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Short Report: West Nile and Usutu Viruses in Mosquitoes in Spain, 2008–2009

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Abstract. West Nile virus lineage 1 (similar to the strains obtained from golden eagles in Spain, 2007) and Usutu virus (similar to the strains obtained from *Culex pipiens* in Spain, 2006) were detected in pools from *Culex perexiguus* collected in southern Spain in 2008 and 2009, respectively. This is the first detection and isolation of West Nile virus lineage 1 from mosquitoes in Spain.

INTRODUCTION

The flaviviruses West Nile virus (WNV) and Usutu virus (USUV) have a complex life cycle, involving several bird species as primary hosts, mosquitoes of the genus *Culex* as primary vectors, and humans, horses, and other mammals as incidental hosts.

West Nile virus has been described in Africa, Europe, the Middle East, Asia, Australia, and America. Studies of the phylogenetic relatedness of WNV strains isolated in different geographic regions show that isolates fall into seven putative genetic lineages of WVN.[1] So far, WNV has been documented in animals and humans in several countries across Europe, mainly in central Europe and in the Mediterranean region. Most of the strains responsible for the European and the Mediterranean Basin outbreaks were lineage 1. Over the last 15 years, outbreaks and/or sporadic cases in horses and/or humans were reported in Romania, Hungary, Russia, Portugal, Czech Republic, France, Italy, Spain, and Greece. In 2008 and 2009 several countries in Europe (Romania, Hungary, Austria, and Italy) reported WNV activity caused by different lineages.[2]

West Nile virus lineage 2, previously described in Africa, was first detected in Europe in goshawk specimens in Hungary in 2004–2005. In Austria in 2008, two outbreaks were detected in wild birds, one in North Austria and the other in Vienna. The virus isolated was WNV lineage 2, homologous to the strains previously found in Hungary.[3] Recently, in August 2010, a WNV outbreak was detected in Central Macedonia (Northern Greece) with an unexpected lethality in humans; the sequences obtained were more than 99% similar to the WNV lineage 2 found in Hungary and Austria in recent years (ProMED-mail, archive no. 20100914.3312).

The USUV had been isolated only once in the Central African Republic in a man with fever and rash, until it emerged in Europe in 2001 in Austria, where USUV was associated with avian mortality for five consecutive summers, affecting predominantly blackbirds (*Turdus merula*). In the last decade, USUV infection was detected in a variety of central European birds in Austria, Hungary, Switzerland, United Kingdom, Spain, and Italy.[4–7] The USUV emerged as a new relevant pathogen for humans in the summer of 2009, when it was associated with neurological disorders in two patients in Italy in an area with co-circulation of WNV and USUV.[7]

In Spain, the first clinical case of WNV infection was reported in 2004 in a patient visiting south-western Spain.[8] Moreover, WNV lineage 1 was detected and further isolated in free-living and captive Spanish Golden Eagles (*Aquila chrysaetos*) in south-central Spain[9] and a new lineage of WNV was described in *Culex pipiens* in the southern part of the country.[1] In this region, recent studies indicate that the virus is circulating in birds, horses, and humans.[10–12] For the first time in Spain, disease by WNV have been described in 2010 in horses (ProMED-mail, archive no.: 20101119.4203) and humans (ProMED-mail, archive no.: 20100925.3478). The USUV was discovered for the first time in Spain in a pool of *Cx. pipiens* from Catalonia in 2006, showing a higher homology to the African USUV isolates than to the central European ones.[10] We collected mosquito samples from southern Spain, from areas where WNV circulation had been seen, to look for the presence of flaviviruses.

