Further evidence of lineage 2 West Nile Virus in Culex pipiens *of North-Eastern Italy*

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Summary
West Nile Virus lineage 1 (WNV lin1) emerged in North-Eastern Italy in 2008 and, since then, it has been detected in animals, humans and mosquitoes. Three years later, in the same area, a lineage 2 (lin2) strain of WNV was found in birds and vectors. On August the 21st, during the 2012 WNV entomological surveillance plan, a WNV lin2 strain was detected by RT-PCR in a pool of *Culex pipiens* mosquitoes captured in Veneto region. According to the alignment of the partial sequences of the NS5 and NS3 genes, no differences between this Italian lineage 2 strain and the Nea Santa-Greece-2010 WNV isolate (Gr-10) were observed. Similarly to the Gr-10 strain, the putative NS3 aminoacid sequences of the Italian strain showed proline in position 249 instead of histidine ($H_{249}P$). Although proline in position 249 has been suggested to increase the virulence of WNV strains, neither human nor veterinary cases associated to this strain have been reported in the region. A prompt mosquito disinfestation was organized to avoid the spread of this potential threatening virus. The simultaneous circulation of both WNV lineage 1 and 2 confirms North-Eastern Italy as a high risk area for WNV emergence and highlights the need for a continuous surveillance.

Ulteriore evidenza del virus West Nile lineaggio 2 nella zanzara Culex pipiens *in Italia nord orientale*

Parole chiave
Culex pipiens,
NS3,
NS5,
Sorveglianza,
Virulenza,
Virus West Nile lineaggio 2,
Zanzare.

Riassunto
Il virus West Nile appartenente al lineaggio 1 (WNV lin1) è comparso in Italia nord-orientale nel 2008 e da allora è stato rilevato negli animali, nell'uomo e nel vettore (la zanzara della specie *Culex pipiens*). Tre anni dopo, nella stessa area, un ceppo di WNV appartenente al lineaggio 2 (WNV lin2) è stato ritrovato in uccelli e nel vettore. Il 21 agosto, durante la sorveglianza entomologica 2012, un pool di *Culex pipiens* catturato nella regione Veneto è risultato positivo alla RT-PCR per WNV lineaggio 2. Le sequenze parziali dei geni NS3 e NS5 sono risultate identiche al ceppo WNV Nea Santa-Greece-2010 (Gr-10). Come quest'ultimo ceppo, anche quello italiano ha mostrato la presenza di prolina in posizione 249 al posto di istidina nella sequenza amminoacidica NS3. Sebbene a questa sostituzione sia stato attribuito l'aumento di virulenza di alcuni ceppi di WNV, nessun caso umano o veterinario ad esso associato è stato rilevato in tutta l'area. Al fine di evitare il diffondersi di un virus potenzialmente più patogeno, si è ritenuto opportuno organizzare un'azione di disinfestazione straordinaria. La simultanea circolazione dei lineaggi 1 e 2 del WNV conferma quest'area del nord Italia come zona ad alto rischio per l'emergenza del virus e sottolinea l'importanza di una sorveglianza continua.

West Nile Virus (WNV) emerged in North-Eastern Italy in 2008 and since then it has been constantly detected in humans, animals and the vector mosquitoes (25, 10, 4, 5, 8, 11, 14). Most of the infections have been caused by WNV lineage 1 (lin1) strains, even though in 2011 a lineage 2 (lin2) strain similar to the Hungarian isolate was detected both in wild birds and local mosquitoes. This article describes the circulation of a WNV lin2 similar to the Nea Santa-Greece-2010 (Gr-2010) (27) strain- in Veneto region, North-Eastern Italy, in 2012.

In order to monitor and control WNV circulation, serological, entomological and virological surveillance programmes for West Nile disease (WND) were implemented both at national and regional level (9, 13, 19, 20, 21). In 2012, thirty-four CO_2-CDC like mosquito traps were placed in rural and suburban sites in North-Eastern Italy. The traps operated from May until October and were activated every 15 days for one night, from sunset to sunrise. It is noteworthy that the entomological monitoring system used for determining mosquito species composition, density, and WNV rate of infection (8) has also shown to be a very valuable tool for the early detection of virus circulation (11, 14).

Collected mosquitoes were immediately refrigerated and transported to the laboratory, counted, identified using standard taxonomic keys (22, 28), and pooled in 50 specimens maximum, according to species, site, and date and then stored at –80 °C.

