ORIGINAL ARTICLE

West Nile Virus Transmission in 2008 in North-Eastern Italy

P. Calistri[1], A. Giovannini[1], G. Savini[1], F. Monaco[1], L. Bonfanti[2], C. Ceolin[2], C. Terregino[2], M. Tamba[3], P. Cordioli[3] and R. Lelli[1]

[1] Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise ''G. Caporale'', Teramo, Italy
[2] Istituto Zooprofilattico Sperimentale delle Venezie, Padova, Italy
[3] Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna ''Bruno Ubertini'', Brescia, Italy

Impacts

- After 10 years, a West Nile virus (WNV) epidemic was observed in Italy.
- The area involved by WNV infection comprises eight Provinces in three Italian Regions.
- It caused encephalitis in horses and humans.

Keywords:
West Nile virus; Italy; horses; viral encephalitis; wild birds

Correspondence:
Paolo Calistri. Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise ''G. Caporale'', Via Campo Boario, 64100, Teramo, Italy. Tel.: +39 0861 332241; Fax: +39 0861 332251; E-mail: p.calistri@izs.it

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Summary

After 10 years, West Nile virus (WNV) re-emerged in Italy in August 2008. As on 31 December 2008, the infection affected eight Provinces in three Regions (Emilia Romagna, Veneto, Lombardy), where a total of 794 cases of WNV infection in 251 equine stables were detected on the basis of the clinical signs and as a result of a serological screening in horses living in the area. Only 4.0% (32/794) of the serologically positive animals showed clinical signs, and the 32 clinical cases were reported in 18 different farms. The observed case-fatality rate was 15.6% (5/32). The confirmed clinical cases were detected from end August to mid October. Significant levels of positivity by RT-PCR were also observed in magpies (*Pica pica*) (9.1%, 95% confidence levels: 6.1–13.4%), carrion crows (*Corvus corone*) (7.4%, 95% confidence levels: 3.6–14.4%) and rock pigeons (*Columba livia*) (12.9%, 95% confidence levels: 7.6–21.2%).

Introduction

West Nile virus (WNV) is a mosquito-borne *Flavivirus* belonging to the Japanese encephalitis antigenic complex in the family *Flaviviridae* (Karabatsos, 1985). The ecological aspects of WNV infection, involving mosquitoes, birds and humans, were first described in the 1950s in Egypt (Taylor et al., 1956). The infection in humans is usually asymptomatic, but 10–20% of people who become infected will develop an acute, influenza-like, self-limiting febrile illness (Mostashari et al., 2001). Neurological disease in both humans and horses was reported for the first time in the late 1950s (Spigland et al., 1958) in Israel. In USA, it has been estimated that less than 1% of infected individuals will develop a more severe form of disease, possibly with a fatal outcome (European Commission, 2003).

In the past 15 years, human cases were reported in Algeria (Le Guenno et al., 1996), in Romania (Tsai et al., 1998; Cernescu et al., 2000), in the Czech Republic (Hubalek et al., 1998), in Russia (Platonov et al., 2001), in Israel (Weinberger et al., 2001) and in Hungary (Bakonyi et al., 2006; Krisztalovics et al., 2008). Outbreaks in horses were also described in Morocco (Tber, 1996; Dauphin et al., 2004), in France (Murgue et al., 2001b) and in Tunisia (Douphin et al., 2004). Since the summer of 1999, the disease was reported in humans, horses and birds in the United States (Centers for Disease Control, Prevention, 1999, World Organisation for Animal Health– OIE, 1999).

An epidemic of WNV encephalitis occurred in Italy during the late summer 1998, among horses in proximity of Padule di Fucecchio marshes, a wetland area in Tuscany (Autorino et al., 2002), where, however, no significant wild bird deaths or rise in human neurological cases were detected. Following the WNV epidemic in Tuscany, the Italian government established a national WNV surveillance programme, which was in place in 15 Italian wetlands since 2001. After the epidemic in Tuscany, no further WNV clinical outbreaks were observed in Italy either in horses or in humans until August–September 2008, although sporadic evidences of WNV introduction and local circulation were detected between 2003 and 2007 by the national surveillance programme in some of the 15 risk areas.

