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Control of Mosquito-Borne Diseases in Northwestern Italy: Preparedness from One Season to the Next

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Abstract

Introduction: Mosquito-borne diseases (MBDs) are spreading worldwide due to globalization and climate change, representing a threat for both humans and animals. Of great concern are the infections caused by viruses belonging to the Flavivirus genus as West Nile virus (WNV) and Usutu virus (USUV) transmitted by Culex sp. or Dengue virus and Zika virus (ZIKV), transmitted by Aedes sp. This work describes the surveillance protocol enforced in Piedmont (Northwestern Italy) to control MBDs spread, focusing on the activities performed on mosquitoes during the 2015 vector season.

Materials and Methods: From July to October, mosquitoes were fortnightly sampled in 50 selected sites according to risk factors with CDC dry ice-baited traps and BG-Sentinel traps baited with BG-Lure and dry ice. Adults were counted, identified to species level, pooled, and screened for flaviviruses using different reverse transcription-PCR protocols and sequencing. Finally, phylogenetic analysis was performed on a dataset including 2014 and 2015 WNV sequences and reference sequences retrieved from GenBank.

Results and Discussion: A total of 17,000 mosquitoes, grouped in 730 pools, were tested. Five pools of *Culex pipiens* were positive for WNV Lineage 2 in Novara, Alessandria, Vercelli, and Torino Provinces. One pool of *C. pipiens* and one pool of *Anopheles maculipennis s.l.* were positive for USUV in Vercelli and Alessandria Provinces. In Vercelli Province one pool of *C. pipiens* resulted positive both for WNV and USUV. Control measures were quickly implemented. Phylogenetic analyses showed that the WNV Lin 2 sequences from Piedmont region cluster with those circulating in Northeastern Italy in the previous years. Given the positive trend in WNV activity compared to 2014 and the emergence caused by other flavivirus as ZIKV, the level of attention for the 2016 vector season may be increased and this surveillance protocol could represent an important tool for public health authorities.

Keywords: flavivirus, mosquito(es), surveillance, vector-borne, West Nile

Introduction

Mosquito-borne diseases (MBDs) are dangerously increasing in prevalence, geographical distribution, and severity, representing a worldwide emerging threat for both humans and animals. Of great concern are the infections caused by viruses belonging to the Flavivirus genus. This genus includes viruses considered endemic in Italy as West Nile virus (WNV) and Usutu virus (USUV) transmitted by *Culex* sp. and viruses as Dengue virus (DENV) and Zika virus (ZIKV), transmitted by *Aedes* sp., not endemic in Italy but with the potential to spread to new areas where the mosquito vector is present.

WNV and USUV are antigenically close flavivirus with similar enzootic birds-mosquitoes transmission cycle whose co-circulation has been reported in several studies (Calzolari et al. 2010, Ben Hassine et al. 2014, Rudolf et al. 2015, Pautasso et al. 2016).

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WNV can be occasionally transmitted, through mosquito bites, to vertebrates other than birds as humans and horses that are considered dead-end host. Human infection through blood transfusion and solid organ transplantation is also demonstrated (Colpitts et al. 2012).

WNV risk for human health is well recognized: the majority (about 80%) of infections in humans are asymptomatic and symptomatic infections are mostly characterized by a mild, self-limiting febrile illness. WNV neuroinvasive disease develops in <1% of WNV-infected persons (Rovida et al. 2015).

USUV is generally correlated with high mortality rates in its bird reservoirs (Weissenbock et al. 2002, Bakonyi et al. 2007, Steinmetz et al. 2011). Mosquitoes infected with USUV can incidentally transmit the virus to other vertebrates, including humans, which can result in neuroinvasive disease (Fros et al. 2015).

Although characterized by a lower pathogenicity than WNV, its pathogenic potential for human is not completely characterized and knowledge about this crucial aspect is constantly evolving (Ashraf et al. 2015, Engel et al. 2016, Grottola et al. 2017).

In Italy, an increasing number of outbreaks of West Nile disease, with occurrences of human cases, have been reported since 2008, mainly in the North East part of the country (Rizzo et al. 2016).