Viral RNA was extracted from pooled mosquitoes (NucleoSpin 96 RNA kit; Machery-Nagel, Duren, Germany) and screened for the presence of flaviviruses by using a one-step SYBR Green-Based rRT-PCR targeting 250bp of the conserved region of the non-structural NS5 gene, with MAMD and cFD2 primers (18, 27). To confirm the presence of WNV, all flavivirus positive samples were tested by an RT-PCR (primer EWN-F and EWN-R) targeting 705bp of NS5 gene (2).

PCR products were analyzed for purity and size by electrophoresis in 2% agarose gel after staining with GelRedTM Nucleic Acid Gel Stain (Biotium, Hayward, CA, USA). Amplicons were subsequently purified with ExoSAP-IT (USB Corporation, Cleveland, OH, USA) and sequenced in both directions using the Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). The products of the sequencing reactions were cleaned-up using the Performa DTR Ultra 96-well kit (Edge BioSystems, Gaithersburg, MD, USA) and analyzed on a 16-capillary ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems). Sequence data were assembled and edited with SeqScape software v 2.5 (Applied Biosystem). They have been aligned and compared with representative sequences available in GenBank.

On the 21st of August 2012, one pool of 50 *Culex pipiens* mosquitoes (Cx.pi.2436/12-RO), collected in the Rovigo province, Veneto region, was found positive for WNV. The BLAST and phylogenetic analysis of the sequence confirmed the strain as WNV lin2. This sample was also amplified by using a specific RT-PCR with the primer pair WN-NS3up1 and WN-NS3do1 (26) that target 778bp of the NS3 gene.

The representative NS5 sequences of the WNV lin1 and lin2 and the NS3 sequences of the WNV lin2 found from 2008 to 2012 in *Cx. pipiens* - animals and humans in the same area of North-Eastern Italy - were compared with reference sequences (Figures 1 and 2). Phylogenetic analyses were carried out for NS5 and NS3 partial gene sequences using the neighbour-joining (N-J) method with 1,000 bootstrap replicates implemented in the MEGA 4 programme (30, 31). NS5 and NS3 sequences were submitted to GenBank database (accession numbers JX878387 and JX878386, respectively).

RNA partial sequence of NS3 and NS5 genes revealed 99-100% identity with WNV isolate Nea Santa-Greece-2010 (Gr-10) (HQ537483). Based on these sequences, the Cx.pi.2436/12-RO strain was closely related to WNV lin2 strains detected in Italy in 2011 and Hungary in 2004 (Figures 1 and 2).

The putative aminoacid sequences of the polyprotein precursor showed the aminoacid substitution histidine vs proline at the 249 site ($H_{249}P$) in the NS3 helicase.

The phylogenetic tree of NS5 sequences of WNV lin1 and lin2 (Figure 2) shows that the North-Eastern strains of WNV lin1 are separated in two clusters, each defined by high bootstrap values (>70%). In particular, the first group contains viruses closely related to the original strains Italy/08 and NY-99, while the second includes viruses closely related to the Livenza strain (JQ928174), isolated in humans (6). To the best of our knowledge this is the first evidence of WNV lin2 similar to the Greek variant Gr-2010 in Italy. The Cx.pi.2436/12-RO strain characterized in this study holds the $H_{249}P$ substitution which was supposed to be related to the higher virulence of WNV lin2 that emerged in Greece in 2010 (15, 16), causing more than 250 neuroinvasive disease cases with 15% fatality (17). Single non-silent mutations in non-structural genes were indicated to modulate WNV virulence, both exacerbating and attenuating infectivity (29, 12, 32). A NS3-Pro249 substitution was present in the virus isolated in the 1951 Egyptian outbreak and in the 1999 New York outbreak, both associated with high fatality rate (7). In experimental studies the introduction of the substitution $T_{249}P$ in the NS3 helicase of a low-virulence WNV strain generated a phenotype highly virulent to American crows with a higher capacity to replicate, and which would

likely result in increased virus transmission rates (7). This particular substitution $T_{249}P$ was detected in all human isolates from the 2008 outbreak (WNV lin1) and was heterogeneously distributed among the 2009 human isolates. The authors concluded that it could not be considered a hallmark of the WNV strain causing the outbreak (23, 24).

