

Rapid communications

GENOME SEQUENCE ANALYSIS OF THE FIRST HUMAN WEST NILE VIRUS ISOLATED IN ITALY IN 2009

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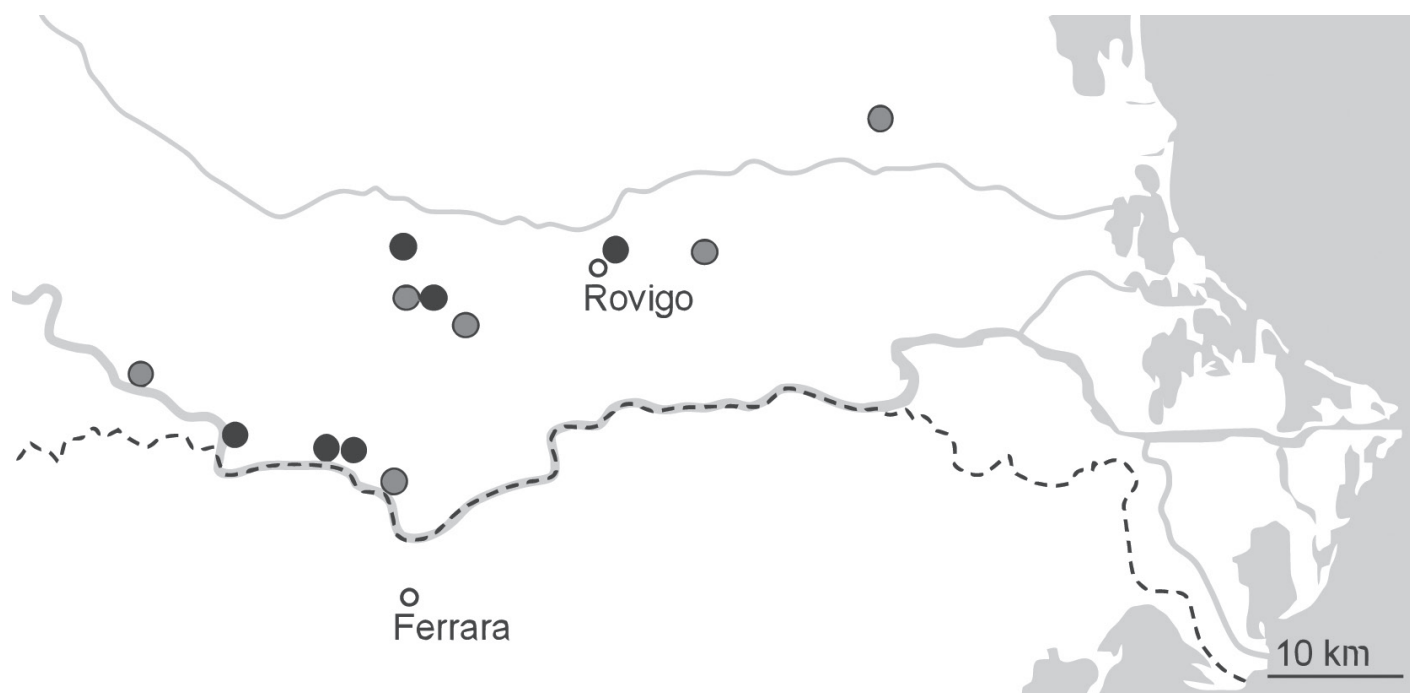
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In 2009, six new human cases of West Nile neuroinvasive disease (WNND) were identified in Veneto region, following the six cases already reported in 2008. A human West Nile virus (WNV) isolate was obtained for the first time from an asymptomatic blood donor. Whole genome sequence of the human WNV isolate showed close phylogenetic relatedness to the Italy-1998-WNV strain and to other

WNV strains recently isolated in Europe, with the new acquisition of the NS3-Thr249Pro mutation, a trait associated with avian virulence, increased virus transmission, and the occurrence of outbreaks in humans.