The study. Mosquitoes were captured in the Guadalquivir marshes and adjoining wetlands (X:37.238347°, Y:–6.130186°; X:36.329369°, Y:–5.837751°; X:36.999241°, Y:–6.499944°; X:37.279287°, Y:–6.909612°) between February 2008 and November 2009. Traps used were Centers for Disease Control and Prevention (CDC)-light traps supplied with CO_2 and Gravid-traps, which were used in the field during the late afternoon and retrieved the following morning. Mosquitoes were pooled by species, sex, collecting site, and date. The number of mosquitoes per pool ranged from 1 to 52 and belong to different species (Table 1). Almost half of the specimens belongs to the species *Ochlerotatus caspius* (45%) followed by *Culex modestus* (21.7%), *Cx. pipiens* (16%), and *Culex perexiguus* (9.4%). The screening was performed with a generic RT-nested-PCR[11] to detect flavivirus genome. We analyzed a total of 61,082 mosquito specimens, grouped into 3,471 pools and WNV was identified in seven pools of *Cx. perexiguus* in 2008 (3 pools collected in August, 3 in September, and 1 in October) and USUV was identified in only one pool in the same vector species collected in November 2009. The vector infection rate was estimated for *Cx. perexiguus* as 0.1% for WNV (95% confidence interval [CI] = 0.04–0.2) and 0.01% for USUV (95% CI = 0–0.06), as calculated with EpiTools epidemiological calculators method (http://epitools.ausvet.com.au/content.php?page=PPVariablePoolSize). *Culex perexiguus* had a seasonal distribution between February and November, and

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Table 1
Number of mosquitoes analyzed in this study

	No. pools	No. mosquitoes	Pools + WNV	Pools + USUV
An. algeriensis	30	102	0	0
An. atroparvus	74	161	0	0
An. plumbeus	1	1	0	0
Cx. pipiens	851	9,747	0	0
Cx. theileri	273	4,259	0	0
Cx. modestus	578	13,222	0	0
Cx. perexiguus	361	5731	7	1
Culex sp.	3	8	0	0
Cs. longiareolata	24	37	0	0
Cs. annulata	54	103	0	0
Oc. berlandi	1	1	0	0
Oc. caspius	1,153	27,451	0	0
Oc. detritus	66	257	0	0
Ur. unguiculata	2	2	0	0

was more abundant during June, July, and August, feeding in the study area mainly on birds (70% of blood meals) (Muñóz J, personal communication).

Finally, we amplified and sequenced 1,671 nucleotides from the NS5 gene from WNV genome (HQ833021 in GenBank) and USUV (HQ833022). Multiple alignments of the Spanish WNV (WNV/08) and USUV (USUV/09) sequences and 50 genomes belonging to different WNV and USUV strains, available in GenBank were carried out. Phylogenetic analysis gave rise to a tree in which these sequences fell under the branch of WNV and USUV, respectively, with a value of certainty of 100% (Figure 1) in both cases. The WNV/08 sequence showed a high relation with all the strains that have been circulating in recent years in Europe. Pairwise alignment of these sequences revealed that they differ in only four nucleotide positions (99% identity) with the sequences obtained in Spain in 2007 from golden eagles, with no change in amino acid residues. The USUV/09 is slightly more similar to USUV from South Africa, than USUV from Vienna 2001 and Budapest 2005 (96.9%, 96.3%, and 96.2%, respectively) when the 1,671 nt fragments were analyzed, and these changes resulted in only two amino acid replacements, with regard to the other strains (alignment positions: 142, R → K, 328 M → I). In the fragment (99 nt) available for the strain from Spain 2006, both Spanish strains are almost identical, and only differ in 1 nt (Figure 2). The homology data suggest that the Spanish strains detected in 2006 and 2009 belong to USUV species and are more related to the African USUV isolates than to the central European isolates.

The homogenate from positive pools were inoculated onto Vero cells cultures. The WNV/08 was isolated from one positive pool and cytopathic effect (CPE) was observed after a single passage at 5-day post-infection. In the case of USUV/09 with a unique pool, neither CPE nor amplification was obtained. The failure in this case of viral isolation, could be caused by cytotoxic effects observed post-inoculation. To attempt the isolation of the USUV/09 again and to minimize the cytotoxicity, the positive pool was diluted 1:10, 1:100 and 1:1000 in the minimal essential medium, and 200 µL were injected onto Vero cell culture. No cytotoxic effect was observed post-inoculation this time. Cell culture was incubated at 37°C for 7 days and three blind passages were carried out. Signs of CPE were checked daily, and the culture supernatants were tested by reverse transcription-polymerase chain reaction, but no virus was isolated again.

