

# WEST NILE VIRUS INFECTION IN VENETO REGION, ITALY, 2008-2009

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We report here an update on human cases of West Nile virus (WNV) infection in Veneto region, northeastern Italy. In addition to two cases of WNV neuroinvasive disease notified through a surveillance programme started in September 2008, further four cases were retrospectively identified (in May 2009) by investigating patients with aseptic meningoencephalitis of unknown aetiology occurring in Veneto region in June-September 2008. All six patients had symptom onset in August-September 2008 and were resident in a wetland area close to the Po river delta in Rovigo province. Further five cases of asymptomatic WNV infection, including four residents of the same area in Rovigo, were identified in a seroprevalence study in farm workers from Veneto region. To date, no human cases have been notified in 2009.

### Introduction

In Italy, the first outbreak of West Nile virus (WNV) infection was reported in the late summer 1998 among horses residing in a wetland area in Tuscany. At that time, 14 horses had neurological illness and six of them died, but no human cases of WNV disease were reported [1]. Subsequently, a national veterinary surveillance plan for WNV was activated in 2002 in Italy, aiming to identify risk areas and to monitor WNV circulation based on observation of wild bird mortality, and on entomological and sentinel chicken surveillance, as well as to check for WNV seroconversion in horses residing in risk areas. Thereafter, sporadic seroconversions have been identified in sentinel chickens and horses [2,3], but no equine or human cases of symptomatic WNV infection had been notified until September 2008, when an outbreak of WNV infection was identified in the northeastern part of Italy [4,5].

The first possible case of WNV neuroinvasive infection in a horse was notified on 8 September 2008 in Emilia-Romagna region, Italy. A special plan for WNV surveillance was subsequently activated in Emilia-Romagna on 16 September, which led to the identification of other horses with WNV neuroinvasive illness [4] and, on 20 September 2008, to the identification of the first human case of meningoencephalitis caused by WNV infection in a female patient who lived in a rural area between Ferrara and Bologna in Emilia-Romagna region, and had symptom onset on 15 September 2008 [5].

In Veneto region, the first WNV-seropositive horse was identified on 24 September 2008 in a stable in Rovigo province, where a horse presented neurological symptoms after being brought back

from Emilia-Romagna region. Thereafter, on 29 September 2008, Veneto region activated a special veterinary surveillance plan in horse stables of Rovigo, Venezia and Padova provinces, and started a seroepidemiological investigation of all workers on farms where infected horses were identified, as well as a surveillance programme for possible human cases of WNV infection in Veneto region. To identify cases that might have occurred before the implementation of these surveillance activities, in May 2009, we performed a retrospective investigation of cases of aseptic meningoencephalitis of unknown aetiology occurring in Veneto region in June-September 2008. Here we describe the results of this retrospective study as well as provide an update on cases reported through the surveillance programme and on those identified in the seroepidemiological study of stable workers, and present the results of screening of blood and organ donations from the affected area.

### Retrospective study of cases of aseptic meningoencephalitis

#### Methods

To identify cases of WNV neuroinvasive disease occurring before the activation of the surveillance programme in Veneto region, we retrospectively analysed cerebrospinal fluid (CSF) samples referred to our Regional Reference Centre from hospitals of Veneto region in the period June-September 2008 for the presence of specific immunoglobulin M (IgM) antibodies against WNV. This study was performed in May 2009.

CSF samples from patients aged  $\geq 15$  years with suspected viral encephalitis, but with negative viral test results (routine PCR and serology tests for herpes simplex virus (HSV), varicella zoster virus (VZV), enteroviruses, tick-borne encephalitis virus (TBEV), Toscana virus (TOSV) and other neurotropic viruses), were selected for the study, according to definition criteria for possible cases of WNV neuroinvasive disease (Table 1).