FIGURE 1

Sites where human cases of symptomatic West Nile neuroinvasive disease occurred in Veneto region, Italy, in 2008 and 2009



Dark grey dots: cases in 2008
Light grey dots: cases in 2009

Introduction

In Italy, the first outbreak of West Nile virus (WNV) infection was reported in 1998 among horses residing in Tuscany region [1]. The virus re-emerged in Italy in 2008, when equine and human cases of West Nile neuroinvasive disease (WNND) were notified in Veneto and Emilia Romagna regions [2,3]. In Veneto region, six clinical cases of WNV infection were identified with disease onset in August-September 2008 and all were from Rovigo province [4]. Three further human cases of WNND were notified in Emilia Romagna region in September-October 2008 [5]. Moreover, the veterinary and entomological surveillance documented that WNV infection was widespread in the same areas in north-eastern Italy [2]. In 2008, WNV strains were isolated from one horse in Rovigo province, Veneto region, and from one donkey, one pigeon and three magpies in Ferrara province, Emilia-Romagna region. Sequencing of 255 bp of the WNV *E* gene showed the virus had 100% amino acid identity with the equine strain isolated in Tuscany in 1998 [6]. The complete genome sequences of two WNV strains isolated from magpies in Italy in 2008 were also deposited in the Genbank database (Accession No. FJ483548 and FJ483549).

In 2009, further 16 human cases of WNND were notified in northern Italy, including six from Veneto region, eight from Emilia-Romagna region and two from Lombardia region, as recently reported in a detailed description of the epidemiological situation in Italy [7].

Here we report the results of genome sequencing of the first human WNV isolate reported in Italy, which provide evidence of the emergence of a strain more virulent than the WNV strain isolated in Italy in 1998. Moreover, we report further clinical and epidemiological details on human cases of symptomatic WNV infection detected in 2009 in Veneto region.

Samples and methods

Human cases of West Nile neuroinvasive disease in Veneto region, 2009

A surveillance programme for possible human cases of WNND has been implemented in Veneto region since September 2008, as reported previously [4]. According to this programme, all possible cases of WNV infection are referred to our Regional Reference Laboratory which performs the following diagnostic tests [4]: detection of WNV RNA in plasma and cerebrospinal fluid (CSF) samples by real-time RT-PCR and detection of IgM and IgG antibodies against WNV in serum and CSF samples by ELISA testing (Focus Diagnostics, Cypress, CA). ELISA-positive samples are further tested by plaque-reduction neutralisation test (PRNT) for confirming specificity of antibody response, while WNV RNA-positive samples are inoculated onto confluent monolayers of Vero E6 cells for virus isolation. Moreover, nucleic acid test (NAT) screening for WNV RNA has been applied to all blood, tissue and organ donations collected from 1 August to 30 October 2009 in the province of Rovigo.

In August-September 2009, six new cases of WNND were identified in Veneto region, following the six cases reported in 2008 [4]. Five of the patients in 2009 were resident in Rovigo province and one in a village in the south of Venice province, not far from Rovigo province (Figure 1).

To date, no cases of West Nile fever have been notified in 2009. Detailed clinical and laboratory data of cases are summarised in Table 1.

Genome sequence analysis of the first human West Nile virus isolate reported in Italy

At the end of August 2009, a WNV strain was isolated in Vero E6 cells from a NAT-positive blood donation of an asymptomatic individual resident in Rovigo province. At the time of blood donation, the donor was WNV IgM- and IgG-negative but after

TABLE 1

Clinical and laboratory data on cases of West Nile neuroinvasive disease notified in Veneto region, Italy, 2009

