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Detection of Usutu Virus Within a West Nile Virus Surveillance Program in Northern Italy

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Abstract

Usutu virus (USUV) is a mosquito-borne flavivirus belonging to the Japanese encephalitis serocomplex, recently related to neurological disease in immunosuppressed patients. In the same area of Northern Italy where USUV human cases occurred in 2009, a regional West Nile virus (WNV) surveillance program based on mosquito monitoring and wild birds screening has been implemented since 2008. Mosquito pools and wild birds were tested using three different polymerase chain reactions (Flavivirus, WNV, and USUV). During summer 2009, 56 pools (54 consisting of *Culex pipiens* and 2 of *Aedes albopictus*) and 27 pools (*Cx. pipiens*) out of 1789 mosquito pools were, respectively, USUV and WNV positive. Moreover, out of 1218 wild birds tested, 44 were WNV positive, whereas only 11 birds were USUV positive by polymerase chain reaction. Data collected during 2009 prove a cocirculation of USUV and WNV in Northern Italy, but these two viruses show different incidence values in both mosquitoes and birds, suggesting involvement of different animals (other bird species or mammals) in their natural cycles. The cocirculation of WNV and USUV poses a new potential threat to human health in this area. The extent of WNV surveillance to other Flaviviruses will require new diagnostic procedures able to process a large number of samples in a limited period of time and highlights the importance of developing more specific serological tests that could be used in field.

Key Words: Birds— Flavivirus—Mosquito(es)—West Nile Virus—Usutu Virus.

Introduction

Usutu virus (USUV) is a mosquito-borne virus of the family Flaviviridae, genus *Flavivirus*, belonging to the Japanese encephalitis serocomplex, together with Japanese encephalitis virus, West Nile virus (WNV), Kunjin virus, Murray Valley encephalitis virus, and Saint Louis encephalitis virus (Kuno et al. 1998).

Isolated for the first time from a bird-biting mosquito (*Culex univittatus*) in South Africa in 1959 (Williams et al. 1964), USUV was first detected in Europe in 2001, where it caused high mortality in blackbirds around Vienna, Austria (Weissenböck et al. 2002). USUV overwintered and has established a local transmission cycle becoming a resident pathogen, with tendency to spread to other geographic areas (Weissenböck et al. 2003). Actually bird mortality due to USUV infection has been reported in several countries of Central Europe such as Austria, Hungary, Switzerland, and Italy (Weissenböck et al. 2002, Bakonyi et al. 2007, Chvala et al. 2007, Manarolla et al. 2009). Little is known about USUV natural cycle, but it probably involves wild birds and mosquitoes. In Africa, the virus circulates between birds and mosquitoes with mammals being asymptomatic hosts (Gratz 2006). In Europe *Culex* mosquitoes have been considered to serve as main vectors (Weissenböck et al. 2010), and USUV sequences have been detected in *Culex pipiens* in Spain (Busquets et al. 2008).

During late summer 2009 in Emilia-Romagna, Northern Italy, two cases of USUV neuroinvasive infection in immunosuppressed patients were reported (Cavrini et al. 2009, Pecorari et al. 2009). Because of WNV circulation with human and horse neuroinvasive cases (Macini et al. 2008, Rossini et al., 2008, Calistri et al. 2009), a regional surveillance plan has been implemented starting from 2008 (Angelini et al. 2010).

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During 2009 WNV surveillance activities have been able to detect USUV circulation in mosquitoes (Calzolari et al. 2009); in this article we report data about simultaneous circulation of both WNV and USUV in mosquitoes and birds in the same area.

Materials and Methods

Survey areas

Emilia-Romagna is a region of Northern Italy that is characterized by an intensive agriculture and animal husbandry. The eastern part of the region is located on the Adriatic Sea and includes one of the largest wetland areas of Europe, the Po River Delta. After WNV circulation was detected in 2008 (Macini et al. 2008), almost every floodplain areas of Emilia-Romagna have been put under surveillance.

