-African routes seems to be less relevant [24].

In conclusion, a novel WNV lin2 strain of the Greek/Hungarian cluster is responsible for a human outbreak of neuroinvasive disease that is ongoing in northern Italy. The virus is co-circulating with the WNV lin1 Livenza strain that caused a large human outbreak in 2012.

*Authors' correction:

At the request of the authors, 'E636K' was replaced with 'E638K'. This change was made on 27 September 2013.

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Conflict of interest

None declared.

Authors' contributions

Luisa Barzon coordinated the study and wrote the manuscript; Monia Pacenti, Riccardo Cusinato, Silvana Pagni, Margerita Cattai, and Laura Squarzon performed surveillance activities and virological tests; Elisa Franchin and Giulia Masi performed WNV genome sequencing; Enrico Lavezzo and Stefano Toppo performed bioinformatics analyses of WNV genome sequences; Francesca Russo coordinated WNV surveillance activities; Giorgio Palù supervised the study and revised the manuscript.

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