WNV lineage 1 (Lin 1) was the only strain detected until 2011 when, for the first time, the presence of both WNV Lin 1 and WNV lineage 2 (Lin 2) was demonstrated. Since 2013, WNV Lin 2 was the main strain detected and a west bound spread of the virus started (Rovida et al. 2015). In 2014 it was detected for the first time in a Northwestern Italian region (Piedmont) in mosquitoes (Pautasso et al. 2016).

MBDs surveillance in Italy allowed also the detection of USUV since 2007 in mosquitoes, birds, and humans (Lelli et al. 2008, Cavrini et al. 2009, Pecorari et al. 2009, Tamba et al. 2011).

A recent study aiming to evaluate the role for public health of USUV in an endemic area of Italy suggested that infection in humans is not a sporadic event as previously thought. Retrospective analyses demonstrated that USUV was the cause of previously unexplained encephalitis cases indicating that USUV should be included in the differential diagnosis of such cases in endemic areas (Grottola et al. 2017).

DENV and ZIKV are characterized by a human-to-mosquito-to-human cycle of transmission.

DENV is the most prevalent arthropod-borne viral disease in tropical and subtropical countries. The disease manifestations range from an influenza-like disease known as dengue fever to a severe, sometimes fatal disease characterized by hemorrhage and shock, known as dengue hemorrhagic fever/dengue shock syndrome (Guha-Sapir et al. 2005).

The classic clinical picture of ZIKV infection resembles that of dengue fever and is manifested by fever, headache, arthralgia, myalgia, conjunctivitis, and maculopapular rash. Although Zika is generally considered a mild MBD, evidence exists of an association between the Zika virus infection during pregnancy and congenital malformations in newborns (Mlakar et al. 2016).

Up until now, all the Dengue and Zika human cases reported in Italy have been related only with returning travellers from endemic countries and not associated with transmission through local potentially competent vectors (Italian Ministry of Health 2015).

However, if vectors are present, infected returning travellers could initiate a local virus transmission as in the Chikungunya (CHIK) outbreak occurred in the Emilia-Romagna region, Italy, in 2007 (Angelini et al. 2007, Liumbruno et al. 2008). Based on the European human cases of DENV- and CHIK virus-associated diseases from 2002 to 2012, short-term travellers (mainly tourists and business travellers) were considered the highest risk group for carrying such diseases (Tomasello and Schlagenhauf 2013).

Given the complex epidemiology of MBDs, determined by the interaction between pathogens, hosts, vectors and ecosystem, cooperation of multiple disciplines (veterinarians, epidemiologists, entomologists, biologists, and doctors) and an accurate risk analysis, is needed for an effective early warning, surveillance, and control (Institute of Medicine (US) Forum on Microbial Threats 2008).

The knowledge of the role of human and animal hosts in the transmission cycles, biology and ecology of competent vectors, and influence of environmental and climatic factors on the different MBDs is important to address the surveillance program and choose the best in terms of type of traps and monitoring areas.

This work describes the surveillance protocol enforced in Piedmont (Northwestern Italy) to control MBDs spread, focusing on the activities performed on mosquitoes during the 2015 vector season.

Materials and Methods

Survey area and mosquito collection

Piedmont, in Northwestern Italy, has an area of 25,401.56 km^2 (population, 4,424,000 habitants; centroid, N 45° 0' 0"; E 8°0' 0") and is divided into eight provinces (Alessandria; Asti; Biella; Cuneo; Novara; Torino; Verbano Cusio-Ossola; Vercelli). About half of the Piedmont territory is mountainous (43.3%), but there are also extensive areas of hills (30.3%) and plains (26.4%).

Entomological surveillance was achieved from the beginning of July to the end of October with fortnightly samplings.

Mosquitoes were sampled in 50 selected sites.

In 40 sites, modified CO_2 CDC dry ice-baited traps (IMT®–Italian Mosquito Trap) operating overnight were placed. The sampling sites were selected as to represent the whole territory under 600 meters above sea level and according to risk-based factors for WNV and USUV cycle establishment: different land use (based on Corine Land Cover classification), proximity to wetland areas, presence of hosts, and habitat features.

CDC dry ice-baited traps are routinely used in WNV surveillance programs in many regions in the world and are the most common sampling method used for adult mosquito collection mainly of *Culex* mosquito species (*e.g.*, *C. pipiens*, *Culex perexiguus*, *Culex modestus*, and *Culex theileri*) (Roiz et al. 2012).