Fortunately, the finding of our WNV lin2 in *Cx. pipiens* in August was not followed by human or animal cases due to this specific strain. However, in order to avoid further spread of a potential threatening virus, the regional authorities decided to implement the routine mosquito disinfestation with an extraordinary campaign, targeting both larval breeding sites within a three kilometres radius from the positive trap and adult mosquitoes close to human settling.

In North-Eastern Italy, WNV lineage 1 and 2 were found to circulate since 2008. Specifically, the WNV lin1 (Italy/08) emerged in 2008 and overwintered through 2010. It was no more detected in hosts and vectors in the following years; the WNV lin1 (Livenza-Italy/11) emerged in 2010 and was still circulating in summer 2012, affecting humans, animals and mosquitoes; while the WNV lin2 (Hungary-2004) circulated among mosquitoes and birds in 2011. Finally, the WNV lin2 (Gr-2010) strain emerged for the first time in 2012 in mosquitoes collected in an area of the region constantly monitored for the presence of WNV and its future circulation is unpredictable.

These findings highlight the fact that new introductions, likely through migratory birds from Africa and within Europe, have occurred in few years (WNV lin1 and lin2) and indicate the capability of these viruses to become endemic and to rapidly evolve and emerge in different sites (3). In conclusions, our results confirm North-Eastern Italy as a high-risk area for WNV emergence and outbreaks, and strongly call for the need of a continuous surveillance in the future.

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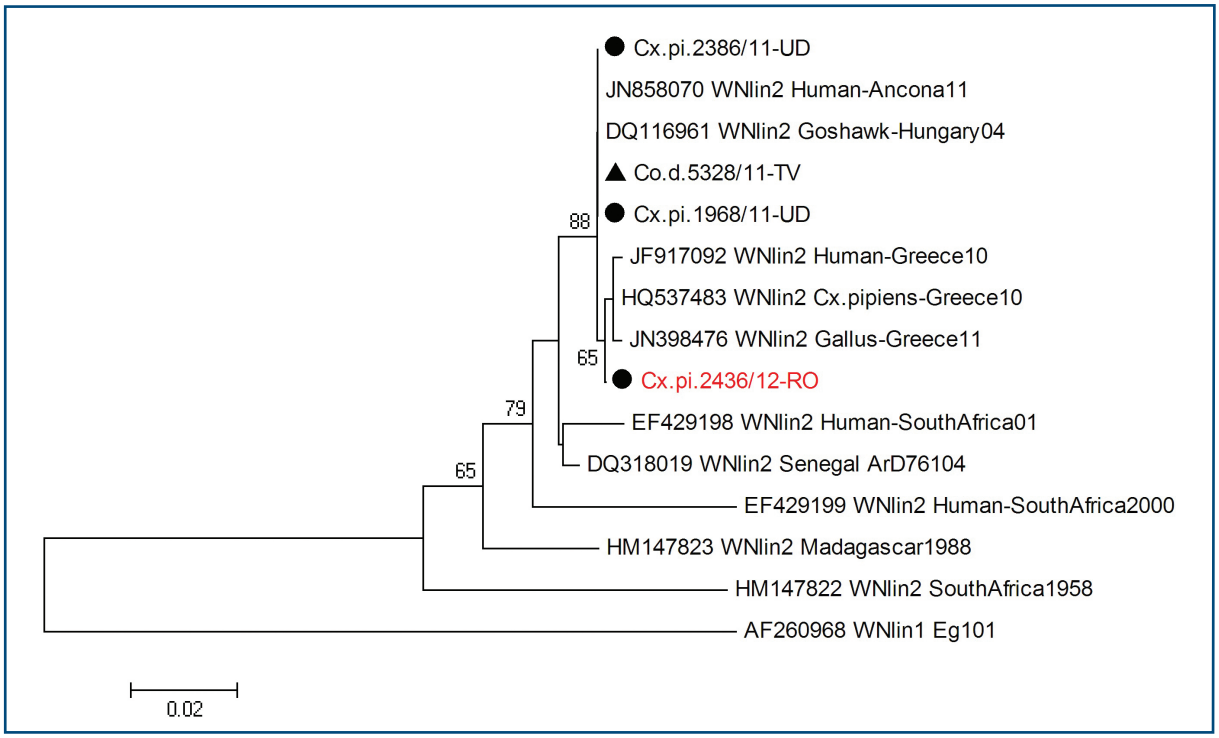

Figure 1. *Phylogenetic tree of WNV lineage 2 NS3 partial sequences.* Phylogenetic tree based on a fragment of 778bp of NS3 gene of West Nile virus. Sequence dataset was analyzed using MEGA 4, the neighbour-joining (NJ) method, and bootstrap analysis (1,000 replicates) based on the ClustalW algorithm. Significant bootstrapping values (>65%) are shown on the nodes.