Mosquito collections

The surveillance system was activated in the period April 15th–October 15th with weekly to monthly collection of mosquitoes in fixed stations and also in the sites where human or horse neurological cases or WNV-positive birds were detected. Mosquitoes were trapped mainly using modified Centers for Disease Control and Prevention traps baited with CO_2 (Petric et al. 1999) in 92 stations, of which 52 stations had been running for a long time for quantitative monitoring purpose in mosquito control programs (21 stations in Bologna province, 27 stations in Ferrara province, and 4 stations in Ravenna province), while 40 ones were newly planned stations to cover the surveillance area using a grid of 100 km^2. Moreover, mosquito collections were promptly performed in sites following the detection of horse and human WNV cases by CO_2 and gravid traps. Mosquitoes were pooled according to date, location, and species, with a maximum number of 200 individuals per pool (Sutherland and Nasci 2007). Pooled mosquitoes were stored in 2 mL polypropylene cryotubes and frozen at −80°C. Two 4.3-mm-diameter copper-plated round balls (Haendler & Natermann Sport GmbH, Münden, Germany) and 1.2 mL of phosphate-buffered saline were added to each tube and samples were grinded for 30 s in a vortex mixer, and then 200-μL aliquots were collected from each grinded samples and submitted to biomolecular analysis.

Wild birds surveillance

Monitoring has been carried out in all the provinces along the Po river, in the plain area of Emilia-Romagna. Every 1600 km^2, a monthly sample of about 40 wild birds caught or shot within specific wildlife population control programs was collected. Monitoring was mainly focused on corvids (*Pica pica*, *Corvus corone cornix*, and *Garrulus glandarius*). After detection of USUV, passive surveillance on wild birds was enforced; thus, also birds of other species, such as blackbird (*Turdus merula*), were tested. Samples of organs (brain, heart, and kidney) of each bird were pooled and submitted to biomolecular analysis.

Virological investigation

RNAs were extracted using Trizol®LS Reagent (Invitrogen, Carlsbad, CA). cDNA synthesis was achieved using random hexamers (Roche Diagnostics, Mannheim, Germany) and SuperScript® II Reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Pools were analyzed using three different polymerase chain reactions (PCRs): (1) traditional PCR, addressed to a fragment of the *NS5* gene, for the detection of *Flavivirus* genus according to Scaramozzino et al. (2001), (2) traditional PCR for the detection of USUV (Weissenböck et al. 2002), and (3) real-time PCR for the detection of part of the *E* gene of WNV according to the method of Tang et al. (2006).

Fragments obtained by *Flavivirus* genus PCR were sequenced by an automated fluorescence-based technique following the manufacturer's instructions (ABI-PRISM 3130 Genetic Analyzer; Applied Biosystems, Foster City, CA). As further confirmation a traditional PCR protocol was performed on WNV real-time PCR-positive samples using primers originally proposed by Lanciotti et al. (2000). Amplicons obtained were sequenced as described above.

These sequences were employed to confirm the reaction specificity by performing a BLAST search in the GenBank library and to evaluate similarity among detected strains.

Calculation of infection rates

We used maximal likelihood estimation (MLE) to evaluate USUV infection rates in mosquito collections (Gu et al. 2004). The rates were fortnightly calculated by grouping together pools of the same species and area (province). We calculated MLE infection rates using the PooledInfRate software (Biggerstaff 2006).

Sequence analysis

Sequence aligning and phylogenetic analysis with neighbor-joining method (*p*-distance) of a portion of the *NS5* gene (260 bp) amplified by Flavivirus PCR with bootstrap test (1000

Table 1. Virological Findings in Mosquitoes Collected in Emilia-Romagna from April to October 2009

Mosquito species	*No. of individuals tested*	*No. of pools tested*	*No. of pools WNV positive*	*No. of pools USUV positive*	*No. of pools WNV and USUV positive*
Aedes albopictus	1227	108		2	
Aedes caspius	29,283	314			
Aedes detritus	5	2			
Aedes dorsalis	13	1			
Aedes geniculatus	8	3			
Aedes vexans	4597	60			
Anopheles maculipennis	82	14			
Anopheles plumbeus	2	2			
Culex modestus	246	26			
Culex pipiens	155,053	1259	19	46	8
Total	190,516	1789	19	48	8

USUV, Usutu virus; WNV, West Nile virus.

replicates) were obtained using evolutionary software MEGA 4.0 (Tamura et al. 2007).