To increase the effectiveness of the entomological surveillance, including the collection of anthropophilic mosquitoes species, in other 10 sampling stations, considered at higher risk level for the introduction of exotic invasive species and exotic pathogens, BG-Sentinel traps baited with BG lure and modified with the addition of CO_2 as attractive were placed and activated for 24 h.

BG-Sentinel traps baited with BG lure are generally used for collecting *Aedes* (Stegomyia) species such as *Aedes aegypti*, *Aedes albopictus*, and *Aedes polinesiensis*, but it is demonstrated that, with the addition of CO_2, they are as effective as CDC-CO_2 traps for the collection of *Culex* mosquito species (Roiz et al. 2012).

Two international airports (Torino and Cuneo Province), the major regional infectious disease hospital (''Amedeo di Savoia'' Hospital, Torino Province) and seven international connection points (Alessandria, Biella, Cuneo, Novara, and Torino Province) were selected. The major risks of introduction considered are given not only by the movements of trades that could carry out eggs, larvae, or adults of exotic mosquitos species, but also by potentially infected travellers considering that, imported cases of exotic MBDs via international travel may potentially result in establishment of an autochthonous disease cycle in previously nonendemic regions if locally established mosquito populations become infected (Gardner and Sarkar 2013).

Adults mosquitoes were identified to the species level using morphological standard classification keys (Stojanovich and Scott 1997).

