Transboundary and Emerging Diseases

Transboundary and Emerging Diseases

ORIGINAL ARTICLE

Mosquito Surveillance in Northwestern Italy to Monitor the Occurrence of Tropical Vector-Borne Diseases

Pautasso A.[1], Desiato R.[1], Bertolini S.[1], Vitale N.[1], Radaelli M.C.[1], Mancini M.[1], Rizzo F.[1], Mosca A.[2], Calzolari M.[3], Prearo M.[1], Mandola M.L.[1], Maurella C.[1], Mignone W.[1], Chiavacci L.[1] and Casalone C.[1]

[1] Istituto Zooprofilattico Sperimentale del Piemonte Liguria e Valle d'Aosta, Torino, Italy
[2] Istituto delle Piante da Legno e l'Ambiente – IPLA, Torino, Italy
[3] Istituto Zooprofilattico Sperimentale della Lombardia ed Emilia Romagna, Reggio Emilia, Italy

Keywords:
arboviruses; surveillance; flavivirus; mosquitoes

Correspondence:
A. Pautasso. Istituto Zooprofilattico Sperimentale del Piemonte Liguria e Valle d'Aosta, Via Bologna 148, Torino 10154, Italy. Tel.: 39 0112686296; Fax: 39 0112686360; E-mail: alessandra.pautasso@izsto.it

Received for publication November 15, 2013

doi:10.1111/tbed.12123

Summary

Mosquito-borne arboviruses (MBV) represent an important health problem, causing diseases and deaths both in human and animals mainly in tropical and subtropical countries. In recent years, they have emerged also in temperate regions where they have caused epidemics. Of mounting concern among public health authorities in Europe are zoonotic mosquito-borne viruses belonging to the *Flavivirus* genus. The aim of this study was to carry out active surveillance on mosquitoes in two regions of northwestern Italy (Liguria and Piedmont) to gain a better knowledge of the mosquito populations by identifying potential vectors of arboviruses and to investigate arbovirus infection. A network of 61 CO_2 CDC traps was placed in the study area; sampling was conducted from May to October 2011. A total of 46 677 mosquitoes was collected, identified to species level, and classified according to their vector competence. Mosquitoes collected from 16 traps, selected according to risk-based factors, were tested by biomolecular analysis to detect flavivirus infection. This study highlights the importance of entomological surveillance in northwestern Italy because most of the mosquitoes collected were found to have high vector competence. Moreover, the risk-based virological surveillance allowed to detect the presence of mosquito flavivirus RNA, phylogenetically closely related to the MMV Spanish isolate, in three pools and USUV RNA in one pool in new areas where it has not been reported previously. The availability of continuous data on mosquito populations provides invaluable information for use in cases of an epidemic emergency. Maintenance of this integrated system for the next years will provide stronger data that can inform the design of a risk-based surveillance for the early detection of the occurrence of outbreaks of tropical MBDs.

Introduction

Mosquito-borne arboviruses (MBV) represent an important health problem, causing diseases and deaths both in human and animals mainly in tropical and subtropical countries. In recent years, they have emerged also in temperate regions where they have caused epidemics (Hubalek, 2008).

Their emergence may be attributable to the impact of climate changes, the increase in human travel and commercialization, and other factors such as urbanization and land-use changes (Gould and Higg, 2009). Several emerging mosquito-borne outbreaks reported recently in the Mediterranean basin were caused by viruses mainly belonging to the family *Togaviridae* (Chikungunya virus) and *Flaviviridae* (Dengue, Usutu (USUV) and West Nile (WNV) viruses). Of increasing concern to public health authorities in Europe is zoonotic mosquito-borne viruses belonging to the *Flavivirus* genus. Outbreaks of WNV have occurred in Italy, Greece, Spain, France, Romania, and

further spread is expected (Calistri et al., 2010). Recently, USUV has emerged in Europe, causing mortality in wild birds. The virus was first detected in Africa from indigenous mosquito species and was not considered pathogenic for humans (Williams et al., 1964). Subsequently, the virus has emerged in Europe, particularly Hungary, Germany, Austria, Spain and Italy (Manarolla et al., 2010; Savini et al., 2011; Vázquez et al., 2011;). Human neurological disease caused by USUV was identified for the first time worldwide in 2007 in Italy in the brain of two immunodeficient patients with neuroinvasive infection (Cavrini et al., 2009; Pecorari et al., 2009). In addition, some flaviviruses have recently been isolated only from mosquitoes with no recognized pathogenic role in humans. Reports of the detection of these viruses and other new mosquito-borne viruses are increasingly reported in Europe (Calzolari et al., 2012; Vázquez et al., 2012).