Results

Mosquitoes

About 190,000 mosquitoes were collected during 2009. They were grouped in 1789 pools and tested by PCR (Table 1). *Cx. pipiens* resulted in the most abundant species (81.4%) followed by *Aedes caspius* (15.4%), *Aedes vexans* (2.4%), *Aedes albopictus* (0.6%), and *Culex modestus* (0.1%). Other species were also collected and tested such as *Aedes geniculatus*, *Aedes dorsalis*, *Aedes detritus*, *Anopheles maculipennis s.l.*, and *Anopheles plumbeus*. Twenty-seven mosquito pools were found WNV positive, and 56 pools were USUV positive. Eight pools gave positive results for both viruses. Except for two pools of *Ae. albopictus* USUV positive, all PCR-positive pools consisted of *Cx. pipiens* (Table 1). The two USUV-positive pools of *Ae. albopictus* were collected in the same municipality during the same week. Positive pools were detected from July 21st to September 9th and from July 8th to October 3rd for WNV and USUV, respectively. Locations of traps with USUV-positive pools are shown in Figure 1.

In the 2008 season about 49,000 mosquitoes were collected in the same area, grouped in 896 pools, and analyzed with the same method, obtaining two *Cx. pipiens* pools positive for WNV, whereas USUV was not detected (Table 2).

Mosquitoes infection rate

We calculated USUV MLE infection rates for *Cx. pipiens* in three areas (provinces of Bologna, Ferrara, and Modena) where 50 out of the 54 USUV-positive *Cx. pipiens* pools were observed (Table 3). In all areas MLE rates peaked in August. Although the number of individuals tested did not allow a statistical analysis, the highest value (4.1) was recorded in Modena area (weeks 32–33), but in Bologna area USUV circulation lasted longer (weeks 26–35). In both areas one USUV neuroinvasive human case was reported (Fig. 1) in August–September 2009 (Cavrini et al. 2009, Pecorari et al. 2009).

Nucleotide sequencing and computer analysis

Twelve USUV *Flavivirus* genus PCR amplicons (a 260 bp portion of the *NS5* gene) were sequenced and aligned. By eliminating gaps and missing data, a total of 233 positions were considered, which showed an identity percentage ranging from 100% to 98.3% among them (differences ranged from 0 to 4 on a total of 233 positions), and all these differences were silent and did not produce variations in the translated amino acid sequence.