CONCLUSIONS

This report shows the circulation and first detection of WNV lineage 1 and USUV in *Cx. perexiguus* mosquitoes and the first isolation of WNV lineage 1 from mosquitoes in Spain. Both viruses have been spreading during the last decade in Europe.

The sequences of WNV and USUV detected in this study from mosquitoes are very similar to WNV detected in golden eagles in 2007 and Spanish USUV detected in *Cx. pipiens* in Catalonia in 2006. Until now, no infections in humans or animals have been detected by USUV in Spain, but cases of WNV disease have been described in humans, birds, and horses[8,9] (ProMED-mail, archive no.: 20101119.4203 and 20100925.3478). Although both viruses could have been reintroduced by migrating birds repeatedly in the Mediterranean region, the fact that the sequences found are highly homogeneous and their frequent detection in the last few years, could suggest an endemic circulation of these viruses.

The USUV and WNV share the same vectors and their co-circulation was demonstrated in Italy (both viruses detected in *Cx. pipiens* mosquitoes) where, at the same time, a human case of USUV infection was wrongly identified as being caused by WNV.[7,12] The USUV may be considered as an emergent and zoonotic virus and both viruses have become resident pathogens in Europe and the differential diagnosis and consequences for public health should be considered.

In Spain this is the first detection and isolation of WNV lineage 1 from *Cx. perexiguus* mosquitoes, previously described as the main WNV vector in Israel,[13] and our findings suggest that *Cx. perexiguus* could be an important vector for WNV lineage 1 in Spain. These events further emphasize the ability of this virus to use different mosquitoes' species in different areas. Further efforts are therefore needed to clarify the ecological and epidemiological patterns of the infection in the Old World. In the same way, enhanced integrated multidisciplinary surveillance activities to monitor the WNV and USUV spread and to take appropriate and timely measures are needed.

