West Nile virus circulation in Emilia-Romagna, Italy: the integrated surveillance system 2009

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Following a large West Nile virus (WNV) epidemic in north-eastern Italy in 2008, human and animal surveillance activities were implemented in Emilia Romagna. Human surveillance was performed by serology or genome detection on blood and cerebrospinal fluid for all suspected cases suffering from acute meningoencephalitis in the regional territory. Animal surveillance consisted of passive and active surveillance of horses and active surveillance of wild birds and mosquitoes. Between 15 June and 31 October 2009, nine of 78 possible cases of West Nile neuroinvasive disease were confirmed (three fatal). From May to October, 26 cases of neurological West Nile disease were confirmed among 46 horses. The overall incidence of seroconversion among horses in 2009 was 13%. In 2009, 44 of 1,218 wild birds yielded positive PCR results for WNV infection. The planned veterinary and entomological surveillance actions detected WNV activity from the end of July 2009, about 2-3 weeks before the onset of the first human neurological case. Passive surveillance of horses seems to be an early and suitable tool for the detection of WNV activity, but it will be less sensitive in the future, because an intensive programme of horse vaccination started in June 2009.

Regional integrated surveillance system

In Italy a national veterinary plan for the surveillance of West Nile virus (WNV) circulation was set up in 2001 under the coordination of the National Reference Centre for Exotic Diseases of animals (CEntro Studi Malattie Esotiche; CESME).

During the late summer of 2008 a large epidemic of WNV infection occurred in north eastern Italy over an area exceeding 7,000 km^2, in three regions, including Emilia-Romagna. Twenty-three horse cases and three human cases of the neuroinvasive form of West Nile disease (WND) were confirmed by laboratory tests [1,2]. After the first evidence of WNV circulation in horses was found, additional surveillance on horses, birds and mosquitoes was activated.

In Emilia-Romagna the WNV surveillance plan 2009 adopted locally the surveillance measures indicated by the national plan. In particular, among the surveillance activities, the choice was to monitor wild non-migratory birds, such as corvids (the crow family), considered the most sensitive indicators among birds, which can be captured easily. As regards equine surveillance, the regional plan emphasised the education of veterinary practitioners, focusing on the inclusion of WNV in differential diagnosis and the achievement of rapid reporting. A major feature of this plan was to establish an extremely sensitive system of passive surveillance. In addition to passive surveillance, active monitoring of horses was implemented in the area involved in the 2008 outbreak, including Ferrara and the neighbouring provinces [3].

Evidence of WNV circulation in 2008 was found in animals [1,4], humans [5], and mosquitoes. This highlighted the need to implement an integrated surveillance system which would describe the phenomenon comprehensively. Such a system facilitates the collection of data to evaluate spatial distribution and time trends of viral circulation and shares information.

For this reason the 2009 Regional Surveillance Plan implemented activities beyond those of the National Plan, revised the surveillance system of human cases, activated intensive entomological monitoring, and enlarged the surveillance area to involve all the provinces along the Po River.

Human surveillance

The aim of the human surveillance system was the early detection of infection in humans and the estimation of its diffusion through the systematic analysis of newly emerging clinical cases, in order to manage specific interventions.

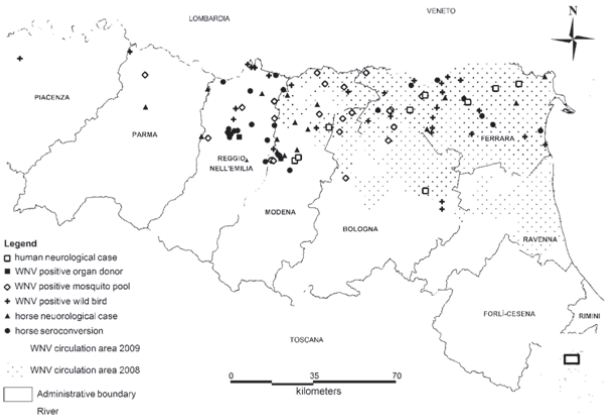
The surveillance was performed throughout the regional territory, from 15 June to 31 October 2009, corresponding to the period of vector activity in Emilia-Romagna and adjusted locally according to weather conditions and vector activity reports. In 2009, the 2008 case definition [6] was extended to include cases of all ages and not only those over 15 years of age.