WNV IgM testing was done by using WNV IgM capture DxSelect™ ELISA (Focus Diagnostics, Cypress, California) according to the manufacturer instruction, with the exception that CSF was diluted 1:2, as recommended by Prince *et al.* [6]. CSF samples which were positive at WNV IgM capture ELISA were tested for neutralising antibodies by plaque-reduction neutralisation test (PRNT) for WNV and for tick-borne encephalitis virus (TBEV), a flavivirus commonly found in northeastern Italy, to rule-out cross-reactivity. PRNT was conducted in a biosafety level 3 lab, according to the protocol

described in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2008 of the World Organisation for Animal Health (OIE). Briefly, heat-inactivated CSF or serum samples were tested at 1:100 final dilution. Equal volume of serum and medium containing 100 plaque-forming units of WNV were incubated for 75 min at 37 °C before inoculation onto confluent monolayers of Vero E6 cells grown in 25 cm<sup>2</sup> flasks. After the inoculum was adsorbed for 1 h at 37 °C, cells were overlaid with agarose-containing medium, and then incubated for 72 h at 37 °C. Then, a second agarose overlay containing 0.003% neutral red dye was applied to each flask for plaque visualisation. Following a further overnight incubation at 37 °C, the number of virus plaques per flask was assessed. Endpoint titres were assigned as the greatest dilution in which >90% neutralisation of the challenge virus was achieved. Samples with reciprocal 90% neutralisation titres of >10 were considered positive. WNV IgM-positive CSF samples were also tested by real-time RT-PCR for WNV-RNA detection using the oligonucleotide primers and TaqMan probe targeting the WNV E gene designed by Lanciotti et al. [7]. For real-time RT-PCR, nucleic acids were purified from 200 µl CSF or plasma samples by using an NucliSENS® easyMAG® system (bioMérieux, Inc., Durham, NC) and eluted in a final volume of 50 µl. Then, 5 µl of RNA was combined with Superscript® One Step RT-PCR System reagents (Invitrogen Ltd, Paisley, UK), primers and probe in a 20-µl total

reaction volume and amplified in a LightCycler® 2.0 Real-Time PCR System (Roche Diagnostics S.p.A., Monza, Italy).

### Results

Of the 74 investigated patients (40 males and 34 females; median age 51.5 years, range 21-94 years) with aseptic meningoencephalitis of unknown aetiology, four (a 69-year-old woman and three men aged 69, 70, and 86 years) had IgM antibodies against WNV in CSF, as demonstrated by IgM capture ELISA (Table 2). The presence of WNV-specific neutralising antibodies in CSF was confirmed in all four cases by PRNT, which showed neutralisation titres >1:40, while WNV-RNA testing gave negative results. The presence of WNV-reactive neutralising antibodies was also demonstrated in a convalescent serum specimen, subsequently provided. For two patients, two consecutive serum samples were available, which showed an increase of WNV-specific antibody titre. All four WNV-positive patients were resident in Rovigo province and were hospitalised in the period from 25 August to 9 September. One of these patients (male, 70 years old), who had encephalitis in early September 2009, was described as a probable case in a previous report, based on the detection of high titre WNV IgG in February 2009 [8].

**TABLE 1**

#### Case definition of West Nile virus (WNV) neuroinvasive disease, surveillance programme in Veneto and Emilia Romagna regions, Italy, 2008-2009

Subjects ≥ 15 yr with fever ≥ 38.5°C and neurological symptoms (e.g., encephalitis, meningitis, Guillain-Barré syndrome or acute flaccid paralysis). Cases were classified as:	
<i>Possible:</i> clinical symptoms and aseptic CSF.	
<i>Probable:</i> clinical symptoms and at least one of the following laboratory criteria:	<ul style="list-style-type: none"> <li>- presence of IgM antibodies against WNV by ELISA;</li> <li>- seroconversion by ELISA;</li> <li>- fourfold increase of IgG antibodies against WNV in two consecutive samplings (&gt;5 days, preferably 15-20 days between the two samples) by ELISA.</li> </ul>
<i>Confirmed:</i> clinical symptoms and at least one of the following laboratory criteria:	<ul style="list-style-type: none"> <li>- isolation of WNV in blood or CSF;</li> <li>- presence of IgM antibodies in CSF (by ELISA);</li> <li>- detection of WNV-RNA by RT-PCR in blood or CSF;</li> <li>- detection of increased levels of WNV IgM and IgG by ELISA and confirmed by PRNT.</li> </ul>