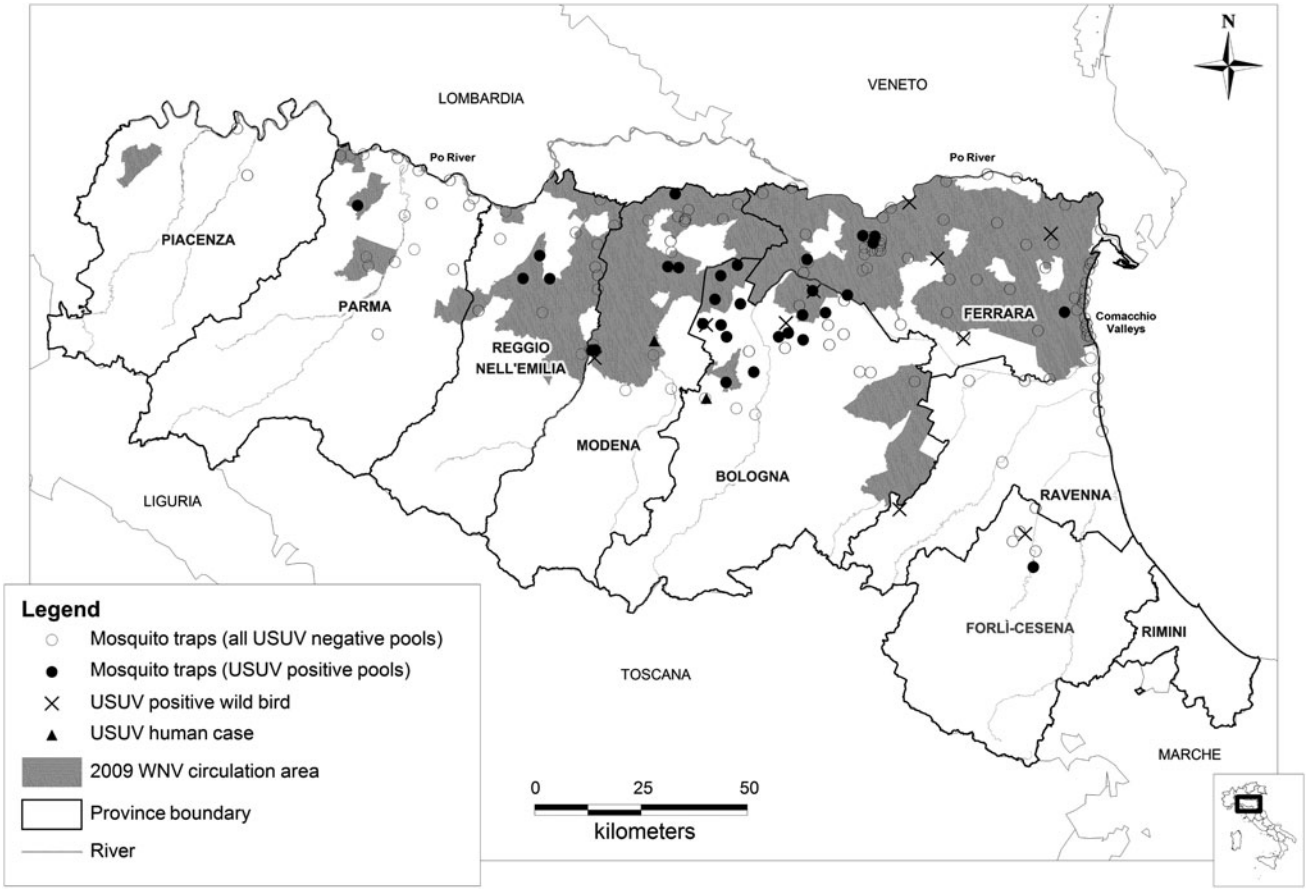

FIG. 1. Map showing locations of mosquito traps, and USUV-positive birds and human cases, Emilia-Romagna, 2009. USUV, Usutu virus.

Table 2. Virological Findings in Mosquitoes Collected in Emilia-Romagna from June to October 2008

Mosquito species	*No. of individuals tested*	*No. of pools tested*	*No. of pools WNV positive*	*No. of pools USUV positive*
Ae. albopictus	821	93		
Ae. caspius	15,128	327		
Ae. detritus	22	6		
Aedes spp.	8	2		
Ae. vexans	1029	30		
An. maculipennis	186	10		
An. plumbeus	1	1		
Cx. modestus	10	4		
Cx. pipiens	31,952	418	2	
Culex spp.	1	1		
Culiseta annulata	3	3		
Culiseta spp.	1	1		
Total	49,162	896	2	0

The highest BLAST scores for the consensus sequence were obtained with USUV isolated in Budapest (EF206350) and USUV strain Vienna 2001 (AY453411). Comparison of consensus sequence obtained showed an identity of 97.4% (6 differences on 263 positions) with these two homologous sequences and identity of 96.1% (9 differences on 263 positions) with a South African strain (AF013412) (Fig. 2).