The human surveillance activity was performed by serology or genome detection on blood and cerebrospinal fluid for all suspected cases suffering from acute meningoencephalitis in the regional territory. Active surveillance of people who live or work in areas of documented viral circulation was also performed. In addition blood and cerebrospinal fluid samples from subjects living or staying for at least one night in the regional area were sent to the Regional Reference Centre for Microbiological Emergencies (Centro di Riferimento Regionale per le Emergenze Microbiologiche; CRREM). In selected cases, positive specimens were confirmed by the National Health Institute (Istituto Superiore di Sanità; ISS) and by the National Institute for Infectious Diseases (Istituto Nazionale Malattie Infettive; INMI).

From October 2008 to April 2009 a serosurvey performed on 9,177 healthy blood donors living in the province of Ferrara detected a total of 62 IgG positive subjects, corresponding to a seroprevalence of 0.68%.

Figure 1

Map of municipalities with confirmed West Nile virus circulation and localisation of human West Nile neuroinvasive disease cases by probable infection site, Emilia-Romagna, Italy, 2009



WNV: West Nile virus

Animal surveillance

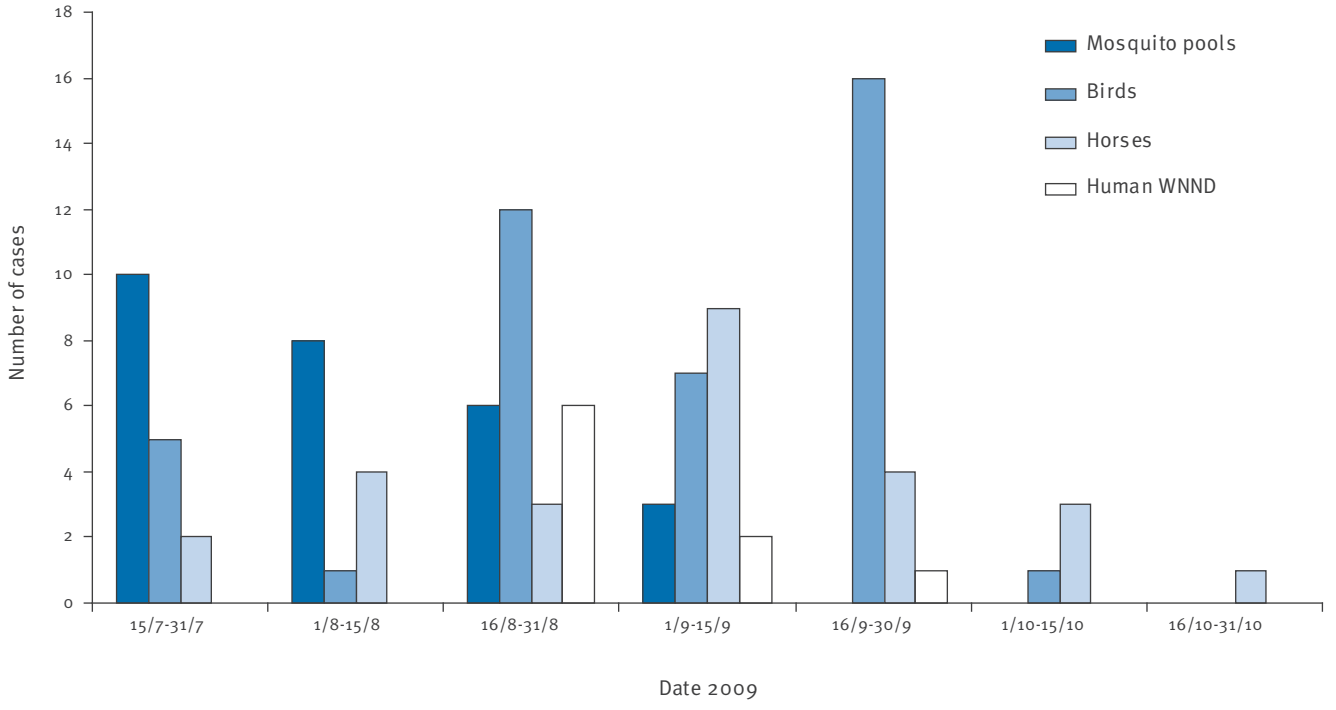
The regional veterinary WNV surveillance system was activated from May to October, performing passive and active surveillance on horses and on non-migratory wild birds.