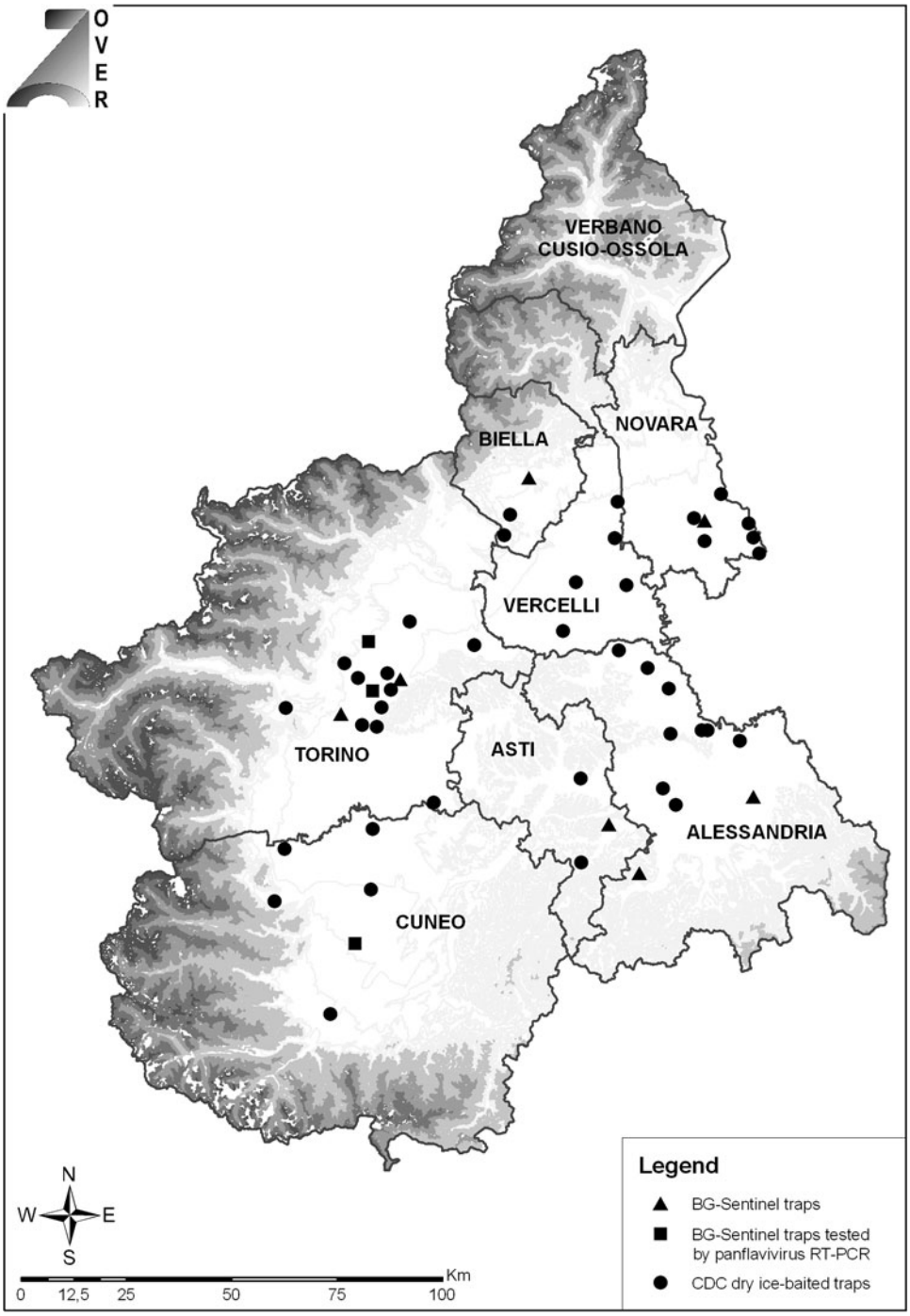

FIG. 1. Division in provinces of Piedmont territory (Northwestern Italy), location of mosquito sampling sites ($n = 50$), and type of traps placed, July–October 2015.

Table 1. Number of Mosquitoes and Number of Pools Counted and Tested by Province in Piedmont (Northwestern Italy), July–October 2015

	Provinces															
	Alessandria		*Asti*		*Biella*		*Cuneo*		*Novara*		*Torino*		*Vercelli*		*Total*	
Species	*Mosq.*	*Pools*	*Mosq.*	*Pools*	*Mosq.*	*Pools*	*Mosq.*	*Pools*	*Mosq.*	*Pools*	*Mosq.*	*Pools*	*Mosq.*	*Pools*	*Mosq.*	*Pools*
Aedes albopictus	137	30	47	5	11	6	37	14	259	27	1251	73	17	10	1759	165
Aedes vexans	34	8	7	1	1	1	1	1	73	4	498	16	37	12	651	43
Anopheles maculipennis s.l.	628	30	0	0	10	4	0	0	1327	31	40	11	971	25	2976	101
Culex modestus	77	3	0	0	0	0	0	0	3	1	0	0	24	5	104	9
Culex pipiens	1420	56	16	3	121	14	398	16	1908	42	933	79	1328	31	6124	241
Culiseta annulata	0	0	0	0	0	0	0	0	0	0	2	1	0	0	2	1
Culiseta longiareolata	0	0	1	1	0	0	0	0	0	0	0	0	0	0	1	1
Ochlerotatus caspius	1800	50	6	1	348	8	5	5	1529	36	627	39	1061	26	5376	165
Ochlerotatus geniculatus	0	0	0	0	0	0	0	0	0	0	1	1	3	2	4	3
ND	0	0	0	0	0	0	0	0	3	1	0	0	0	0	3	1
Total	4096	177	77	11	491	33	441	36	5102	142	3352	220	3441	111	17,000	730

Mosq., mosquitoes; ND, not determined.

Table 2. Virological Findings in Mosquitoes Collected in Piedmont (Northwestern Italy), July–October 2015

Province	*Collection date*	*Traps*	*Coordinates*	*Species*	*Specimens per pool*	*Results*
Novara	July 29, 2015	CO_2 CDC	45.464718 N; 8.626483 E	*C. pipiens*	100	West Nile Lineage 2
Alessandria	August 6, 2015	CO_2 CDC	45.099872 N; 8.553320 E	*C. pipiens*	100	West Nile Lineage 2
Novara	August 26, 2015	CO_2 CDC	45.464718 N; 8.626483 E	*C. pipiens*	80	West Nile Lineage 2
Vercelli	August 26, 2015	CO_2 CDC	45.325820 N; 8.269479 E	*C. pipiens*	98	West Nile Lineage 2
Torino	September 23, 2015	CO_2 CDC	45.188761 N; 7.963259 E	*C. pipiens*	11	West Nile Lineage 2
Vercelli	September 23, 2015	CO_2 CDC	45.22086 N; 8.231179 E	*C. pipiens*	21	West Nile Lineage 2/Usutu virus
Vercelli	August 21, 2015	CO_2 CDC	45.499326 N; 8.394868 E	*A. maculipennis s.l.*	5	Usutu virus
Alessandria	September 9, 2015	CO_2 CDC	44.885624 N; 8.536641 E	*C. pipiens*	44	Usutu virus