(●) Italian WNV lineage 2 strains detected in pools of *Culex pipiens* in North-Eastern Italy: Cx.pi.2436/12-RO caught in Rovigo province in 2012 and Cx.pi.1968/11-UD, caught in Udine Province in 2011. (▲) Italian WNV lineage 2 strain detected in animals: Co.d.5328/11-TV, a collared dove found dead in Treviso province in 2011.

WNV lineage 2 strains from GenBank (GenBank accession number, isolation source, country, year): JN858070 human, Italy (Ancona), 2011 (1); DQ116961 goshawk, Hungary, 2004; JF917092 human, Geece, 2010; HQ537483 *Culex pipiens*, Greece, 2010; JN398476 gallus, Greece, 2011; EF429198 human, South Africa, 2001; DQ318019 Senegal; EF429199 human, South Africa, 2000; HM147823 Madagascar, 1988; HM147822 South Africa, 1958. WNV lineage 1 strain from GenBank AF260968, Egypt.

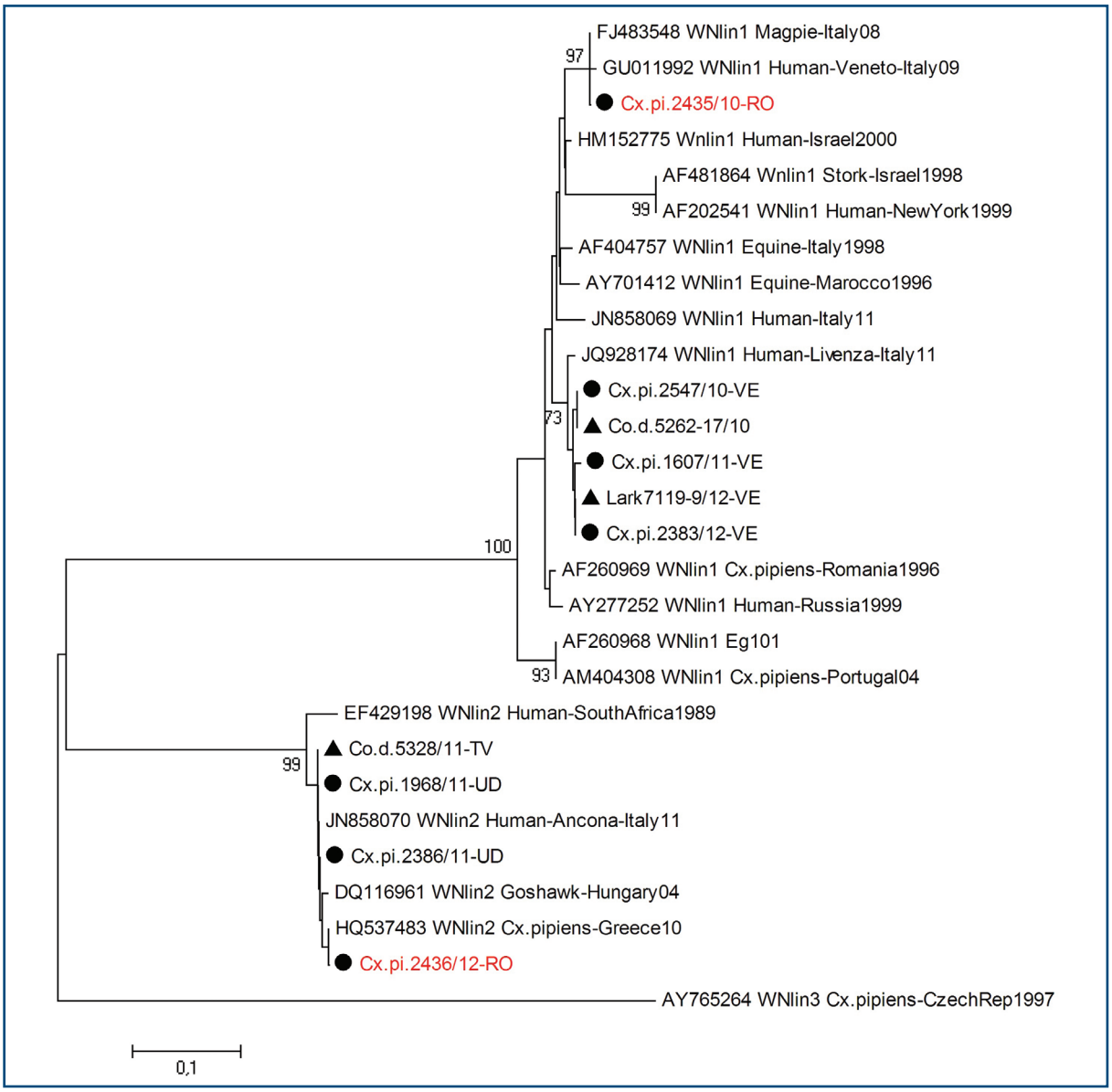

Figure 2. *Phylogenetic tree of WNV lineage 1 and 2 NS5 partial sequences.* Phylogenetic tree based on a fragment of 705bp of the NS5 gene of West Nile virus. Sequence dataset was analyzed using MEGA 4, the neighbour-joining (NJ) method, and bootstrap analysis (1,000 replicates) based on the ClustalW algorithm. The tree obtained was rooted with WNlin 3 (AY 765264) strain. Significant bootstrapping values (>70%) are shown on the nodes.