Horse passive surveillance

In Italy all suspected signs of WND in horses must be notified to the official veterinary services. Suspected cases were confirmed if resulted positive by reverse transcription – polymerase chain reaction (RT-PCR) performed on central nervous system [7] or to a WNV virus

Figure 2

Distribution of West Nile virus confirmed cases (mosquito pools, birds, horses, and human West Nile neuroinvasive disease) by date, Emilia-Romagna, Italy, July-October 2009



WNND: West Nile neuroinvasive disease

Note: The figure does not include a magpie (found in early May) or a jay (found in early November).

neutralisation (VN) test (cut-off titre 1:10) in microtitre plates and to IgM enzyme linked immunosorbent assay (ELISA) [8,9].

Horse active surveillance

In the provinces of Ferrara, Bologna, Modena, Ravenna, and Reggio Emilia, every 1,600 km², 28 seronegative unvaccinated equine sentinels, sufficient to detect an incidence above 10% (CI 95%), were selected in the spring of 2009. They were serologically tested twice after the selection, at the beginning of August and the beginning of September. Samples collected were screened by a home-made competitive ELISA [10]. Positive samples were confirmed by VN and IgM ELISA at the CESME in Teramo. A seroconversion was confirmed if VN titre was at least 1:10 and there was evidence of IgM antibodies.

Wild bird surveillance

Monitoring was carried out in all the provinces along the Po River, in the plain area of Emilia-Romagna. Every 1,600 km², a monthly sample of about 40 wild birds caught or shot within specific wildlife population control programmes was collected. Samples of organs (brain, heart, and kidney) of each bird were pooled and examined by RT-PCR [7].

Entomological surveillance

The surveillance system was based on the weekly to monthly (frequency depends on local resources) collection of mosquitoes in fixed stations and in the sites where birds, humans, or horses signalled WNV activity. Mosquito collections for WNV screening were conducted in six provinces: Ferrara, Ravenna, Bologna, Modena, Reggio Emilia, and Parma, using 92 CO_2 baited traps positioned in fixed stations. Moreover, mosquito collections were performed promptly using CO_2 and gravid traps in sites where positive horses and human cases had been detected.

The surveillance system was activated in the period 15 April to 10 October. Collected mosquitoes were pooled (maximum 200) by species, date, and site of collection and examined by RT-PCR [7]. In addition, overwintered mosquito females were collected during the period 3 March to 8 April by manual aspirator in rural buildings in the area where WNV was active in 2008.

Virological analysis

Human samples

The detection of WNV genome in human plasma, cerebrospinal fluid, and serum samples obtained from patient suffering from clinical symptoms of meningoencephalitis was performed by an RT-PCR assay based on specific TaqMan probes [7].

Animal samples

In horses RNA was extracted starting from 200 µl of serum or whole blood with EDTA as anticoagulant. In birds RNA was extracted from 200 µl of phosphate buffer saline homogenate (about 20% tissue g / buffer ml) of pooled brain, heart and kidney of each analysed bird. In mosquitoes RNA was extracted from 200 µl of a maximum of 200 pooled mosquitoes manual grinded by using copper stained beads, in 500-800 µl of PBS.

cDNA was submitted to RT-PCR according to the method of Tang and colleagues [7]. Positive samples were confirmed by sequencing of partial nucleocapsin and premembrane protein M amplified according to [11]. Finally from each RT-PCR positive sample WNV was isolated on Vero and RK13 cell lines.