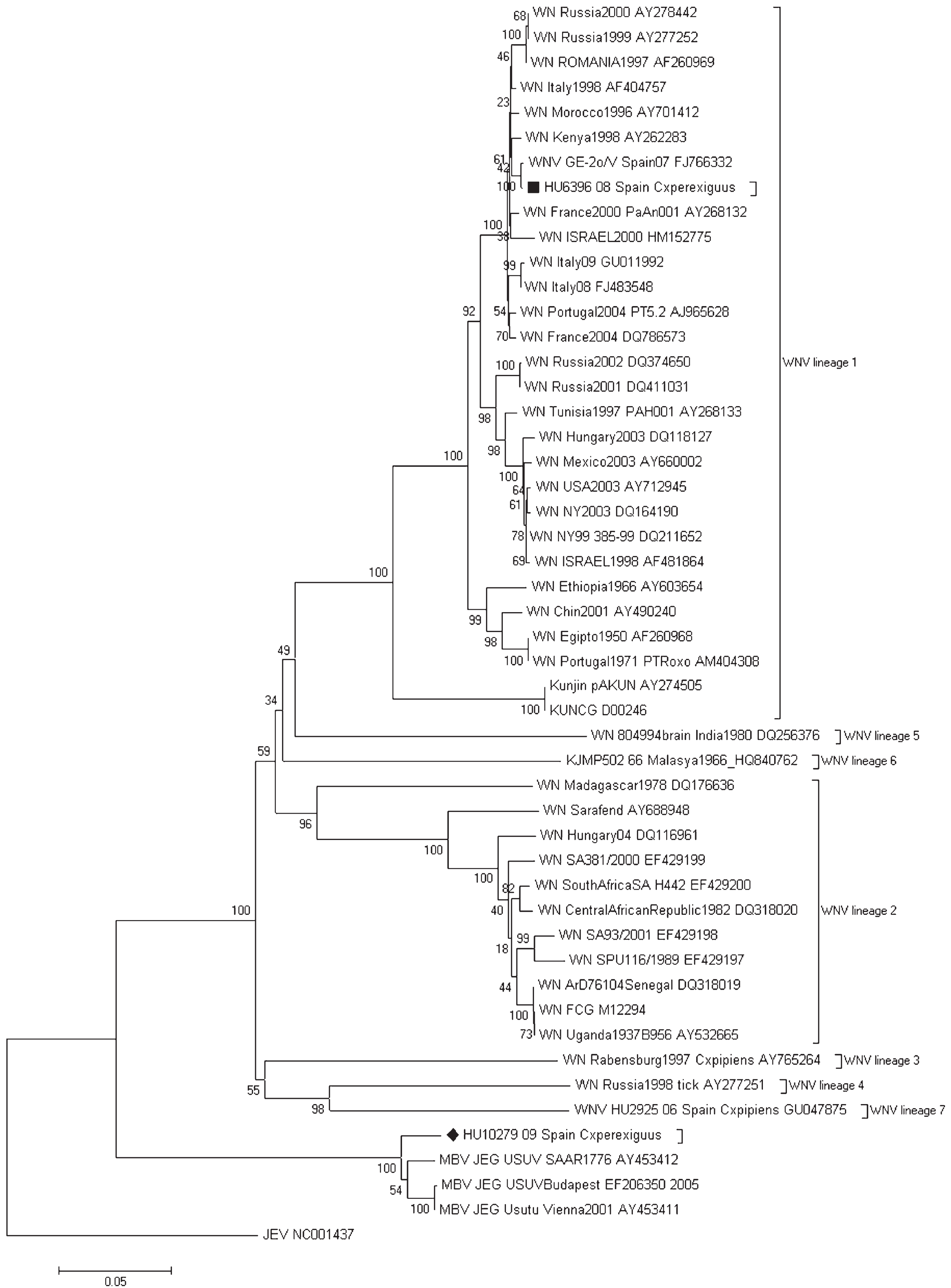

FIGURE 1. Phylogenetic tree of 50 flaviviruses sequences (including two sequences obtained in this study: HU6365/08 Spain and HU10279/09 Spain). The phylogenetic analyses were carried out by the neighbor-joining method and kimura-2 model on Mega4. The percentage of successful bootstrap replicates ($N = 1,000$) is indicated at nodes. Japanese encephalitis virus (JEV) is used as an outgroup. The accession numbers of the sequences used in this phylogenetic study are indicated in the phylogenetic tree.