Referral date	Sex/age (years)	Symptoms	Laboratory data	Outcome*	Province
31 Aug	M/76	Fever, severe meningoencephalitis	IgM+/IgG+ in serum and CSF, PRNT confirmed, WNV RNA-negative.	Amelioration of symptoms, discharged from hospital on 13 October	Rovigo
8 Sep	F/78	Fever, severe meningoencephalitis	IgM+/IgG+ in serum and CSF, PRNT confirmed, WNV RNA-positive in plasma.	Still hospitalised, amelioration of symptoms	Venice
10 Sep	M/82	Fever, headache, severe meningoencephalitis	IgM+/IgG+ in serum and CSF, PRNT confirmed, WNV RNA-negative.	Death on 17 September	Rovigo
11 Sep	M/62	Fever, headache, severe meningoencephalitis	IgM+/IgG- in serum and CSF, PRNT confirmed, WNV RNA-negative.	Amelioration of symptoms, discharged from hospital on 25 September	Rovigo
24 Sep	M/78	Guillain-Barré syndrome	IgM+/IgG+ in serum and CSF, PRNT confirmed, WNV RNA-positive in plasma.	Still hospitalised with severe disease	Rovigo
28 Sept	F/84	Fever, arthritis, severe meningoencephalitis	IgM+/IgG- in serum and CSF, PRNT confirmed, WNV RNA-negative.	Still hospitalised with symptoms	Rovigo

WNV: West Nile virus; CSF: cerebrospinal fluid; PRNT: plaque-reduction neutralisation test.

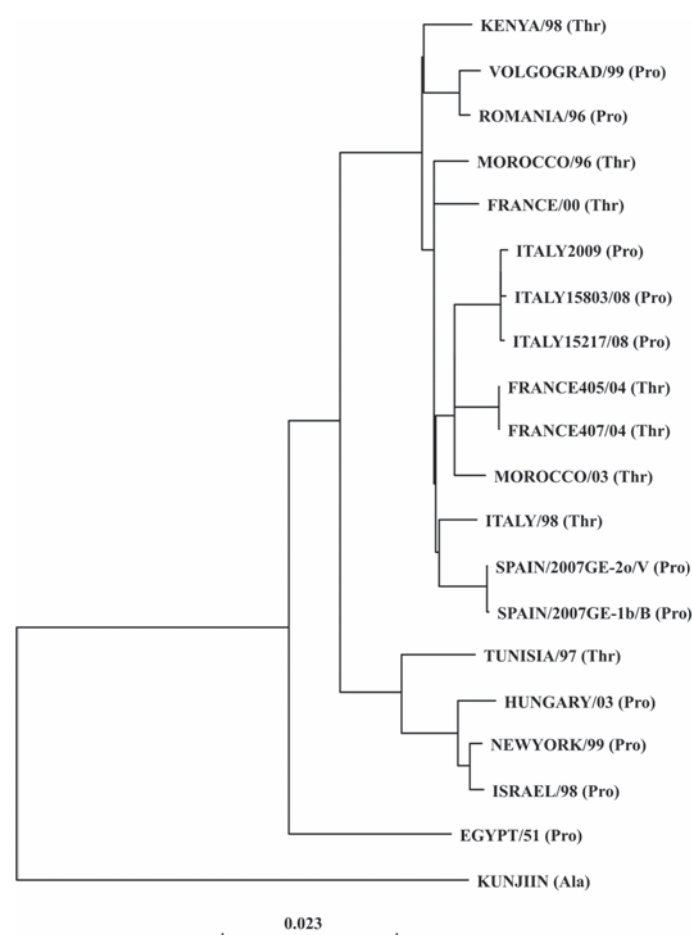
*As of 5 November 2009 (date of publication).

a few days showed seroconversion and remained asymptomatic. WNV growth in cell cultures was demonstrated by the presence of cytopathic effect in the monolayer and detection of WNV-RNA at real-time RT-PCR testing.