Results

Results of the integrated surveillance system are mapped in figure 1, with the sequence of events summarised in figure 2 (July-October), and discussed below.

Human cases

As of 31 October 2009, nine out of 78 possible cases of West Nile neuroinvasive disease (WNND) notified in Emilia-Romagna have been confirmed (8/9 males; median age 72 years, range: 62-78). Three patients

Table 1

Species distribution of wild birds tested for West Nile virus (n=1,218), Emilia-Romagna, Italy, May-October 2009

Species	Birds tested	WNV RT-PCR positive	% WNV positive
European magpie (*Pica pica*)	607	27	4.4
Carrion crow (*Corvus corone cornix*)	350	5	1.4
European starling (*Sturnus vulgaris*)	98	5	5
Eurasian jay (*Garrulus glandarius*)	96	2	2
Common blackbird (*Turdus merula*)	30	0	-
Strigiformes	11	2	18
Charadriiformes	8	3	38
Other Passeriformes	7	0	-
Other bird orders	5	0	-
Piciformes	4	0	-
Columbiformes	2	0	-
Total	**1,218**	**44**	**3.6**

RT-PCR: reverse transcription-polymerase chain reaction; WNV: West Nile virus

Table 2

Species of mosquitoes tested for West Nile virus (n=190,516), Emilia-Romagna, Italy, May-October 2009

Province	Bologna			Forlì			Ferrara			Modena			Piacenza			Parma			Ravenna			Reggio Emilia			Total		
Species	Mos-quito	Pool	Pool +	Mos-quito	Pool	Pool +	Mos-quito	Pool	Pool +	Mos-quito	Pool	Pool +	Mos-quito	Pool	Pool +	Mos-quito	Pool	Pool +	Mos-quito	Pool	Pool +	Mos-quito	Pool	Pool +	Mos-quito	Pool	Pool +
Ae. albopictus	169	13		11	4		392	31		227	23		58	11		228	6					142	20		1,227	108	0
Ae. caspius	1,713	51		14	5		9,953	114		16,915	121		2	1		12	2		606	11		68	9		29,283	314	0
Ae. detritus																1	1					4	1		5	2	0
Ae. dorsalis										13	1														13	1	0
Ae. geniculatus										6	2											2	1		8	3	0
Ae.vexans	2	1		11	2		84	3		4,090	41		1	1		363	3					46	9		4,597	60	0
An. maculipennis	4	1					59	3		14	5		1	1								4	4		82	14	0
An. plumbeus										2	2														2	2	0
Cx. modestus	7	3					114	10		117	12											8	1		246	26	0
Cx. pipiens	84,225	645	9	158	17		52,973	396	5	6,664	78	7	331	13		6,926	50	1	911	10		2,865	50	5	155,053	1,259	27
Total	**86,120**	**744**	**9**	**194**	**28**	**0**	**63,575**	**557**	**5**	**28,048**	**285**	**7**	**393**	**27**	**0**	**7,530**	**62**	**1**	**1,517**	**21**	**0**	**3,139**	**95**	**5**	**190,516**	**1,789**	**27**

died, two living in Ferrara Province and one in Modena. In addition, not reported in figures, the local health units of Parma and Modena notified two other confirmed cases, both 72 year-old women resident in Mantua province (Lombardy region), treated in hospital in Emilia-Romagna. Another case of infection was that of a 78 year-old female liver donor. Before her death, she had spent two weeks visiting relatives in the WNV affected area (Reggio Emilia).