The first clinical signs of disease were observed on the 20th of August 2008 in a racehorse, aged 2 years, living in a stable in Ferrara Province, closer to Po river and less than 50 km far from the Comacchio wetlands, one of the risk areas selected in the framework of the WNV national surveillance programme. The suspicion of West Nile encephalitis was confirmed on the 8th of September by virus neutralization test, RT-PCR and virus isolation.

Immediate veterinary investigations in the surrounding geographical areas permitted to detect other suspected horse cases. An emergency veterinary surveillance programme was, therefore, put in place with the aim of defining the geographical extension of the infection and the animal populations involved. In the public health sector, passive surveillance was strengthened and active surveillance was implemented in stables workers, which resulted in the detection of nine cases of WNV infection, four of which were characterized by neuroinvasive symptoms (Rossini et al., 2008; Gobbi et al., 2009).

Aim of this study is to describe the main epidemiological findings of the WNV infection observed in animals during 2008 in Italy.

Materials and Methods

National surveillance programme in place from 2001 to 2008

A national surveillance programme to detect the introduction of WNV infection was in place in 15 Italian wetlands areas since 2001. These areas were identified according to the presence of a significant number of water fowls, including species of migratory birds which can represent a possible risk of virus introduction. The national programme was mainly based on surveillance of birds and horses for detecting WNV circulation (Toma et al., 2008). The surveillance in birds was based on (i) a viral screening on carcasses of dead wild birds found in the areas at risk and on (ii) flocks of sentinel chickens bled every 15 days throughout the transmission season, from May to November. Positive birds were euthanatized and organs were submitted for viral isolation. In addition, a number of equines (sentinels), able to detect with a 95% confidence level the presence of WNV infection if it occurs at a prevalence of 10%, was selected in each monitoring site. Overall, in the 15 risk areas, 450 horses had to be tested twice a year, immediately before (April–May) and after the transmission season (October–November). Sentinel horses were to be uniformly distributed inside the areas and, whenever possible, kept in proximity of sentinel chickens. The programme included also the clinical surveillance of all neurological signs in equines.

Emergency veterinary programme

Following the first cases of disease in August 2008, clinical investigations on equine stables were performed in the affected province and in the surrounding areas. Following the detection of further neurological cases and the evidence of virus transmission in resident wild birds (Table 1), the veterinary authorities implemented an emergency veterinary programme in an area encompassing around 16000 km^2 in Emilia-Romagna, Lombardy and Veneto regions (Table 1 and Fig. 1).

The programme was carried out until the 31st of December, when epidemic was clearly over and the results obtained were considered exhaustive for delimiting the infected area. The emergency veterinary programme was based on the following activities:

1 Syndromic surveillance of all neurological cases in equines. A broad suspect case definition was used to enhance the sensitivity of the surveillance system. Any case of ataxia or sudden death in equines in the area at risk, in fact, was considered as suspected as well as any equine animal showing at least two of the following signs: circle movements, weakness of posterior legs, motor paralysis or paresis, muscular fasciculation, blindness, ptosis of lower lip, gnashing. In absence of clinical signs, even a serological positive result was considered a suspected case of WNV infection. Confirmation is made with serological and virological tests: IgG virus neutralization, plaque reduction neutralization tests, RT-PCR and virus isolation.

2 Serological examination of horses. All animals that travelled outside the area at risk (Fig. 1) in the last 3 months were excluded from the sampling. Part of the blood samples were taken during the activities carried out in the framework of the Italian control programme for Equine infectious anaemia, which includes the annual testing of all equines more than 6 months of age. Samplings are equally distributed from January to December.

3 Serological screening of cattle selected as sentinel in the framework of the Italian bluetongue surveillance plan (Giovannini et al., 2004).

4 Extensive monitoring of wild birds found dead or captured in the framework of other surveillance programmes or in application of campaigns for the reduction in noxious birds populations (the target species of such campaigns was mainly Corvids).

5 Entomological surveillance in horse stables where infected animals were detected, with the aim of identifying the main vectors involved in virus transmission. Details on the entomological activities carried out in the area as well as the main results are reported in another study in preparation.