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#USUV_09Spain_Cxperexiguus                   GAA GCG TGA GAA GAA GCT CGG AGA GTT TGG CAA AGC GAA AGG CAG TCG AGC CAT ATG GTT TAT GTG GCT AGG CGC CAG ATT CCT GGA GTT TGA AGC CCT
#USU_SpainMB119_06_Cxpipiens_AM909649        ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... .T.
#USU-BO-10/2009_Cxpipiens_HM138718           ... .A. G.. ... ... ... ... ... ... ... ... ... ... ... T.. ... ... ... ... ... C.. ... ... ... ... ... ... ... ... ... ... ... T..
#USUV_SAAR1776_AY453412                      ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... ... C.. ... ... ... ... ... ... ... ... ... ... ... ...
#Usutu_Vienna2001_AY453411                   ... ... ... ... ... ... ... ... ... ... T.. ... ... ... T.. ... ... ... ... ... C.. ... ... ... ... ... ... ... ... ... ... ... T..
#USUVBudapest_EF206350_2005                  ... ... ... ... ... ... ... ... ... ... T.. ... ... ... T.. ... ... ... ... ... C.. ... ... ... ... ... ... ... ... ... ... ... T..
#WN_Ita09_GU011992                           A.. .A. A.. ... A.. A.C ... ... ... C.. A.. G.. C.. G.. A.. CA. ... ... ... ... C.. ... ... C.. A.. TC. C.. T.. ... ... C.. ... T..
#WNV_GE-2o/V_Spain07_FJ766332                A.. .A. A.. ... A.. A.C ... ... ... C.. A.. G.. C.. G.. A.. CA. ... ... ... ... C.. ... ... C.. A.. TC. C.. T.. ... ... C.. ... T..
#WN_Portugal04_PT5.2_AJ965628                A.. .A. A.. ... A.. A.C ... ... ... C.. A.. G.. C.. G.. A.. CA. ... ... ... ... C.. ... ... C.. A.. TC. C.. T.. ... ... C.. ... T..
#WN_France04_magpye_DQ786573                 A.. .A. A.. ... A.. A.C ... ... ... C.. A.. G.. C.. G.. A.. CA. ... ... ... ... C.. ... ... C.. A.. TC. C.. T.. ... ... C.. ... T..
#WN_gooseHungary03_DQ118127                  A.. .A. A.. ... A.. A.C ... ... ... C.. A.. G.. C.. G.. A.. CA. ... ... T.. ... C.. ... ... C.. A.. TC. C.. T.. ... ... C.. G.. T..
#WN_France00_PaAn001_AY268132                A.. .A. A.. ... A.. A.C ... ... ... C.. A.. G.. C.. G.. A.. CA. ... ... ... ... C.. ... ... C.. A.. TC. C.. T.. ... ... C.. ... T..
#WN_LEIVVlg00_27924_AY278442                 A.. .A. A.. ... A.. A.C ... ... ... C.. A.. G.. C.. G.. A.. CA. ... ... ... ... C.. ... ... C.. A.. TC. C.. T.. ... ... C.. ... T..
#WN_ISR00_0304h_HM152775                     A.. .A. A.. ... A.. A.C ... T.. ... C.. A.. G.. C.. G.. A.. CA. ... ... ... ... C.. ... ... C.. A.. TC. C.. T.. ... ... C.. ... T..
#WN_LEIVVlg99_27889_AY277252                 A.. .A. A.. ... A.. A.C ... ... ... C.. A.. G.. C.. G.. A.. CA. ... ... ... ... C.. ... ... C.. A.. TC. C.. T.. ... ... C.. ... T..
#WN_Italy1998_equineAF404757                 A.. .A. A.. ... A.. A.C ... ... ... C.. A.. G.. C.. G.. A.. CA. ... ... ... ... C.. ... ... C.. A.. TC. C.. T.. ... ... C.. ... T..
#WN_Kenya1998CxunivitatusKN3829_AY262283     A.. .A. A.. ... A.. A.C ... ... ... C.. A.. G.. C.. G.. A.. CA. ... ... ... ... C.. ... ... C.. A.. TC. C.. T.. ... ... C.. ... T..
#WN_IS98STD_staork_AF481864                  A.. .A. A.. ... A.. A.C ... ... ... C.. A.. G.. C.. G.. A.. CA. ... ... T.. ... C.. ... ... C.. A.. TC. C.. T.. ... ... C.. G.. T..
#WN_Tunisia97_PAH001_AY268133                A.. .A. A.. ... A.. A.C ... ... ... C.. A.. G.. C.. G.. A.. CA. ... ... ... ... C.. ... ... C.. A.. TC. C.. T.. ... ... C.. ... ...
#WN_RO97_50_Cxpipiens_AF260969               A.. .A. A.. ... A.. A.C ... ... ... C.. A.. G.. C.. G.. A.. CA. ... ... ... ... C.. ... ... C.. A.. TC. C.. T.. ... ... C.. ... T..
#WN_08Spain_Cxperexiguus                     A.. .A. A.. ... A.. A.C ... ... ... C.. A.. G.. C.. G.. A.. CA. ... ... ... ... C.. ... ... C.. A.. TC. C.. T.. ... ... C.. ... T..
#WN_Morocco1996horse93_111_AY701412          A.. .A. A.. ... A.. A.C ... ... ... C.. A.. G.. C.. G.. A.. CA. ... ... ... ... C.. ... ... C.. A.. TC. C.. T.. ... ... C.. ... ...
#WN_Portugal71_PTRoxo_AM404308               ... .A. A.. ... ... A.C T.. ... ... C.. A.. G.. T.. G.. A.. CA. ... ... ... ... C.. ... ... C.. A.. TC. C.. T.. ... ... C.. ... T..
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FIGURE 2. Comparison at the nucleotide level of sequenced fragment of the Spanish USUV/09 and WNV/08 among related USUV and WNV. Dot indicates coincident nucleotide.