Wild birds

In 2008 we did not perform PCR to detect USUV, but 45 (9.5%) out of 475 birds tested by PCR were WNV positive. Detailed results have been reported in another article (Calistri et al. 2009). Out of the 1218 birds tested by PCR in 2009, 11 (0.9%) were positive for USUV and 44 (3.6%) for WNV. Three birds (two magpies and one gull) gave positive results for both viruses. Most of infected wild birds were corvids collected between August and October within population control programs, but WNV and USUV were also detected in other bird species mainly found dead in wildlife recovery centers (Table 4). USUV-positive birds were sampled from August 19th (a magpie caught) to October 26th (a blackbird found dead). Except for a magpie caught in May, WNV-positive wild birds were detected starting from the end of July. The last WNV-positive bird was a jay shot on November 4th. Through passive surveillance USUV was detected also in areas not covered by the WNV surveillance program. Figure 1 shows where USUV-positive birds were collected.

Discussion

Discussion will be especially focused on USUV, because we have just reported results of WNV monitoring in another article (Angelini et al. 2010), in which the whole surveillance system has been described.

USUV has been detected in recent years in Northern Italy (Rizzoli et al. 2007, Lelli et al. 2008, Manarolla et al. 2009), where, as in Central Europe, it can cause neurological lesions and death in birds (Weissenböck et al. 2002, Manarolla et al. 2009). Because of immunosuppressive treatments, USUV

Table 3. Usutu Virus Maximal Likelihood Estimation Infection Rates in *Culex pipiens* in Three Provinces of Emilia-Romagna, May–October 2009

		Bologna				*Ferrara*				*Modena*			
			No. of pools				*No. of pools*				*No. of pools*		
Month	*Weeks*	*MLE (CI 95%)*	*Tested*	*Positive*	*No. of individuals*	*MLE (CI 95%)*	*Tested*	*Positive*	*No. of individuals*	*MLE (CI 95%)*	*Tested*	*Positive*	*No. of individuals*
May	18–19	0.0 (0.0–1.8)	25	0	1968	0.0 (0.0–8.6)	3	0	274				
	20–21	0.0 (0.0–0.4)	74	0	9860								
June	22–23	0.0 (0.0–0.3)	88	0	13,289	0.0 (0.0–1.1)	26	0	3285				
	24–25	0.0 (0.0–0.7)	80	0	12,807	0.0 (0.0–0.2)	111	0	20,101				
July	26–27	0.3 (0.1–0.7)	92	4	15,015	0.0 (0.0–0.7)	41	0	4856	0.0 (0.0–24.7)	1	0	63
	28–29	0.5 (0.2–1.1)	76	6	11,663	0.1 (0.0–0.5)	88	2	13,742	0.0 (0.0–6.0)	5	0	408
August	30–31	2.1 (1.1–3.8)	44	10	5559	0.7 (0.0–3.7)	15	1	1391	0.8 (0.0–4.0)	9	1	1208
	32–33	2.9 (1.6–5.0)	47	13	5473	0.7 (0.1–2.2)	33	2	3080	4.1 (1.4–10.7)	11	4	1260
September	34–35	1.3 (0.5–3.0)	40	5	4052	0.6 (0.1–3.0)	27	1	1670	0.0 (0.0–4.3)	13	0	707
	36–37	0.0 (0.0–0.9)	44	0	4138	0.0 (0.0–13.5)	18	0	238	0.5 (0.0–2.6)	20	1	1928
October	38–39	0.0 (0.0–1.8)	31	0	1935	0.0 (0.0–1.6)	22	0	2156	0.0 (0.0–3.1)	15	0	1013
	40–41	0.0 (0.0–4.6)	16	0	646					0.0 (0.0–31.8)	4	0	77

CI, confidence interval; MLE, maximal likelihood estimation.