Table 1. Chronology of the main relevant events after the detection of the first neurological case of West Nile in a horse in Ferrara Province

Date	Events and actions taken
20 August 2008	Detection of the index case: a racehorse living in a stable in Ferrara Province showed sings of posterior weakness, ataxia and loss of equilibrium
8 September 2008	The first case of equine encephalitis due to West Nile virus was confirmed in a racehorse in Ferrara Province. Veterinary services initiated to visit all horse stables in the whole Ferrara Province
16 September 2008	A sample constituted by a pool of organs (brain, spleen, kidneys and heart) from a magpie (*Pica pica*) captured in Ferrara Province gave a positive result by RT-PCR. Virological tests were extended to other samples collected from pigeons (*Columbia livia*) in the same province
17 September 2008	Other 11 neurological cases in horses in Ferrara, Rovigo and Bologna Provinces were confirmed. Clinical surveillance activities were extended to all surroundings provinces
23 September 2008	The activities of the emergency veterinary programme started in Emilia Romagna region. The whole territory of Ferrara province and the northernmost areas (lying north to the 'Emilia' national road, which separates the flatland from the mountainous part of the region) of Modena, Bologna and Ravenna provinces were included in the programme (Fig. 1)
26 September 2008	A sample taken on 15th September 2008 and constituted by a pool of organs (brains, spleen, kidney and heart) of an euthanized sentinel chicken on Ferrara province resulted positive by RT-PCR
29 September 2008	The activities of the emergency veterinary programme started in Rovigo, Padova and Venezia provinces (Veneto region) (Fig. 1)
7 October 2008	The activities of the emergency veterinary programme started in the southernmost part of Mantova province (Lombardy region) (Fig. 1)
8 October 2008	Four serum samples taken on 15th September 2008 from previously tested negative sentinel horses in Ferrara province resulted positive by virus neutralization
13 October 2008	The first edition of an e-learning training course on West Nile disease for Italian official veterinarians started. Five editions were provided until the 20th of December 2008
13 October 2008	One serum sample taken on 23rd September 2008 from a previously tested negative sentinel horse in Ravenna province resulted positive by virus neutralization
30 October 2008	The last case was confirmed in a horse in Bologna province

Characteristics of the area involved by WNV circulation

The area is characterized by the presence of the Po river, the longest Italian river, which creates a large delta and wetlands in proximity of its outlet into the Adriatic sea. Around 137 wild birds regular nesting species and 139 wild birds wintering species have been observed in the Po delta area (Anonymous, 2004). This area is connected by several

Fig. 1. Areas included in the emergency veterinary programme established by the Italian veterinary Authority after the detection of first cases of West Nile virus infection in horses in Ferrara Province.

pathways of migratory birds with other natural reservoirs in Africa, in the Mediterranean Basin and in north-eastern Europe (Atkinson et al., 2006). Several equines stables are present in the area, with a total of around 26 000 animals.

An indirect evidence of the suitability of the region for the transmission of arboviruses is represented by the chikungunya epidemic occurred in August–September 2007 less than 30 km far from the area involved by the WNV transmission in 2008 (Angelini et al., 2008) and the serological evidence of Usutu virus circulation in 2007 (Lelli et al., 2008).

From the climatic point of view, the area involved by WNV circulation is characterized by the following daily average temperatures (minimum and maximum) during seasons (Fig. 2):

- December–February: 0.5–8.2°C
- March–May: 8.1–20.2°C
- June–August: 18.4–31.6°C
- September–November: 9.9–19.9°C.

Results

In Table 1, the main events and findings are chronologically presented. Following the first case of West Nile encephalitis, 32 clinical cases were reported in 18 different horse stables. The epidemic curve of clinical cases is reported on Fig. 2. The observed case-fatality rate was 15.6% (5/32). Clinical cases were detected from the end of August to mid October.

The serological screening of sentinel horses in Ferrara and Ravenna Provinces permitted to identify the seroconversion of 11 animals in seven stables (six in Ferrara and one in Ravenna Provinces).

As on the 31st of December, additional 751 cases of WNV infection in 226 equine stables were detected on serological basis, as a result of the screening in sentinel horses or through the monitoring of horses which remained in the area at risk during the last 3 months (Table 2). The overall observed percentage of infected animals in the positive stables was 39.1% (794/2030) and only 4.0% (32/794) of the serologically positive animals showed clinical signs (Table 2). The geographical distribution of WNV cases is reported in Fig. 3.