(●) Italian WNV strain detected in pools of *Culex pipiens* in North-Eastern Italy: Cx.pi.2587/10-VE, Cx.pi.1607/11-VE and Cx.pi.2383/12-VE caught in Venice province in 2010, 2011 and 2012; Cx.pi.2435/10-RO and Cx.pi.2436/12-RO caught in Rovigo province in 2010 and 2012; Cx.pi.1968/11-UD, caught in Udine Province in 2011. (▲) Italian WNV strain detected in animals: Lark-7119-9/12VE found dead in Venice Province in 2012, Co.d.5328/11-TV, a collared dove found dead in Treviso province in 2011.

WNV lineage1 strains from GenBank (GenBank accession number, isolation source, country, year): AF202541 human, New York, 1999; AF481864 stork, Israel, 1998; HM152775 human, Israel, 2000; GU011992 human, Italy (Veneto Region), 2009; FJ483548 magpie, Italy, 2008; AF404757 equine, Italy, 1998; AY701412 equine, Morocco, 1996; JN858069 human, Italy, 2011; JQ928174 human, Italy (Livenza), 2011; AF260969 *Culex pipiens*, Romania, 1996; AY277252 human, Russia, 1999; AF260968 Egypt; AM404308 *Culex pipiens*, Portugal, 2004. WNV lineage 2 strains from GenBank: EF429198 human, South Africa, 1989; JN858070 human, Italy (Ancona), 2011; DQ116961 goshawk, Hungary, 2004; HQ537483 *Culex pipiens*, Greece, 2010. WNV lineage 3 strain from GenBank: AY765264 *Culex pipiens*, Czech Republic, 1997.