Table 3. Number of Mosquitoes and Number of Pools Collected in BG-Sentinel Traps, July–October 2015

	International airports[a]		*International connection point*		*Infectious diseases hospital*[a]		*Total*	
Species	*Mosq.*	*Pools*	*Mosq.*	*Pools*	*Mosq.*	*Pools*	*Mosq.*	*Pools*
A. albopictus	189	14	752	30	377	7	1318	51
A. vexans	0	0	48	3	2	1	50	4
A. maculipennis s.l.	3	1	454	9	0	0	457	10
C. pipiens	261	13	567	26	18	5	846	44
C. modestus	0	0	17	1	0	0	17	1
Culiseta longiaerolata	0	0	1	1	0	0	1	1
O. caspius	78	6	652	17	13	2	743	25
Total	531	34	2491	87	410	15	3432	136

[a]Mosquitoes collected in international airports (Torino and Cuneo Province) and near the infectious diseases hospital ''Amedeo di Savoia'' (Torino Province) were also analyzed according to Scaramozzino et al. (2001).

Molecular analyses

Adult mosquitoes, maintained under cold chain conditions to preserve virus viability in the samples, were grouped into pools of maximum 100 individuals (by species, date, and location of collection) and stored at −80°C until tested for the presence of virus. After homogenation in phosphate-buffered saline (PBS) sterilized solution, viral RNA was extracted using reagents of the RNeasy Mini kit (Qiagen) with an automated QIAcube protocol and subsequently analyzed by a multiplex real-time reverse transcription-PCR for the simultaneous detection and differentiation of WNV Lin 1 and 2 (Del Amo et al. 2013) and by a real-time RT-PCR specific for USUV (Cavrini et al. 2011) using the QuantiTect Probe RT-PCR kit (QIAGEN, Valencia, CA).

On positive pools two traditional RT-PCR for the amplification of WNV (Lanciotti et al. 2000) and USUV (Bakonyi et al. 2004) were carried out using the SuperScript® III Platinum® One-Step qRT-PCR Kit (Thermo Fisher Scientific).

Amplicons of the expected size (408- and 425-bp, respectively) were sequenced using the Big Dye Terminator kit v 3.1 (Lifetecnologies) and run on a ABI3130 Genetic Analyzer (Applied Biosystems). The related sequences were employed to perform a basic local alignment search tool (BLAST) in the GenBank library to confirm the specificity of positive reaction and to estimate the degree of identity of detected strains.

Moreover, mosquitoes collected in International airports (Torino and Cuneo Province) and near the infectious diseases hospital ''Amedeo di Savoia'' (Torino Province) were analyzed with a panflavivirus end point RT-PCR targeting the conserved region of the NS5 gene sequences (Scaramozzino et al. 2001) using the SuperScript® III Platinum® One-Step qRT-PCR Kit (Thermo Fisher Scientific) (Fig. 1).

All positive pools were sent to the National Reference Centre for Animal Exotic Diseases (CESME, Teramo, Italy) for confirmation and determination of Lineage.

Phylogenetic and statistical analyses

WNV Lin 2 phylogeny was obtained by an alignment of ~400 nucleotides. Phylogenetic analysis was performed on a dataset including 2014 and 2015 WNV sequences and WNV Lin 2 reference sequences retrieved from the GenBank. Nucleotide substitution models were evaluated using jModelTest2 (Darriba et al. 2012) and the best model was selected according to Akaike Information Criterion analysis. MEGA version 6.0 (Tamura et al. 2013) was used for calculating p-distance matrices and phylogeny inference according to the maximum likelihood criterion. The nucleotide substitution model was set according to jModelTest2 output and was General Time Reversible plus Gamma distributed (GTR+G) model. The robustness of the hypothesis was tested in 1000 nonparametric bootstrap analyses. WNV sequences reported in this study were submitted to GenBank under accession numbers sequences KT877358–KT877360; KU962940–KU962945.