A sentinel chicken flock located in Ferrara Province resulted positive by RT-PCR on the 15th of September. The results of the monitoring activities on wild birds are reported in Table 3. Samples collected from 490 birds captured in Emilia Romagna and Lombardy Regions were tested by RT-PCR until the 31st of December 2008. More than 87% of tested animals are represented by three species: magpies (*Pica pica*), carrion crows (*Corvus corone*) and rock pigeons (*Columba livia*). Significant levels of positivity by RT-PCR were observed in these species: 9.1% (6.1–13.4%) in magpies, 7.4% (3.6–14.4%) in carrion crows and 12.9% (7.6–21.2%) in rock pigeons. Ninety percentage of animals were collected in three Provinces (Ferrara, Ravenna and Bologna), and the utmost positive level was observed in Ferrara Province. The geographical distribution of RT-PCR positive specimens of birds is represented in Fig. 4. The positive results were obtained on samples collected from mid August to mid October.

Table 4 reports the results of serological screening of sentinel cattle, selected in the framework of the Italian bluetongue surveillance plan. Up to the 31st of December 2008, 152 bovine sentinel herds (with a total of 1401 cattle) were tested by virus neutralization test in the whole area. The sentinel cattle system confirmed the virus circulation mainly in Ferrara Province, similarly to what observed in wild birds and horses. The geographical distribution of sentinel cattle herds tested is shown in Fig. 4.

West Nile virus strains were isolated from blood samples of one clinically affected horse in Rovigo Province

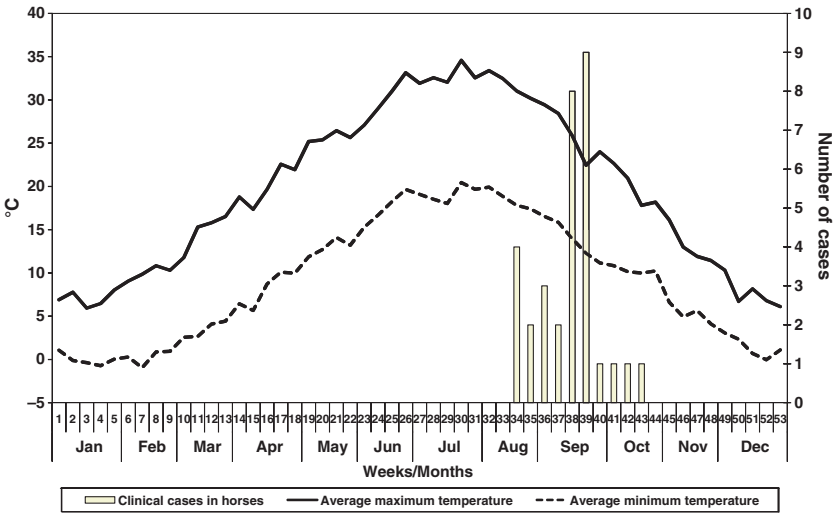

Fig. 2. Temporal distribution of West Nile virus clinical cases in horses (n = 32) and average weekly maximum and minimum air temperature in Ferrara Province (data source: Italian Air Force Meteorological Service, 2002–2008).

Table 2. Number of West Nile virus (WNV) serologically positive stables and confirmed neurological equine cases detected in the area under surveillance

Regions	Provinces	Total no. positive stables (1)	No. stables where WNV clinical signs were observed (2)	No. equine animals				% positive animals (4/3)	% positive animals showing clinical s igns (5/4)	Case-fatality rate (%) (6/5)
				Tested (3)	Positive (4)	Showing clinical signs (5)	Dead (6)			
Emilia	Ferrara	64*	10	698	320[a]	16	2	45.8	5.0	12.5
Romagna	Ravenna	5*	0	59	10[a]	0	0	16.9	0.0	
	Bologna	23	4	448	128	6	1	28.6	4.7	16.7
	Modena	17	1	241	33	1	0	13.7	3.0	0.0
Veneto	Rovigo	77	1	278	161	1	0	57.9	0.6	0.0
	Padova	21	1	92	37	1	0	40.2	2.7	0.0
	Venezia	8	0	58	20	0	0	34.5	0.0	
Lombardy	Mantova	36	1	156	85	7	2	54.5	8.2	28.6
Total		251	18	2030	794	32	5	39.1	4.0	15.6

*Eleven seroconverted sentinel horses in seven stables (Ferrara: 10 horses in six stables; Ravenna: one horse in one stable) are included.