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

**TABLE 2**

#### Summary of data on cases of West Nile virus (WNV) infection in Veneto region, Italy, 2008-2009

Province	Retrospective analysis of cases of meningoencephalitis of unknown aetiology (June-September 2008) Number of confirmed/total investigated (%)	WNV disease surveillance (October 2008 - July 2009) Number of confirmed/total suspected (%)	Seroepidemiological survey of farm workers (October-December 2008) Number of confirmed/total investigated (%)
Rovigo	4/15 (26.7%)	2/24 (8.3%)	4/212 (1.9%)
Padova	0/21 (0%)	0/17 (0%)	0/92 (0%)
Venezia	0/11 (0%)	0/2 (0%)	1/17 (5.9%)
Vicenza	0/1 (0%)	0/4 (0%)	-
Verona	0/1 (0%)	0/4 (0%)	-
Treviso	0/13 (0%)	0/10 (0%)	-
Belluno	0/12 (0%)	-	-
TOTAL	4/74 (5.4%)	2/61 (3.3%)	5/321 (1.6%)

## WNV infection surveillance in Veneto region, 2008-2009

### Methods

A surveillance programme for possible human cases of WNV infection was activated in Veneto region on 29 September 2008, after the notification of the first equine case on 24 September 2008. All infectious disease units of hospitals in Veneto region were asked to report suspected cases of aseptic encephalitis and/or meningitis of unknown aetiology from all provinces of the region and cases of fever and rash from areas where WNV infection had been documented in horses (initially only Rovigo, eventually also Venice and Padua provinces), and to collect blood and CSF samples from these patients. Specimens of blood and CSF were sent to our Regional Reference Centre and investigated for IgM and IgG antibodies against WNV by ELISA testing (Focus Diagnostics), PRNT to confirm ELISA-positive samples, and WNV real-time RT-PCR, as above described.

### Results

Within this ongoing surveillance programme, to date, 61 patients from Veneto region (33 males and 28 females; median age 47 years, range 19-85 years) were reported with suspected WNV infection and referred for further investigation. Of these, 37 were referred in October-November 2008, and 24 were reported in June-July 2009. Of these, only two cases in 2008 were confirmed for WNV infection, as described in a previous report [8]. The first was an 81-year-old woman from Rovigo hospitalised in the end of August 2008 for suspected viral meningoencephalitis with fever, headache, and altered mental status. On October 16, serology testing demonstrated the presence of IgM and IgG antibodies against WNV, confirmed by PRNT; retrospective analysis of a CSF sample collected on 6 September demonstrated the presence of IgM antibodies against WNV, while WNV RNA testing was negative. The second case was a 48-year-old female patient resident in Rovigo province, who had an episode of fever, severe headache, maculopapular rash, pharyngitis, adenopathy, and arthralgia starting in early August 2008. WNV serology testing, performed in the end of November for persistence of symptoms, was positive for IgM and IgG antibodies, confirmed by PRNT.

To date, no human cases of WNV infection have been identified in 2009.

## Seroepidemiological survey

### Methods

A seroepidemiological survey was started in Veneto region on 29 September 2008 involving all workers employed in farms where WNV-positive horses were identified by the veterinary surveillance. The aim of the study was to investigate the prevalence of WNV infection and to promote the awareness of the disease in this at-risk population. In the survey, local Public Health Services conducted interviews with farm workers to ascertain their risk for WNV infection and collected serum samples, which were sent for analysis to our Regional Reference Centre. We tested the samples for IgM and IgG antibodies against WNV by ELISA and used PRNT for confirmation, as above described.