Control measures following positivity

Following confirmation of WNV circulation in one determined province during the surveillance activities, as provided by national legislation, Nucleic Acid Test (NAT) to screen for WNV in donors of blood was introduced to ensure safety in blood transfusion (CNS 2015). Furthermore, to control WNV mosquito vectors, as provided by local disinfestations protocols, adulticiding and larviciding programs were applied.

Results

A total of 17,000 mosquitoes, divided in 730 pools, were collected during 17 sampling sessions (Table 1). The most abundant species was *C. pipiens* (6124/17,000, 36%) followed by *Ochlerotatus caspius* (5376/17,000, 31.6%), *Anopheles maculipennis s.l.* (2976/17,000, 17%), and *A. albopictus* (1759/17,000, 10.34%). Details of BG-Sentinel sampling session divided for type of site (International airports, international connection points, and infectious diseases hospital) are shown in Table 3. BG-Sentinel traps collected the majority of *A. albopictus* (1318/1759, 75%).

All 730 pools were analyzed according to Cavrini et al. (2011) and Del Amo et al. (2013). WNV Lin 2 was detected in six pools of *C. pipiens*. They were collected with CDC dry ice-baited traps from July in four provinces: Novara (July 29, 2015 and August 26, 2015), Alessandria (August 6, 2015), Vercelli (August 26, 2015), and Torino (September 23, 2015). Interestingly, in Vercelli Province (August 21, 2015) one pool of *C. pipiens* resulted positive both for WNV and USUV. Other two pools, respectively, one of *C. pipiens* in Alessandria

Province (September 9, 2015) and one of *A. maculipennis s.l.* in Vercelli Province (August 21, 2015) tested positive for USUV (Table 2). Furthermore 49/730 pools were also analyzed according to Scaramozzino et al. (2001), but no other flavivirus of medical interest was found (Table 3).

WNV RT-PCR amplicon sequencing and BLAST analysis confirmed maximum similarity with WNV Lin 2 sequences.

Phylogenetic analyses (Fig. 2) showed that the WNV Lin 2 sequences identified in Piedmont Region belong to the Central/Southern European cluster, having Hungary/04 as prototype strain. Some sequences (of both years) form a sub-clade with a human sequence from the 2014 WNV outbreak cases in Pavia.

Following confirmation of WNV Lin 2, 39,623 human blood bags were tested by Human Public Service for hemovigilance. No blood bags resulted infected.

Discussion

Results obtained highlight the importance of choosing the type of traps to be used and their placement when designing a field study considering the different cycles of flaviviruses, the different vectors and the significant differences in capture efficiencies between methods (Roiz et al. 2012). CO_2 CDC dry ice-baited traps allowed the collection of a great number and variety of host-seeking mosquitoes, most of *Culex* species, considered the main vector of WNV and USUV.

Furthermore, the addition of BG-Sentinel traps in sites at higher risk level for the introduction of exotic invasive species and exotic pathogen implemented the number of *Aedes* species samples, competent vectors of DENV and ZIKV.

WNV was detected in Piedmont for the first time in 2014, in Alessandria Province, in 2 (0.3%) of 723 pools of mosquitoes tested. In 2015 it was detected, in four different provinces (Alessandria, Novara, Vercelli, and Torino Province), in 6 (0.8%) of 730 pools of mosquitoes tested. Even if low, the percentage of positivity is doubled compared to previous year, highlighting a positive trends in vector activity. Moreover, the first neuroinvasive human case occurred in the region (Torino Province) at the end of August (Istituto Superiore di Sanità 2015).

Phylogenetic analyses showed that the WNV Lin 2 sequences from Piedmont region cluster with those circulating in Northeastern Italy in the previous years, supporting the west bound spread of the virus started in 2013. The analyses excluded a genetic correlation with the Volgograd 2007 strain, representing a separate introduction of the virus to Europe from Africa, and never reported outside eastern Europe until 2014. Recently, an isolate, phylogenetically related to Volgograd 2007 strain, was reported also in Northeastern Italy (Friuli Venezia Giulia region) by Ravagnan et al. (2015).