References

1. Bagnarelli P., Marinelli K., Trotta D., Monachetti A., Tavio M., Del Gobbo R., Capobianchi M.R., Menzo S., Nicoletti L., Magurano F. & Varaldo P.E. 2011. Human case of autochthonous West Nile virus lineage 2 infection in Italy, September 2011. *Euro Surveill*, **16** (43), pii=20002. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20002.
2. Bakonyi T., Ivanics E., Erdelyi K., Ursu K., Ferenczi E., Weissenbock H. & Nowotny N. 2006. Lineage 1 and 2 strains of encephalitic West Nile virus, central Europe. *Emerg Infect Dis*, **12** (4), 618-23.
3. Bakonyi T., Ferenczi E., Erdélyi K., Kutasi O., Csörgö T., Seidel B., Weissenböck H., Brugger K., Bán E. & Nowotny N. 2013. Explosive spread of a neuroinvasive lineage 2 West Nile virus in Central Europe, 2008/2009. *Vet Microbiol*, **165** (1-2), 61-70.
4. Barzon L., Pacenti M., Cusinato R., Cattai M., Franchin E., Pagni S., Martello T., Bressan S., Squarzon L., Cattelan A.M., Pellizzer G., Scotton P., Beltrame A., Gobbi F., Bisoffi Z., Russo F. & Palù G. 2011. Human cases of West Nile Virus Infection in North-Eastern Italy, 15 June to 15 November 2010. *Euro Surveill*, **16** (33), pii=19949. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19949.
5. Barzon L., Pacenti M., Franchin E., Squarzon .L, Lavezzo E., Toppo S., Martello T., Cattai M., Cusinato R. & Palù G. 2012. Novel West Nile virus lineage 1a full genome sequences from human cases of infection in North-Eastern Italy, 2011. *Clin Microbiol Infect*, **18** (12), E541-4.
6. Barzon L., Pacenti M., Franchin E., Lavezzo E., Martello T., Squarzon L., Toppo S., Fiorin F., Marchiori G., Russo F., Cattai M., Cusinato R. & Palù G. 2012. New endemic West Nile virus lineage 1a in northern Italy, July 2012. *Euro Surveill*, **17** (31), pii=20231. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=20231.
7. Brault A.C., Huang C.Y., Langevin S.A., Kinney R.M., Bowen R.A., Ramey W.N., Panella N.A., Holmes E.C., Powers A.M. & Miller B.R. 2007. A single positively selected West Nile viral mutation confers increased virogenesis in American crows. *Nat Genet*, **39**, 1162-1166.
8. Busani L., Capelli G., Cecchinato M., Lorenzetto M., Savini G., Terregino C., Vio P., Bonfanti L., Pozza M.D. & Marangon S. 2011. West Nile virus circulation in Veneto region in 2008-2009. *Epidemiol Infect*, **139** (6), 818-825.
9. 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 Virology Journal*, **4**, 29-37.
10. Calistri P., Giovannini A., Savini G., Monaco F., Bonfanti L., Ceolin C., Terregino C., Tamba M., Cordioli P. & Lelli R. 2010. West Nile virus transmission in 2008 in North-Eastern Italy. *Zoonoses Public Health*, **57** (3), 211-219.
11. Gobbi F., Barzon L., Capelli G., Angheben A., Pacenti M., Napoletano G., Piovesan C., Montarsi F., Martini S., Rigoli R., Cattelan A.M., Rinaldi R., Conforto M., Russo F., Palù G. & Bisoffi Z. 2012. Veneto Summer Fever Study Group. Surveillance for West Nile, dengue, and chikungunya virus infections, Veneto Region, Italy, 2010. *Emerg Infect Dis*, **18** (4), 671-673.
12. Liu W.J., Wang X.J., Clark D.C., Lobigs M., Hall R.A. & Khromykh A.A. 2006. A single amino acid substitution in the West Nile virus nonstructural protein NS2A disables its ability to inhibit alpha/beta interferon induction and attenuates virus virulence in mice. *J Virol*, 80, 2396-2404.
13. Ministero della Salute. 2008. Approvazione del Piano di sorveglianza nazionale per la encefalomielite di tipo West Nile (West Nile disease). [Approval of the national surveillance plan for West Nile encephalomyelitis]. *Official Journal* n. 36 of 12 february 2008. http://www.trovanorme.salute.gov.it/dettaglioAtto?id=25243.
14. Mulatti P., Bonfanti .L, Capelli G., Capello K., Lorenzetto M., Terregino C., Monaco F., Ferri G. & Marangon S. 2013. West Nile Virus in North-Eastern Italy, 2011: Entomological and Equine IgM-Based Surveillance to Detect Active Virus Circulation. *Zoonoses Public Health*, **60** (5), 375-382.
15. Papa A., Bakonyi T., Xanthopoulou K., Vázquez A., Tenorio A. & Nowotny N. 2011. Genetic characterization of West Nile virus lineage 2, Greece, 2010. *Emerg Infect Dis*, **17** (5), 920-922.
16. Papa A., Xanthopoulou K., Gewehr S. & Mourelatos S. 2011. Detection of West Nile virus lineage 2 in mosquitoes during a human outbreak in Greece. *Clin Microbiol Infect*, **17** (8), 1176-1180.
17. Papa A. 2012. West Nile virus infections in Greece: an update. *Expert Rev Anti Infect Ther*, **10** (7), 743-750.
18. Ravagnan S., Granato A., Porcellato E., Cazzin S., De Nardi R., Mazzacan E., De Battisti C., Marangon S. & Capelli G. 2011. Development of a one-step real-time PCR assay for detection of flaviviruses in pooled mosquitoes. *In* 5th Annual Meeting EPIZONE, 11–14 April 2011, The Netherlands.
19. Regione Veneto. 2011. Deliberazione della giunta regionale N. 1094 del 26/07/2011 (BUR n. 63 del 23/08/2011). Progetto Sorveglianza sulle zoonosi trasmesse da vettori e valutazione del rischio di diffusione sul territorio della Regione Veneto. http://bur.regione.veneto.it/BurvServices/Pubblica/DettaglioDgr.aspx?id=234061.
20. Regione Veneto. 2012. Deliberazione della giunta regionale N. 1519 del 31/07/2012 (BUR n. 71 del 28/08/2012). Progetto Sorveglianza sulle zoonosi trasmesse da vettori e valutazione del rischio di diffusione sul territorio della Regione Veneto. http://bur.regione.veneto.it/BurvServices/Pubblica/DettaglioDgr.aspx?id=241875.
21. Regione Veneto. 2011. Deliberazione della giunta regionale N. 2562 del 29/12/2011 (BUR n. 10 del 31/01/2012). Piano Regionale Prevenzione del Veneto - anni 2010-2012. http://bur.regione.veneto.it/BurvServices/Pubblica/DettaglioDgr.aspx?id=237293.