Mosquito-borne diseases (MBD) have a complicated transmission cycle in which vectors, pathogens and animal hosts interact under the strong influence of environmental conditions. This makes monitoring and surveillance tools necessary to identify, assess and control vector-borne diseases. Mosquito-based surveillance, as the primary tool for quantifying the intensity of virus transmission in an area, should be a mainstay in most surveillance programmes for arboviruses. Indeed, active surveillance based on vector-sampling programmes is essential to evaluate the presence and density of vectors and the pathogen prevalence in a vector population (CDC Guidelines, 2003; Braks et al., 2011).

The aim of this study was to carry out active surveillance on mosquitoes in two regions in northwestern Italy (Liguria and Piedmont) to gain a better knowledge on the mosquito populations by identifying potential vectors of arboviruses and to investigate arbovirus infection.

Mosquito population surveillance started in Piedmont in 1997 and was carried out by the Istituto per le Piante da Legno e l'Ambiente (IPLA) to monitor mosquito populations and implements stategies for the reduction in mosquito abundance and nuisance. Moreover, since 2009, Piedmont has been involved in the national WNV surveillance programme (Istituto Zooprofilattico Sperimentale di Teramo): entomological surveillance was performed in a small area defined as a 'risk area' in the provinces of Alessandria and Asti to detect the introduction of WNV infection. Recent investigations on mosquito population ecology in the northeast part of Piedmont showed that the area's environmental features are conducive to mantaining competent vectors for MBDs (Bisanzio et al., 2011). Moreover, the detection of USUV and insect-specific flavivirus in mosquitoes collected in 2009 and 2010 in the area suggested a low infection prevalence and a possible continuous circulation (Cerutti et al., 2012).

By contrast, data on mosquito populations in Liguria were insignificant and fragmentary and no area was included in the national WNV surveillance programme for entomological surveillance.

Materials and Methods

Study area

The study area comprised of Piedmont and Liguria in northwestern Italy. Piedmont has an area of 25 401.56 km^2 (population, 4 457 335 habitants; centroid, N 45°0′ 0″; E 8°0′ 0″) and is divided into eight provinces (Alessandria; Asti; Biella; Cuneo; Novara; Torino; Verbania; Vercelli).

Liguria is an important commercial and touristic area and has an area of 5420.97 Km^2 (population, 1616.788 habitants; centroid, N 44°30′ 0″;E 8°50′ 0″) and is divided into four provinces (Genoa, Imperia, La Spezia, Savona).

Adult mosquito collection

Entomological surveillance was activated from 2 May to 29 October 2011, with weekly or fortnightly collections of adult mosquitoes. Mosquitoes were trapped using 61 modified CO_2 CDC dry ice-baited traps to collect a great number and variety of host-seeking females (Bellini et al., 2002). All traps were georeferenced and worked at night from 4 : 00 p.m. to 10 a.m. A total of 51 traps were placed in Piedmont and 10 in Liguria in areas under 600 m above sea level (Fig. 1).

Trap sites were chosen so as to represent the whole territory of both regions according to proximity to wetland zones, presence of animal hosts and different land use [according to the Corine Land Cover classification (CLC)] and habitat features.

Mosquitoes were identified to species level according to morphological characteristics by means of standard classification keys (Stojanovic and Scott, 1997; Severini et al., 2009). Mosquito species were classified according to their vector competence (Scientific submitted to EFSA, 2009).

Virus survey

The virological surveillance was performed in Piedmont between 15 June and 29 September, focusing on 16 CO_2-baited traps located in sites selected according to risk-based factors including proximity to wetlands, international airports and stopover sites for migratory birds (Fig. 1).