The surveillance system adopted allowed the early detection of WNV in vectors in many provinces before the occurrence of human cases permitting the application of national plan procedures for blood screening. Even if in Piedmont not one blood bag resulted positive in 2015, the trend in virus activity expected in 2016 could potentially increase the risk of infection through blood transfusion.

The only human case occurred in Torino Province (end of August) before entomological surveillance signaled WNV circulation (end of September). Nevertheless, if administrative provinces borders had not been considered but surrounding territories, the entomological surveillance would have been able to detect WNV circulation before human cases, as verified in other Italian provinces during 2015 (Rizzo et al. 2016). Moreover, a more widespread distribution of the traps in Torino Province, not homogeneously covered, would have allowed to detect the virus before.

USUV, circulating in Piedmont since 2009, was detected in 2015 in two provinces (Alessandria and Vercelli). The cocirculation WNV/USUV in Vercelli province confirms the overlap of WNV and USUV transmission cycles, not only geographically but also in terms of host and vector ranges reported in different countries (Nikolay 2015).

Furthermore, the detection of USUV is not to undervalue, given the supposed pathogenicity not only in immunocompromised patients (Cavrini et al. 2009, Pecorari et al. 2009), but recently also in healthy ones (Santini et al. 2015).

Even if the virological surveillance excluded the introduction of other flavivirus as DENV or ZIKV so far, the entomological surveillance shows clearly the presence of *Aedes* mosquitoes able to acquire and transmit them.

Conclusions

This work shows as the diagnostic approach for mosquitoes screening used in Piedmont allowed to early detect the introduction of MBDs and to monitor their spread, providing useful information to public authorities, to apply control measures.

Given the enlargement of the WNV circulation area in 2015, the mosquitoes overwintering and the occurrence of the first human case, the level of attention for the 2016 vector season may be increased.

The long experience of neighboring regions shows that entomological surveillance can detect the virus in mosquitoes much earlier than the occurrence of the human cases, supporting the importance of enforcement of a mosquito-based surveillance (Calzolari et al. 2013). However, the selection of most suitable mosquito-trapping methods is crucial and surveillance should be planned in response to a recognised risk and carried out to support subsequent actions.

Finally, considering the emergence caused by other flaviviruses in which humans could be reservoir such as the recent ZIKV infection in South America and the call addressed to European countries from the World Health Organization (WHO), to implement the entomological surveillance for virus

FIG. 2. Maximum Likelihood tree of partial polyprotein gene sequences for WNV Lin 2 detected in mosquitoes collected in Piedmont (Northwestern Italy), during vector season 2014–2015 together with homologous sequences available in GenBank. Footnote Figure 2 WNV Lin 2 phylogeny was obtained by an alignment of ~400 nucleotides. The phylogenetic tree includes the Italian WNV isolates indentified in 2014 (*circle*) and 2015 (*diamond*) together with homologous sequences available in GenBank. Host, country of origin, year of collection and accession number are indicated for each sequence. Bootstrap (1000 replicates) values >50 are shown at the internal nodes. The length of each pair of branches represents the distance between sequence pairs. The scale bar represents the percentage of nucleotide differences. ML, Maximum Likelihood; WNV, West Nile virus. Figure 2 can be viewed in greater detail online at www.liebertpub.com/vbz