### Results

Of 321 investigated subjects (178 males and 143 females, median age 45 years; range 4-84 years), two men (71 and 76 years old) and three women (51, 60, and 67 years old), all asymptomatic, were IgM and IgG WNV-reactive (two cases) or only IgG WNV-reactive (three cases) and confirmed by PRNT. Four of

these persons were resident in Rovigo province and one in Venice province (Table 2). Four have been previously reported [8].

## Screening of blood and organ donations

### Methods

Following the notification of the first human case of WNV infection in Veneto region, in accordance with the European Union blood safety directive [9], a nucleic acid test (NAT) for WNV RNA screening was started on 28 October 2008 in all blood, stem cells, tissue, and organ donations collected in the period from 1 September to 5 December 2008 from donors who were resident in Rovigo province or who stayed for at least one night in Rovigo province during the last 28 days before donation. In 2009, based on estimates of WNV circulation in Italy, WNV-RNA NAT screening will be done on all donations collected from 1 August to 31 October in Rovigo province, as well as in the provinces of Ferrara (Emilia-Romagna region) and Mantova (Lombardia region).

### Results

During 2008, our Regional Reference Centre individually screened a total of 5,500 donations by using the Procleix WNV Assay (Chiron, Novartis). All donations resulted WNV RNA-negative.

## Discussion

Surveillance of suspected cases of WNV infection and retrospective investigation of cases of meningoencephalitis of unknown aetiology occurring in Veneto region led to the identification of six patients with WNV neuroinvasive disease. All cases were resident in a wetland area of about 40 km in diameter in Rovigo province and had symptom onset in the period ranging from early August to mid-September 2008. The incidence of WNV disease in this area could be estimated at 12 cases per 100,000 population, but this is probably an underestimation because based in part on retrospective data.

In the neighbouring provinces of Ferrara and Bologna in Emilia-Romagna region, three human cases of WNV neuroinvasive disease were reported, with symptom onset in early, mid-, and late September 2008 [5,8].

The seroprevalence study in farm workers from Veneto region demonstrated a low prevalence (<2%) of WNV infection, but, notably, four of the five cases with asymptomatic infection were resident in the above mentioned wetland area in Rovigo province. Moreover, the veterinary survey in horse stables reported the highest seroprevalence in Rovigo province, where 58% horses had WNV-neutralising antibodies [10]. WNV infection appears to be widespread among horses in northeastern Italy. In fact, in 2008, several equine outbreaks of WNV infection were identified in Veneto, Emilia-Romagna, and Lombardia regions, with a total of 794 seropositive horses out of 2,030 investigated (39.1%), including 32 horses with WNV neuroinvasive disease [10,11]. On 28 July 2009, a case of equine WNV disease was notified in Reggio Emilia province (Emilia-Romagna region), which is located outside the area where WNV circulation was identified [12].

We could not recover and characterise the virus responsible for the human cases described here. It was isolated from birds, a horse, and a donkey by the National Reference Veterinary Laboratory [11,13]. Genome sequencing and phylogenetic analysis showed that the virus isolated in 2008 was closely related to the WNV strain isolated during the equine outbreak, which occurred in Tuscany

region in 1998, and to other European strains [11,13,14]. So, both 1998 and 2008 Italian outbreaks could be related to a continuous endemic circulation of WNV, although a recent new introduction of WNV by migratory birds cannot be excluded, since the location of the current outbreak is very close to a migratory bird resting area.

To date, no human cases have been notified in 2009, but it is conceivable that new cases will present this year. In fact, the virus has been frequently isolated from local birds and mosquitoes [10] thus indicating it has established an endemic infection cycle.

In conclusion, a relatively high incidence of WNV infection was observed in August-September 2008 in Veneto region, in an area close to the Po river delta. The burden of WNV infection in this area is probably still underestimated. To clarify this issue, Veneto region has recently started a seroepidemiological study in blood donors from Rovigo province. This will be done on samples obtained from 2,550 blood donors (about 1/3 of all donations) from Rovigo province, for 17 weeks, starting on 15 July 2009.

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