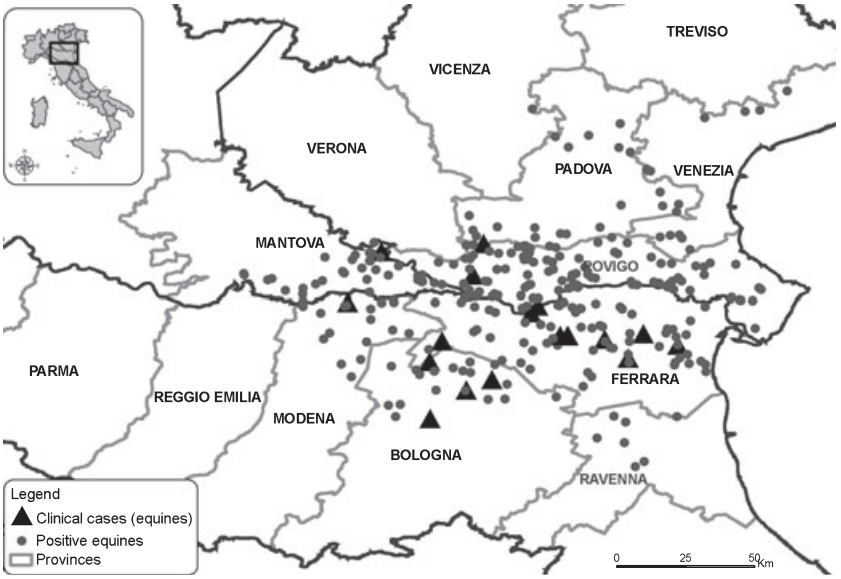

Fig. 3. Geographical distribution of cases of West Nile virus infection in equines. A distinction between cases with signs of WN encephalitis and serological positive cases is made.

and one donkey kept in a stable in Ferrara Province, as well as from pools of brain, kidneys, heart and spleen of one rock pigeon (*C. livia*) and three magpies (*P. pica*) caught in the same territory.

Discussion

The area where the first signs of encephalitis due to WNV were observed in August 2008 is close to Po river and less than 50 km far from the Comacchio wetlands (Fig. 1), which was a geographical area already selected within the WNV national surveillance programme as area at risk for WNV introduction and spreading. Sporadic suspects of WNV introduction and local circulation were observed during the past 5 years in this area, but they did not result in a wide spread of infection as in 2008. In the period between 2003 and 2007, in fact, the WNV surveillance activities in the Comacchio wetlands resulted in sporadic serological positive findings, indicating a certain level of WNV circulation in the area. In particular, positive serological results were obtained by ELISA IgG test in sentinel chicken flocks in 2003, 2005, 2006 and 2007, while horses tested positive by plaque reduction neutralization tests were found in 2005 and 2007. Clinical signs of diseases, however, were never reported in horses of the area.

In relation to the capacity of the WNV national surveillance programme to early detect the virus transmission, the detection of the infection in 2008 by the observation of clinical signs in horses may induce to

Table 3. Birds tested and positives by RT-PCR in the area under surveillance

Species	Bologna Tested	Bologna Positives	Bologna % (95% c.l.)	Ferrara Tested	Ferrara Positives	Ferrara % (95% c.l.)	Mantova Tested	Mantova Positives	Mantova % (95% c.l.)	Modena Tested	Modena Positives	Modena % (95% c.l.)	Ravenna Tested	Ravenna Positives	Ravenna % (95% c.l.)	Total Tested	Total Positives	Total % (95% c.l.)
Rock Pigeon (*Columba livia*)				93	12	12.9 (7.57–21.2)										93	12	12.9 (7.57–21.2)
Cormorant (*Phalacrocorax carbo*)				7	1	14.3 (3.19–52.7)										7	1	14.3 (3.19–52.7)
Carrion Crow (*Corvus corone*)	39	0	0 (0.0–8.8)	23	6	26.1 (12.62–46.7)	14	0	0 (0.0–21.8)	19	1	5.3 (1.23–24.9)				95	7	7.4 (3.67–14.4)
Yellow-legged Gull (*Larus michahellis*)				3	1	33.3 (6.76–80.6)										3	1	33.3 (6.76–80.6)
Magpie (*Pica pica*)	50	1	2 (0.48–10.4)	40	19	47.5 (32.88–62.6)	1	0	0 (0.0–84.2)	4	0	0 (0.0–52.2)	146	2	1.4 (0.42–4.8)	241	22	9.1 (6.12–13.4)
Eurasian Jay (*Garrulus glandarius*)	4	0	0 (0.0–52.2)	2	1	50 (9.43–90.6)				5	1	20 (4.33–64.1)	3	0	0 (0.0–60.2)	14	2	14.3 (4.33–40.5)
Other species*	7	0	0 (0.0–36.9)	30	0	0 (0.0–11.2)										37	0	0 (0.0–9.3)
Total	100	1	1 (0.24–5.4)	198	40	20.2 (15.21–26.3)	15	0	0 (0.0–20.6)	28	2	7.1 (2.19–22.8)	149	2	1.3 (0.41–4.7)	490	45	9.2 (6.94–12.1)