Every week, field-collected mosquitoes were pooled according to species, date and site, with a maximum number of 200 individuals per pool (Sutherland and Nasci,

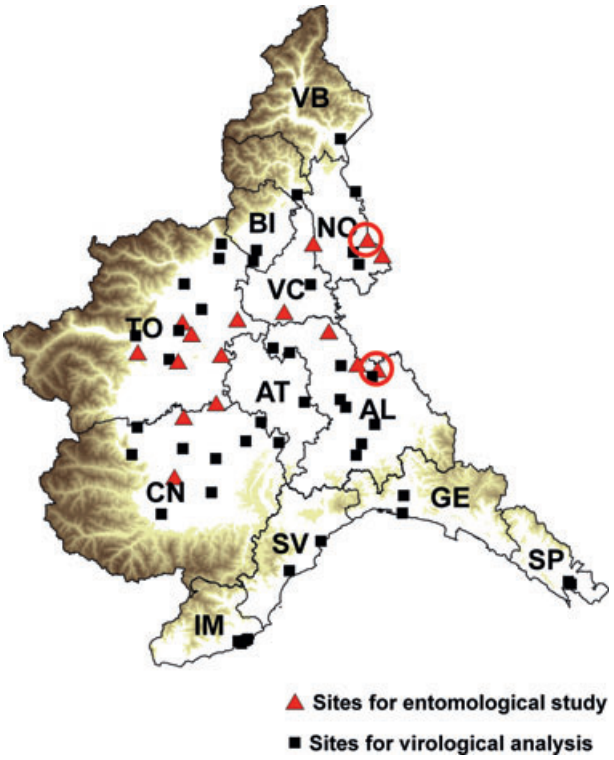

Fig. 1. Map showing the trap sites in the survey.
Squares denote sites selected only for entomological study; triangles denote sites selected for virological analysis. Circles indicate sites where pooled samples tested positive on PCR for: USUV (Novara province) and mosquito-borne flavivirus (Alessandria province). Province abbreviations: AL Alessandria; AT Asti; BI Biella; CN Cuneo; NO Novara; TO Torino; VB Verbania; VC-Vercelli; GE Genoa; IM Imperia; SP La Spezia; SV Savona.

2007) and disrupted in PBS using TissueLyser LT (Qiagen, Hilden, Germany). Total RNA from each pool was extracted using TRI reagent (Sigma-Aldrich, St. Louis, MO, USA) and eluted in a final volume of 50 µl of RNase DNase free water. A retrotranscription step was performed on 7 µl of RNA with a High-capacity c-DNA Reverse Transcription kit (AB, Foster City, CA, USA), and cDNA from each pool was amplified simultaneously with an end-point flavivirus-genus PCR assay for a conserved region of the NS5 protein gene according to Scaramozzino et al. (2001) and with a real-time PCR assay for a 92-bp fragment of 3′ non-coding region of WNV according to Tang et al. (2006). Confirmation of the positive/doubtful samples was performed following two PCR protocols for Usutu virus (Manarolla, 2004) and for WNV (Lanciotti et al., 2000), respectively. PCR fragments were sequenced on an automated ABI-PRISM 3130 Genetic Analyzer (AB) to identify the detected virus, and sequence homology was calculated using the basic local alignment search tool (BLAST, US National Library of Medicine, Bethesda, MD, USA) in GenBank and expressed as nucleotide identity percentage.

Statistical analysis

Descriptive analysis was performed on data to display vector population in the study area. Flavivirus infection prevalence was estimated considering the pooled samples. The related minimum infection rate (MIR) and maximum likelihood estimation (MLE) were calculated using the PooledInfRate statistical software package (Biggerstaff, 2006).

Results

Adult mosquito collection

A total of 46 677 mosquitoes were collected from 61 traps at different sampling rates and in different periods (Table 1).

A total of 45 842 adult mosquitoes (98.2% of total mosquitoes), belonging to 14 different species, were collected in Piedmont, and 835 individuals (1.79%) belonging to four different species in Liguria. Of these, 100 damaged individuals (0.2%) were identified only to the genus level due to a lack of taxonomic characteristics. The most abundant species was *Culex pipiens* (49.13%), collected both in Piedmont and Liguria, followed by *Ochlerotatus caspius* (32.83%) and *Culex modestus* (11.10%), trapped only in Piedmont. According to their vector competence, four species (28 453 mosquitoes; 60.9%) were classified as high competent, three species (18 036 mosquitoes; 38.6%) as moderate and seven species (77 mosquitoes; 0.5%) as low (Table 2).

The geographical distribution of the most common mosquito species trapped is shown in Fig. 2.

Virus survey

A total of 7833 mosquitoes, split in 266 pools, underwent biomolecular analyses. Only one *Culex pipiens* pool (id 92603), sampled within the Ticino River Natural Park (Novara province N 45°31′003 and E 8°42′ 48), tested positive at the Usutu virus PCR assay; the E protein gene sequence obtained (427 bp fragments) showed the highest homology, with a 99% nucleotide identity, with a strain identified in *Culex pipiens* in Emilia Romagna in 2010 (GeneBank accession number: JF834676), as well as with a 2004 Austrian strain from blackbirds (GB:EF078299).