For WNV genome sequencing, the supernatant of infected Vero E6 cells at the first passage was collected for RNA and PCR amplification with a set of 21 primer pairs targeting overlapping sequences of ~600 nucleotides in WNV genome. Primer sequences are available upon request. Amplicons underwent bi-directional sequencing by using the BigDye® Terminator Sequencing Kit on

FIGURE 2

Maximum likelihood phylogenetic tree of aligned complete genome sequences of 20 West Nile virus strains, including the strain isolated from a blood donation in the province of Rovigo, Veneto region, in 2009, and two strains isolated from magpies in Italy, in 2008



ITALY2009: human, from blood donation in Rovigo province, Italy, 2009 (Genbank Accession No. GU011992)

ITALY15217/08 and ITALY15803/08: magpies, Italy, 2008

The amino acid at each NS3-249 site is indicated between brackets. Branch lengths are drawn to scale to indicate the number of nucleotide changes (genetic distances). Scale bar shows the number of base substitution per site. All WNV strains belong to lineage 1, clade 1a, with the exception of Kunjin virus (outgroup) which belongs to clade 1b.

a 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). After alignment and assembling with the SeqScape v2.5 software (Applied Biosystems), the consensus sequence (Genbank Accession No. GU011992) was aligned using ClustalW and Blastp with genome sequences of the following WNV strains: Kunjin 1973 (MRM61C; Westaway; Accession No. D00246), Egypt 1951 (Eg101; Accession No. AF260968), Romania 1996-mosquito (R097-50, *Culex pipiens*, Bucharest, Romania; Accession No. AF260969), Italy 1998-equine (PaAn981, Tuscany, Italy; Accession No. AF404757), Volgograd 1999-human (Accession No. AF317203), NY 1999-human (Accession No. AF202541), Spain 2007 GE-1b/B and GE-2o/V (golden eagle *Aquila chrysaetos*; Accession No. FJ766331 and FJ766332, respectively), France 407/2004 and 405/2004 (house sparrow *Passer domesticus* and common magpie *Pica pica*; Accession No. DQ786573 and DQ786572, respectively), Morocco 2003-equine (Accession No. AY701413), France 2000-equine (PaAn001, Accession No. AY268132), Morocco 1996-equine (Accession No. AY701412), Kenya 1998-mosquito (KN3829; Accession No. AY262283), Tunisia 1997-human (PaH001; Accession No. AY268133), Hungary 2003-goose (*Anser anser domesticus*; Accession No. DQ118127), Israel 1998-goose (*Anser anser domesticus*; Accession No. AF481864). Two WNV strains, 15217 and 15803, isolated from magpies in Italy in 2008 (Genbank Accession No. FJ483548 and FJ483549, respectively) were also included in the analysis. The aligned nucleic acid sequences were used to construct a phylogenetic tree using the maximum likelihood algorithm within Phylo_Win v2.0 software with bootstrap resampling analysis (500 iterations) (Figure 2).

Results

Phylogenetic tree analysis of the complete genome sequence of 20 WNV strains shows that the human WNV strain isolated in Italy in 2009 belongs to lineage 1, clade 1a, and is closely related to the two WNV strains isolated from magpies in Italy in 2008 (average nucleotide and amino acid divergence of 0.14% and 0.07%, respectively) (Figure 2). Both the human 2009 WNV isolate and the WNV strains isolated from magpies in Italy in 2008 were phylogenetically related to strains isolated since 1996 in the western Mediterranean area, including the Italy 1998-equine WNV strain (Figure 2). In particular, nucleotide and amino acid divergence of the 2009-human WNV isolate from the Italy 1998-equine WNV strain was 1.62% and 0.25%, respectively. All amino acid changes among Italian WNV isolates are detailed in Table 2.

The 2008-2009 Italian WNV isolates had a higher degree of divergence from the eastern European strains isolated in Romania in 1996 and in Russia in 1999 and from the American/Israeli cluster (Figure 2). Our findings obtained with WNV complete genome sequences, which confirm the results of a recently reported detailed genetic analysis of Mediterranean WNV strains [8], provide a more detailed picture of WNV evolution in Italy and in the Mediterranean area than the phylogenetic analysis performed on a partial sequence of the WNV *E* gene obtained from veterinary samples in Italy in 2008 [6].