*Which comprises: two little owl (*Athene noctua*), six common raven (*Corvus Corax*), four pheasant (*Phasianus Colchicus*), one little egret (*Egretta garzetta*), two long-eared owl (*Asio otus*), six goose (*Anser anser*), one grey partridge (*Perdix perdix*), eleven common starling (*Sturnus vulgaris*), three eurasian crow (*Corvus monedula*), and one turtledove (*Streptopelia turtur*).

re-consider the criteria followed in the formulation of the national programme. However, it should be not forgotten that the subsequent testing of sentinel chicken flocks on the 15th of September resulted in the evidence of animals that seroconverted in one flock in the Comacchio area.

The observation of such a wide West Nile fever epidemic in 2008 induced the veterinary authorities to analyse the critical points in the surveillance of this infection. Immediate actions for strengthening the surveillance system were put in place by the veterinary authorities, including the implementation of five editions of an e-learning training course with the participation of 506 veterinarians, the preparation and dissemination of informative leaflets, and of a daily bulletin on the evolution of WNV epidemic, prepared by the National Reference Centre for Foreign Animal Diseases, Istituto Zooprofilattico Sperimentale, Teramo, Italy (IZS A&M) and made available on its website (http://www.izs.it).

In addition, the serological surveys performed on horses resident in the area under surveillance permitted to define the extension of the territories likely to be infected. The survey was not fully completed and it was stopped on the 31st of December. The results obtained, in fact, were considered sufficient to delimit the area involved by the WNV transmission.

An interesting result of the emergency veterinary programme performed in the infected area was the use of bovine sentinel animals to trace the infection on the territory. In which circumstances the existing Italian bovine sentinel network might be of benefit for WNV surveillance should be explored more in depth in the future. In particular, sensitivity and specificity of the system need to be defined.

The levels of infection observed in some species of wild birds confirmed the high susceptibility of these species to WNV infection (Komar et al., 2003), although a significant increase in mortality in birds was not observed. The role of some bird species, like magpies, carrion crows and rock pigeons needs to be more investigated, both in relation to their capacity of supporting a WNV epidemic, and to represent a possible link between rural and urban or suburban areas. The latter aspect should be deeply explored, given its possible relevance for the public health.

Further surveillance activities to be performed during 2009 are currently under examination. In particular, what is needed is: (i) the definition of a system able to detect the virus circulation as early as possible in the infected and surrounding areas, and (ii) the assessment of the degree of possible endemization of infection in the local wild birds and vectors populations.

The observed case-fatality rate (five deaths out of 32 clinical cases, equal to 15.6%) appears to be much lower

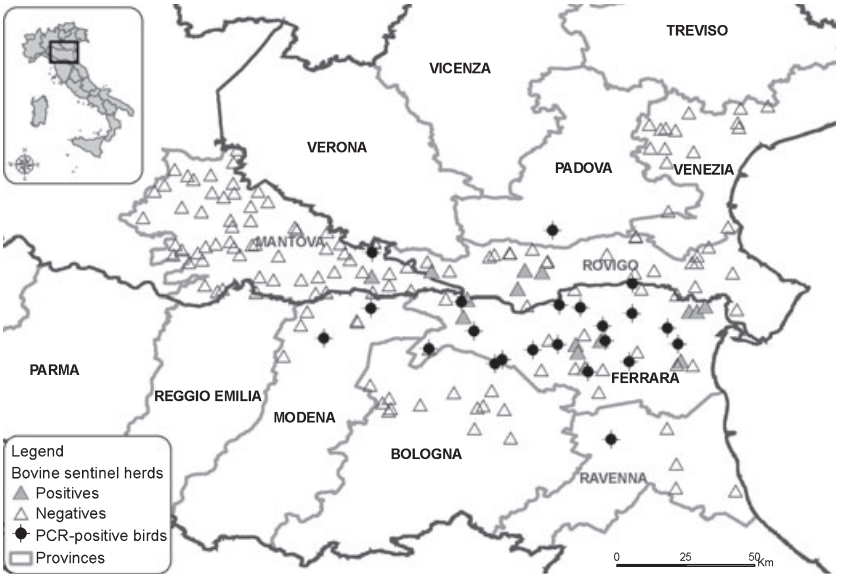

Fig. 4. Geographical locations where birds tested positive by RT-PCR were captured and of bovine sentinel herds tested by virus neutralization test.