Alessandria 77796/3 Culex pipiens Italy 2014
Genova 83361/3/1 Culex pipiens Italy 2014
Alessandria 74636/3 Culex pipiens Italy 2014
Torino 788 Culex pipiens Italy 2015
Vercelli 579 Culex pipiens Italy 2015
Pavia5 human Italy 2014 KP789958
Cremona2 human Italy 2014 KP789954
Kavala 1 human Greece 2013 KJ883342
578/10 horse Hungary 2010 KC496015
Xanthi 2 human Greece 2013 KJ883344
Novara 359 Culex pipiens Italy 2015
Alessandria 438 Culex pipiens Italy 2015
Austria/2008 gh goshawk Austria 2008 KF179640
Verona/35.2 human Italy 2014 KP789956
Xanthi 4 human Greece 2013 KJ883341
Kavala/39.1 human Greece 2012 KF179639
Xanthi 1 human Greece 2013 KJ883343
AN-2 human Italy 2011 JN858070
Cremona4 human Italy 2014 KP789957
Mantova/40.1 human Italy 2013 KF823806
Xanthi 5 human Greece 2013 KJ883349
Padova/34.1 humnan Italy 2013 KF647251
Rovigo/33.2 human Italy 2013 KF647249
Rovigo/33.1 human Italy 2013 KF647250
Rovigo/35.1 human Italy 2013 KF647252
Rovigo/34.1 human Italy 2013 KF647248
Rovigo/32.1 human Italy 2013 KF588365
Rovigo/35.2 human Italy 2013 KF823805
Mantova/36.1 human Italy 2013 KP789960
Sad/12 goshawk Serbia 2012 KC407673
Xanthi 3 human Greece 2013 KJ883345
Vercelli 784 Culex pipiens Italy 2015
Nea Santa Culex pipiens Greece 2010 HQ537483
BD-AUT human Austria 2014 KM659876
blood donor/Vienna/2014 human Austria 2014 KP109691
C105 human Greece 2013 KJ577738
C147 human Greece 2013 KJ577739
Thessaloniki 3 human Greece 2013 KJ883350
Thessaloniki 4 human Greece 2013 KJ883346
Cz 13-104 Culex modestus Czech Republic 2013 KM203860
Kavala 2 human Greece 2013 KJ883348
Verona/35.1 human Italy 2014 KP789955
1270/14 Culex pipiens Italy 2014 KT207792
Cz 13-502 Culex modestus Czech Republic 2013 KM203863
Pavia1 human Italy 2014 KP789953
Pavia4 human Italy 2014 KP789959
Cz 13-479 Culex modestus Czech Republic 2013 KM203862
Serres 1 human Greece 2013 KJ883347
Novara 565 Culex pipiens Italy 2015
Cz 13-329 Culex modestus Czech Republic 2013 KM203861
Novi Sad-2010 Culex pipiens Serbia 2010 KC496016
04 goshawk Hungary 2004 DQ116961
Central/Southern European cluster
B956 human Uganda 1937 AY532665
ArD76104 Senegal DQ318019
WNFCG derivate of Uganda 1937 M12294
LEIV-3266Ukr bird Ukraine 1980 JX041631
SA93/01 human South Africa 2001 EF429198
SPU116/89 human South Africa 1989 EF429197
HS101 08 horse South Africa 2008 JN393308
SA381/00 human South Africa 2000 EF429199
WNV Congo 1958 HM147824
149/77 horse South Africa 1977 KM052152
H442 human South Africa 1958 EF429200
ArB3573/82 Culex tigripes Central African Republic 1972 DQ318020
Reb VLG 07 H human Russia 2007 FJ425721
WNV tick Romania 2013 KJ934710
792/14 Culex pipiens Italy 2014 KT207791
Eastern European cluster
WNV Madagascar 1988 HM147823
WNV South Africa 1958 HM147822
Lineage 2
NSW2011 horse Australia 2011 JN887352
Clade 1A
04.05 horse Morocco 2003 AY701413
GE-2o/V golden eagle Spain 2007 FJ766332
15803 magpie Italy 2008 FJ483549
LEIV-Vlg00-27924 human Russia 2000 AY278442
LEIV-Vlg99-27889 human Russia 1999 AY277252
T-1304 Tajikstan JX070655
LEIV-1628Az bird Azerbaijan 1967 JX041629
LEIV-72Az Ornithodoros capensis Azerbaijan 1970 JX041628
EthAn4766 Ethiopia AY603654
Egypt101 Egypt EU081844
NY99 USA DQ211652
385-99 USA AY842931
MQ5488 Culex pipiens USA 2000 AF404754
Clade 1B
Lineage 1
804994 human India 1980 DQ256376
Lineage 3
Rabensburg 97-103 Culex pipiens Austria AY765264
Lineage 5
LEIV-Krnd88-190 tick Russia 1998 AY277251
Lineage 4
0.05

spread prevention (WHO 2016), this surveillance protocol could represent a good example for public health authorities to set up effective preparedness and control strategies, complementary to syndromic surveillance on human cases.

Author Disclosure Statement

No competing financial interests exist.

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