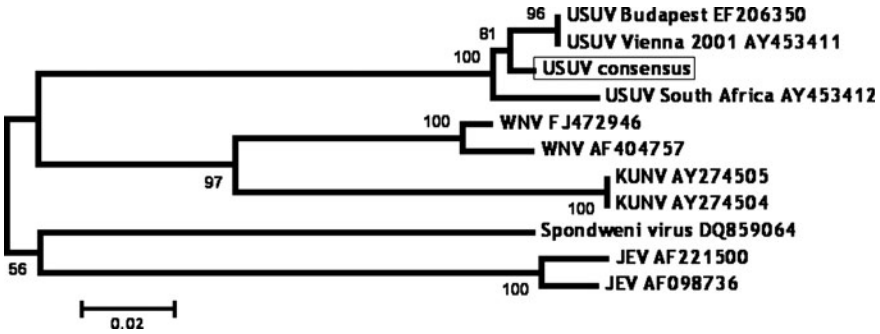

FIG. 2. Neighbor-joining phylogeny tree of a 260-bp portion of the *NS5* gene. The consensus sequence originating from USUV strains detected in mosquito pools in Italy is placed in the tree.

could produce similar disease in humans and could become a new threat for public health (Cavrini et al. 2009, Pecorari et al. 2009). In Emilia-Romagna this virus probably shares with WNV the same vector, *Cx. pipiens*, so its detection during WNV surveillance activities is not amazing. In literature USUV and WNV seem to have similar cycles, but comparing our monitoring results some differences may be evidenced: while we detected more mosquitoe pools PCR positive for USUV than for WNV, more WNV-infected birds were detected than USUV ones, which led to suspect a possible involvement of other animals, such as other bird species or mammals (man included), in the USUV natural cycle. The last hypothesis is enforced by the detection of USUV genome in *Ae. albopictus* (for the first time to our knowledge), a mosquito considered primarily a mammalian feeder, although shown to fed on a wide variety of hosts. The role of *Ae. albopictus* in USUV epidemiology has to be considered and further investigated. As reported by Gratz (2006), in Africa USUV was isolated from rodents (Gen. *Promys*), but in Europe there is a lack of data about a possible involvement of mammals in the virus cycle. However, other hypothesis could explain the differences obtained: for instance, different duration of infection in bird species examined could lead to a different proportion of PCR-positive results. WNV is able to cause persistent infection in birds, in house sparrow (*Passer domesticus*) viral RNA persisted in tissues through 65 days (Nemeth et al. 2009). In addition Komar et al. (2003) noted that several orders of birds showed different viraemia duration and titer after experimental infection with WNV. Duration of USUV infection in wild birds is still unknown. Moreover, a higher frequency of vertical transmission or a higher vector competence for USUV could explain the higher prevalence detected in *Cx. pipiens* mosquito pools. All these items will require specific investigation in the next future.

As WNV, USUV detection in mosquitoes has happened before occurrence of human cases, so preventive measures, such as mosquito control or blood donors testing, could be possibly organized and performed.

As just suggested by Chvala et al. (2007), mosquito monitoring and screening of wild birds are suitable to detect USUV circulation and could replace dead bird surveillance when bird mortality drops because of herd immunity. Virological surveillance may be preferable against serological monitoring, because of cross reactions with other Flaviviruses. Sera reacting to both WNV and USUV were detected in other studies, especially by using low specific tests such as hemagglutination inhibition test (Meister et al. 2008) or en-

Table 4. Virological Findings in Wild Birds Collected in Emilia-Romagna from May to November 2009

Bird species	*No. of birds tested*	*No. of USUV PCR positive*	*No. of WNV PCR positive*	*No. of WNV and USUV PCR positive*	*% of USUV positive*	*% of WNV positive*
European Magpie (*Pica pica*)	607	2	25	2	0.7	4.4
Carrion Crow (*Corvus corone cornix*)	350		5			1.4
Eurasian Jay (*Garrulus glandarius*)	96	2	2		2.1	2.1
European Starling (*Sturnus vulgaris*)	98	1	5		1.0	5.1
Eurasian Blackbird (*Turdus merula*)	30	3			10.0	
Other Passeriformes	7					
Columbiformes	2					
Strigiformes	11		2[a]			18.2
Piciformes	4					
Charadriiformes (*Larus* sp.)	8		2	1	12.5	37.5
Other orders	5					
Total	1218	8	41	3	0.9	3.6