Table 4. Sentinel cattle tested and positives by virus neutralization test in the area under surveillance

		Sentinel herds			Sentinel cattle		
Regions	Provinces	Tested	Positives	% (95% c.l.)	Tested	Positives	% (95% c.l.)
Emilia Romagna	Bologna	13	0	0 (0.0–23.2)	185	0	0 (0.0–2)
	Ferrara	23	10	43.5 (25.55–63.4)	400	24	6 (4.07–8.8)
	Modena	3	0	0 (0.0–60.2)	36	0	0 (0.0–9.5)
	Ravenna	4	0	0 (0.0–52.2)	56	0	0 (0.0–6.3)
Veneto	Padova	1	1	100 (15.81–98.7)	7	1	14.3 (3.19–52.7)
	Rovigo	27	4	14.8 (6.06–32.7)	197	6	3 (1.43–6.5)
	Venezia	22	0	0 (0.0–14.8)	166	0	0 (0.0–2.2)
Lombardy	Mantova	59	1	1.7 (0.41–8.9)	354	1	0.3 (0.07–1.6)
Total		152	16	10.5 (6.61–16.4)	1401	32	2.3 (1.63–3.2)

than that reported in the 1998 Italian outbreak (42.9%) (Autorino et al., 2002) and in other epidemics in the Mediterranean Basin (Murgue et al., 2001a). The calculation of the death rate might be affected by a certain degree of underreporting of clinical cases due to some instances of incorrect application of the case definition by the different regional veterinary and to other sources of underreporting. Anyway, as underreporting does not involve fatalities, its net effect would be to inflate the case-fatality rate. Further studies are needed to investigate the possible correlations between the epidemiological features of this WNV strain (low number of clinical cases and deaths in horses, absence of significant mortality in wild birds, apparent high capacity of spreading) and its genetic characteristics.

The phylogenetic analysis of the isolates indicates that these viruses belong to the lineage I among the European strains, with a 98.8% nucleotide similarity with the strain isolated in Tuscany during the 1998 outbreak and a complete similarity (100%) of the deduced amino acid sequence (Savini et al., 2008; Monaco et al., 2009). According to the partial sequences of protein E, the 2008 Italian strain was similar to the Romanian 1996–1997, Volvograd 1999, Senegal 1993 and Kenyan 1998 strains (Savini et al., 2008; Monaco et al., 2009). Although the similarities between viruses isolated in Tuscany in 1998 and in Ferrara 10 years later, there is no evidence of any direct epidemiological link between the two Italian epidemics. The fact that in both episodes the areas where the infection took place were close to wetlands and marshes, where a large population of wild migratory birds rests and nests, might support the hypothesis of a recent introduction of the virus by migratory birds. The spreading of the WNV infection could be then sustained by the favourable ecological factors like the vector densities and climate conditions. The chikungunya virus transmission

and the Usutu virus presence 1 year before in the same region prove the existence in the region of ideal conditions for supporting and spreading arboviral infections.

Thomas and Urena (Thomas and Urena, 2001) state that it is difficult to identify the factors triggering an outbreak of WNV. The researchers believe that a combination of climate, bird and mosquito dynamics and other variable factors can lead to initial outbreaks of the infection. However, although additional observations and epidemiological studies are needed, the reasons behind the wide spread of WNV infection in 2008 in Italy still remain unknown.

In conclusion, further investigations are needed to attempt to clarify the current unknown aspects related to the WNV transmission in Italy. The studies on WNV epidemiology need to be conducted in strict collaboration among experts of veterinary and public health sector, with the involvement of epidemiologists, entomologists, ornithologists and experts on climate mechanisms. Only with a broad multidisciplinary approach will the several facets of this multifactorial zoonosis may be analysed on a proper way.

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