Horse cases

Passive surveillance

From May to October, 26 cases of neurological WND were confirmed among 46 horses in which it was suspected. Four of the eight provinces involved in the regional surveillance system had WND cases in horses. The first symptoms in horses were detected in the second half of July in the provinces of Ferrara and Reggio Emilia, but the most cases were notified between mid-August and mid-September (figure 2).

Active surveillance

Seroconversions in sentinel horses were detected in three provinces (Ferrara, Modena, and Reggio Emilia). Early seroconversions were registered among the controls examined at the beginning of August. Serological controls around the stables with WND cases also confirmed recent WNV infections in the province of Parma. The overall incidence of seroconversion among horses in 2009 was 13% (95% CI: 10% to 16%), but in Ferrara it was 28% (95% CI: 19% to 39%).

Wild birds

Six of the eight provinces that took part in the regional surveillance system reported positive birds. In 2009, 44 wild birds out of 1,218 (tested by PCR) yielded positive results for WNV infection. With the exception of a magpie caught in May, positive wild birds were detected from the end of July (figure 2). Most of the infected wild birds were corvids (*Pica pica, Corvus corone cornix, Garrulus glandarius*), collected within population control programmes in August and September, but WNV was detected also in other species, mainly found dead in wildlife recovery centres (table 1).

Table 1. Species distribution of wild birds tested for West Nile V (n=1,218), Emilia-Romagna, Italy, May-October 2009

Mosquitoes

About 190,000 mosquitoes were collected, pooled and tested using PCR (1,789 pools of ≤200 individuals/pool). Culex pipiens were the most abundant species (81.4%) followed by *Aedes caspius* (15.4%) and Aedes vexans (2.4%). Other collected species are shown in table 2.

Twenty-seven pools, all consisting of *Culex pipiens*, yielded positive results for WNV. Early positive pools were collected in the province of Reggio Emilia at the end of July. Minimum infection rates (MIR: (no. of

positive pools/no. of mosquitoes tested) x 1,000) [12] were calculated, with higher MIR values recorded in August in the provinces of Reggio Emilia (3.08) and Modena (1.44).

Referring to the collection of overwintering mosquitoes, three mosquito species were collected: *Culex pipiens* (516 females, 52%), *Anopheles maculipennis* s.l. (475 females, 48%), *Culiseta annulata* (4 females, <1%): all specimens were tested and yielded negative results.

Conclusions

The planned veterinary and entomological surveillance actions detected WNV activity from the end of July 2009, about 2-3 weeks before the onset of the first human neurological case. Figure 2 shows that mosquitoes and birds were the first indicators of WNV circulation. The same figure makes it clear that human cases occurred later in the season, as reported elsewhere [6]. Passive surveillance of horses also seems to be an early and suitable tool for the detection of WNV activity, but it will be less sensitive in the future, because an intensive programme of horse vaccination started in June 2009.

More human cases of WNND occurred in 2009 than in 2008, and three were fatal. It is important to note that in 2008 the epidemic became evident in the late summer (beginning of September). In 2009 the first human cases were detected earlier than 2008. It is likely that increased attention of clinicians to this emerging disease improved the surveillance system sensitivity in 2009.

The circulation of WNV in a large area of the Po plain in two consecutive years shows that this territory is becoming suitable to support WNV establishment and possible endemicity. This indicates a need to organise standard surveillance measures to detect WNV activity early and assess risk to public health.

The results of the entomological surveillance confirm that the CO_2 trap is a reliable and valuable tool for early detection of WNV. *Culex pipiens*, the most abundant mosquito species in the region, is the only vector species incriminated, since no other species collected in the field were found to be infected.

The quick and intensive spread of WNV in the past two years suggests that the whole Po plain may be affected in the future. In forthcoming years, surveillance of wild birds and insects will be used to forecast the extension and spread of WNV. The information gathered will be used to direct or optimise actions intended to prevent virus transmission, such as vector monitoring and control, information campaigns to improve personal protection, and deploy screening tests on blood, tissue, and organs for transplant.

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