[a]*Asio otus*, *Athene noctua*.
PCR, polymerase chain reaction.

zyme-linked immunosorbent assay (Lelli et al. 2008). Plaque reduction neutralization test has to be performed to confirm positive sera, but this test is complex, costly, and time consuming.

In conclusion, our findings prove a cocirculation of USUV and WNV in Emilia-Romagna region in the 2009 summer season. Our work suggests that it is important to consider other Flaviviruses, such as USUV, for the evaluation of WNV surveillance. Both viruses seem to be able to cause neurological disease in humans in particular conditions; taken together with the above-reported epidemiological data, the circulation of USUV poses a new potential threat to human health in this area. The expansion of surveillance to other Flaviviruses will require new diagnostic procedures able to process a large number of samples in a limited period of time and highlights the importance of developing more specific serological tests that can be used in field.

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Disclosure Statement

No competing financial interests exist.

References

Angelini, P, Tamba, M, Finarelli, AC, Bellini, R, et al. West Nile virus circulation in Emilia-Romagna, Italy: the integrated surveillance system 2009. Euro Surveill 2010; 15:pii: 19547.

Bakonyi, T, Erdelyi, K, Ursu, K, Csorgo, E, et al. Emergence of Usutu virus in Hungary. J Clin Microbiol 2007; 45:3870–3874.

Biggerstaff, B. PooledInfRate, version 3.0. An Excel Add.In to compute infection rates from pooled data. CDC 2006. Available at www.cdc.gov/ncidod/dvbid/westnile/software.htm. Accessed August 29, 2009.

Busquets, N, Alba, A, Allepuz, A, Aranda, C, Núñez, JI. Usutu virus sequences in *Culex pipiens* (Diptera: Culicidae), Spain. Emerg Infect Dis 2008; 14:861–863.

Calistri, P, Giovannini, A, Savini, G, Monaco, F, et al. West Nile virus transmission in 2008 in north-eastern Italy. Zoonosis Public Health 2010; 57:211–219.

Calzolari, M, Bonilauri, P, Bellini, R, Maioli, G, et al. Evidence of circulation of Usutu virus in mosquitoes in Emilia-Romagna region (Italy). *Proceedings of the 5th International SOVE Congress Antalya*, Turkey, 11–16 October 2009.

Cavrini, F, Gaibani, P, Longo, G, Pierro, AM, et al. Usutu virus infection in a patient who underwent orthotropic liver transplantation, Italy, August–September 2009. Euro Surveill 2009; 14:pii: 19448.

Chvala, S, Bakonyi, T, Bukovsky, C, Meister, T, et al. Monitoring of Usutu virus activity and spread by using dead bird surveillance in Austria, 2003–2005. Vet Microbiol 2007; 122: 237–245.

Gratz, N. *Vector- and Rodent-Borne Diseases in Europe and North America*. Cambridge, UK: Cambridge University Press, Part I, Chapter 4, 2006:28–30.

Gu, W, Lampman, R, Novak, RJ. Assessment of arbovirus vector infection rates using variable size pooling. Med Vet Entomol 2004; 18:200–204.

Komar, N, Langevin, S, Hinten, S, Nemeth, N, et al. Experimental infection of North America birds with the New York 1999 strain of West Nile Virus. Emerg Infect Dis 2003; 9:311–322.

Kuno, G, Chang, GJ, Tsuchiya, KR, Karabatsos, N, Cropp, CB. Phylogeny of the genus *Flavivirus*. J Virol 1998; 72:73–83.