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REFERENCES

1. Vázquez A, Sanchez-Seco MP, Ruiz S, Molero F, Hernandez L, Moreno J, Magallanes A, Tejedor CG, Tenorio A, 2010. Putative new lineage of West Nile virus, Spain. *Emerg Infect Dis 16:* 549–552.
2. Calistri P, Giovannini A, Hubalek Z, Ionescu A, Monaco F, Savini G, Lelli R, 2010. Epidemiology of West Nile in Europe and in the Mediterranean basin. *Open Virol J. 22:* 29–37.
3. Bakonyi T, Ivanics E, Erdélyi K, Ursu K, Ferenczi E, Weissenböck H, Nowotny N, 2006. Lineage 1 and 2 strains of encephalitic West Nile virus, central Europe. *Emerg Infect Dis 12:* 618–623.
4. Bakonyi T, Erdélyi K, Ursu K, Ferenczi E, Csorgo T, Lussy H, Chvala S, Bukovsky C, Meister T, Weissenböck H, Nowotny N, 2007. Emergence of Usutu virus in Hungary. *J Clin Microbiol 45:* 3870–3874.
5. Steinmetz HW, Bakonyi T, Chvala S, Weissenböck H, Eulenberger U, Hatt JM, Robert N, Hoop R, Nowotny N, 2007. Emergence of Usutu virus in Switzerland. *Proceedings 43rd International Symposium on Diseases of Zoo and Wild Animals*. *Edinburgh*, 129–131.
6. Buckley A, Dawson A, Moss SR, Hinsley SA, Bellamy PE, Gould EA, 2003. Serological evidence of West Nile virus, Usutu virus and Sindbis virus infection of birds in the UK. *J Gen Virol 84:* 2807–2817.
7. Calzolari M, Bonilauri P, Bellini R, Albieri A, Defilippo F, Maioli G, Galletti G, Gelati A, Barbieri I, Tamba M, Lelli D, Carra E, Cordioli P, Angelini P, Dottori M, 2010. Evidence of simultaneous circulation of West Nile and Usutu viruses in mosquitoes sampled in Emilia-Romagna region (Italy) in 2009. *PLoS ONE 5:* e14324.
8. Kaptoul D, Viladrich PF, Domingo C, Niubó J, Martínez-Yélamos S, de Ory F, Tenorio T, 2007. West Nile virus in Spain: report of the first diagnosed case (in Spain) in a human with aseptic meningitis. *Scand J Infect Dis 39:* 70–71.
9. Jiménez-Clavero MA, Sotelo E, Fernandez-Pinero J, Llorente F, Blanco JM, Rodriguez-Ramos J, Perez-Ramirez E, Höfle U, 2008. West Nile virus in golden eagles, Spain, 2007. *Emerg Infect Dis 14:* 1489–1491.
10. Busquets N, Alba A, Allepuz A, Aranda C, Núñez JI, 2008. Usutu virus sequences in *Culex pipiens* (Diptera: Culicidae), Spain. *Emerg Infect Dis 14:* 861–862.
11. Sánchez-Seco MP, Rosario D, Domingo C, Hernandez L, Valdes K, Guzmán MG, Tenorio A, 2005. Generic RT-nested-PCR for detection of flaviviruses using degenerated primers and internal control followed by sequencing for specific identification. *J Virol Methods 126:* 101–109.
12. Gaibani P, Pierro AM, Cavrini F, Rossini G, Landini MP, Sambri V, 2010. False-positive transcription-mediated amplification assay detection of West Nile virus in blood from a patient with viremia caused by a Usutu virus infection. *J Clin Microbiol 48:* 3338–3339.
13. Orshan L, Bin H, Schnur H, Kaufman A, Valinsky A, Shulman L, Weiss L, Mendelson E, Pener H, 2008. Mosquito vectors of West Nile fever in Israel. *J Med Entomol 45:* 939–947.