Based on these results, we believe that the WNV strain responsible for the recent outbreaks might have originated from the Italy 1998-equine strain, since the virus seems to have had

a continuous low level, endemic circulation in Italy from 1998 to 2008. The virus might have also evolved somewhere else in western Mediterranean area and then it might have been reintroduced in Italy, for instance by migratory birds. The rapid spread in the last two years in Italy, with the occurrence of human cases of WNND, might be due to the positive selection of amino acid mutations in viral proteins conferring increased virulence and transmission capacity. In this regard, it is interesting to note that, in comparison with the Italy 1998-equine strain and with other western Mediterranean strains, the recent Italian WNV isolates have acquired the Thr249Pro mutation in the helicase domain of the NS3 protein, a trait associated with avian virulence [9]. In fact, this mutation is predicted to confer higher stability to the NS3 protein at high temperature conditions, such as in avian hosts, where the mutated virus can efficiently replicate leading to high levels of viraemia in birds that may facilitate the infection of new mosquito vectors. In support of this hypothesis, high mortality rates were reported among birds in the United States (US) and Israel, whereas seroprevalence studies in Romania indicated significant infection of resident birds [9,10]. It is important to note that the NS3 Thr249Pro mutation has emerged on at least three independent occasions (i.e., in the 1951 Egyptian isolate, in the 1996 Romanian isolate and within the Israeli/North American clade) and, in each case, viruses carrying this substitution have been associated with human disease outbreaks [9]. The WNV strains isolated from golden eagles in Spain in 2007 also carry the NS3 Thr249Pro change [8]. Studies in mice showed that the Spanish isolates do not have increased pathogenicity as compared with other strains, but virulence in birds has not been investigated [8].

Conclusions

Since 2008, an outbreak of WNV infection is ongoing in north-eastern Italy, in areas surrounding the Po river delta. The Italian outbreak is characterised by the occurrence of cases of severe meningoencephalitis [3-5,7], as also described in the recent outbreaks in the US [11], Romania [12], Israel [13], and Russia [14]. The number of human cases of WNND identified in the province of Rovigo represents about 1% of all cases of WNV infection occurring in 2009 in Rovigo province as estimated from

the preliminary results of an ongoing seroepidemiological survey on blood donors.

Genome sequencing of WNV isolates is providing insight into the mechanism of re-emergence of this virus in Italy. In fact, the human WNV strain isolated this year and the strains isolated from magpies in 2008 are closely related to the Italy 1998-equine strain and to other western Mediterranean strains, with the acquisition of new amino acid mutations in non-structural proteins. These mutations include the Thr249Pro change in WNV-NS3 helicase, a trait associated with avian virulence and rapid geographic diffusion of WNV in North America [9]. In this regard, the veterinary and entomologic surveillance demonstrates that the virus is endemic in Italy and that it is rapidly spreading to other regions [15]. However, at variance with the WNV outbreaks in the US and Israel [16], the Italian outbreak does not seem to be associated with a particularly high mortality rate among birds [15]. The mechanisms of susceptibility of different bird species for WNV virulence is still unknown and might be related both to the genetic and immunological characteristics of the avian hosts and to the particular genetic backbone of each WNV strain [16].

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TABLE 2

Description of amino acid differences among Italian West Nile virus strains

AA position in WNV polyprotein	AA position in WNV proteins	Italy- 98 AF404757 (equine)	Italy-08 FJ483548 (magpie)	Italy-08 FJ483549 (magpie)	Italy-09 GU011992 (human)
851	NS1-60	Val	Val	Val	Ala
1228	NS2A-85	Ile	Val	Val	Val
1248	NS2A-105	Ile	Ile	Thr	Ile
1494	NS2B-120	Ile	Ile	Val	Val
1754	NS3-249	Thr	Pro	Pro	Pro
2209	NS4A-85	Val	Ile	Ile	Ile
2224	NS4A-100	Pro	Ser	Ser	Ser
2581	NS5-53	His	His	Tyr	His
2786	NS5-258	Val	Ala	Ala	Ala
2950	NS5-422	Arg	Lys	Lys	Lys

AA: amino acid; NS: non-structural protein.

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