Lanciotti, RS, Kerst, AJ, Nasci, RS, Godsey, MS, et al. Rapid detection of West Nile Virus from human clinical specimens, field-collected mosquitoes, and avian samples by a TaqMan reverse transcriptase-PCR assay. J Clin Microbiol 2000; 38:4066–4071.

Lelli, R, Savini, G, Teodori, L, Filipponi, G, et al. Serological evidence of Usutu virus occurrence in north-eastern Italy. Zoonoses Public Health 2008; 55:361–367.

Macini, P, Squintani, G, Finarelli, AC, Angelici, P, et al. Detection of West Nile virus infection in horses, Italy, September 2008. Euro Surveill 2008; 13:pii: 18990.

Manarolla, G, Bakonyi, T, Gallazzi, D, Crosta, L, et al. Usutu virus in wild birds in Northern Italy. Vet Microbiol 2009; 141:159–163.

Meister, T, Lussy, H, Bakonyi, T, Sikutova, S, et al. Serological evidence of continuing Usutu virus (Flaviviridae) activity and establishment of herd immunity in wild birds in Austria. Vet Microbiol 2008; 127:237–248.

Nemeth, N, Young, G, Ndaluka, C, Bielefeldt-Ohmann, H, et al. Persistent West Nile virus infection in the house sparrow (*Passer domesticus*). Arch Virol 2009; 154:783–789.

Pecorari, M, Longo, G, Gennari, W, Grottola, A, et al. First human case of Usutu virus neuroinvasive infection, Italy, August–September 2009. Euro Surveill 2009; 14:pii: 19446.

Petric, D, Zomba, M, Bellini, R, Veronesi, R, et al. Validation of CO_2 trap data in three European regions. *Proceedings of the International Conference of Urban Pest*, Prague, 1999:437–445.

Rizzoli, AP, Rosa, R, Rosso, F, Buckley, A, Gould, E. West Nile virus circulation detected in Northern Italy in sentinel chickens. Vector Borne Zoonot Dis 2007; 7:411–417.

Rossini, G, Cavrini, F, Pierro, A, Macini, P, et al. First human case of West Nile virus neuroinvasive infection in Italy, September 2008—case report. Euro Surveill 2008; 13:pii: 19002.

Scaramozzino, N, Crance, JM, Jouan, A, Debriel, DA, et al. Comparison of Flavivirus universal primer pairs and development of a rapid, highly sensitive heminested reverse transcription-PCR assay for detection of flaviviruses targeted to a conserved region of the NS5 gene sequences. J Clin Microbiol 2001; 39:1922–1927.

Sutherland, GL, Nasci, RS. Detection of West Nile virus in large pools of mosquitoes. J Am Mosq Control Assoc 2007; 23:389–395.

Tamura, K, Dudley, J, Nei, M, Kumar, S. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 2007; 24:1596–1599.

Tang, Y, Hapip, CA, Liu, B, Fang, CT. Highly sensitive TaqMan RT-PCR assay for detection and quantification of both lineages of West Nile virus RNA. J Clin Virol 2006; 36:177–182.

Weissenböck, H, Hubalek, Z, Bakonyi, T, Nowotny, N. Zoonotic mosquito-borne flaviviruses: worldwide presence of agents

with proven pathogenicity and potential candidates of future emerging diseases. Vet Microbiol 2010; 140:271–280.

Weissenböck, H, Kolodziejek, J, Fragner, K. Usutu virus activity in Austria, 2001–2002. Microbes Infect 2003; 5:1132–1136.

Weissenböck, H, Kolodziejek, J, Url, A, Lussy, H, et al. Emergence of Usutu virus, an African mosquito-borne flavivirus of the Japanese encephalitis virus group, central Europe. Emerg Infect Dis 2002; 8:652–656.

Williams, MC, Simpson, DI, Haddow, AJ, Knight, EM. The isolation of a West Nile virus from man and Usutu virus from the bird-biting mosquito Mansonia aurites (Theobald) in the Entebbe area of Uganda. Ann Trop Med Parasitol 1964; 58:367–374.

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