22. Romi R., Pontuale G. & Sabatinelli G. 1997. Le zanzare italiane: generalità e identificazione degli stadi preimaginali (Diptera, Culicidae). *Fragmenta entomologica*, **29**, 1-141.

23. Rossini G., Carletti F., Bordi L., Cavrini F., Gaibani P., Landini M.P., Pierro A., Capobianchi M.R., Di Caro A. & Sambri V. 2011. Phylogenetic analysis of West Nile virus isolates, Italy, 2008-2009. *Emerg Infect Dis*, **17** (5), 903-906.

24. Rossini G., Carletti F., Rigoli R., Piga S., Bagnarelli P., Gaibani P., Pierro A., Nanni Costa A., Grossi P., Ippolito G., Landini M.P., Di Caro A., Capobianchi M.R. & Sambri V. 2013. Heterogeneity of West Nile virus genotype 1a in Italy, 2011. *J Gen Virol*, **94** (Pt 2), 314-317.

25. Savini G., Monaco F., Calistri P. & Lelli R. 2008. Phylogenetic analysis of West Nile virus isolated in Italy in 2008. *Euro Surveill*, **13** (48), pii=19048. http://www.eurosurveillance.org/ViewArticle.aspx?ArticleId=1904.

26. Savini G., Capelli G., Monaco F., Polci A., Russo F., Di Gennaro A., Marini V., Teodori L., Montarsi F., Pinoni C., Pisciella M., Terregino C., Marangon S., Capua I. & Lelli R. 2012. Evidence of West Nile virus lineage 2 circulation in Northern Italy. *Vet Microbiol*, **158** (3-4), 267-273.

27. Scaramozzino N., Crance J.M., Jouan A., Debriel Da Stoll F. & Garin D. 2001. 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*, **39**, 1922-1927.

28. Severini F., Toma L., Di Luca M. & Romi R. 2009. Le zanzare italiane: generalità e identificazione degli adulti (Diptera, Culicidae). *Fragmenta entomologica*, **41** (2), 213-372.

29. Shirato K., Miyoshi H., Goto A., Ako Y., Ueki T., Kariwa H. & Takashima I. 2004. Viral envelope protein glycosylation is a molecular determinant of the neuroinvasiveness of the New York strain of West Nile virus. *J Gen Virol*, **85**, 3637-3645.

30. Tamura K., Dudley J., Nei M. & Kumar S. 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 40. *Mol Biol Evol*, **24**, 1596-1599.

31. Thompson J.D., Higgins D.G. & Gibson T.J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res*, **22**, 4673-4680.

32. Wicker J.A., Whiteman M.C., Beasley D.W., Davis C.T., Zhang S., Schneider B.S., Higgs S., Kinney R.M. & Barrett A.D. 2006. A single amino acid substitution in the central portion of the West Nile virus NS4B protein confers a highly attenuated phenotype in mice. *Virology*, **349** (2), 245-253.