Two mosquito pools of *Ochlerotatus caspius* (id 81618.1 and .2) and one pool of *Culex pipiens* (id 81618.3), collected from the same trap alongside the Po river (N 44°59′297 and E 8°46′ 109), tested positive at *Flavivirus*-genus PCR screening (Table 3).

Table 1. Trap sites and mosquito count by province. CI denotes confidence interval at 95%

Province	Region	No. of traps	No. of trap sessions	No. of mosquitoes (%)	Mean	Mean 95% CI	
AL	Piedmont	11	185	10 770 (23.07)	58.22	43.96	72.48
AT	Piedmont	2	36	3797 (8.13)	105.47	57.32	153.63
BI	Piedmont	2	22	1289 (2.76)	58.59	31.51	85.67
CN	Piedmont	12	72	1545 (3.31)	21.46	16.62	26.30
NO	Piedmont	5	60	11 773 (25.22)	196.22	64.59	327.85
TO	Piedmont	14	233	4113 (8.81)	17.65	11.45	23.85
VB	Piedmont	1	17	117 (0.25)	6.88	3.22	10.54
VC	Piedmont	4	41	12 438 (26.65)	303.37	95.20	511.53
Total Piedmont		**51**	**666**	**45 842 (98.21%)**	**95.98**	**40.48**	**151.48**
GE	Liguria	2	15	138 (0.30)	9.20	8.02	10.38
IM	Liguria	4	36	518 (1.11)	14.39	13.26	16.52
SP	Liguria	2	12	78 (0.17)	6.50	5.13	7.87
SV	Liguria	2	12	101 (0.22)	8.42	6.67	10.17
Total Liguria		**10**	**75**	**835 (1.79)**	**12.10**	**11.13**	**13.07**

In bold characters total values in Piedmont and Liguria Regions.

Table 2. Mosquito species collected in Piedmont and Liguria and their vector competence

		Mosquitoes in Piedmont		Mosquitoes in Liguria		Mosquitoes total		
Mosquito species	Genera	No.	%	No.	%	No.	**%**	Vector competence
Culex pipiens	*Culex*	22 367	47.92	566	1.21	22 933	49.13	High
Ochlerotatus caspius	*Ochlerotatus*	15 325	32.83	0	0.00	15 325	32.83	Moderate
Culex modestus	*Culex*	5181	11.10	0	0.00	5181	11.10	High
Anopheles maculipennis s.l.	*Anopheles*	2057	4.41	0	0.00	2057	4.41	Moderate
Aedes vexans	*Aedes*	654	1.40	0	0.00	654	1.40	Moderate
Culex sp.	*Culex*	94	0.20	0	0.00	94	0.20	Not evaluated
Aedes albopictus	*Aedes*	65	0.14	254	0.54	319	0.68	High
Aedes cinereus	*Aedes*	30	0.06	0	0.00	30	0.06	Low
Culex theileri	*Culex*	20	0.04	0	0.00	20	0.04	High
Not evaluable	Other	11	0.02	0	0.00	11	0.02	Not evaluated
Anopheles plumbeus	*Anopheles*	10	0.02	0	0.00	10	0.02	Low
Culiseta subochrea	*Culiseta*	10	0.02	0	0.00	10	0.02	Low
Aedes geniculatus	*Aedes*	6	0.01	0	0.00	6	0.01	Low
Culiseta longiareolata	*Culiseta*	5	0.01	12	0.03	17	0.04	Low
Anopheles claviger	*Anopheles*	0	0.00	3	0.01	3	0.01	Low
Aedes sp.	*Aedes*	3	0.01	0	0.00	3	0.01	Not evaluated
Culiseta sp.	*Culiseta*	3	0.01	0	0.00	3	0.01	Not evaluated
Culiseta annulata	*Culiseta*	1	0.00	0	0.00	1	0.00	Low
Total		45 842	92.21	835	1.79	46 677	100.00	

The BLAST search for partial NS5 gene sequence (263 bp fragment) from the three samples showed a nucleotide identity of 97% with a sequence detected in *Ochlerotatus caspius* in Emilia Romagna in 2009 (HQ441866) (Calzolari et al., 2010) and a nucleotide identity of 93% with a novel *Flavivirus*, recently isolated from a pool of *Ochlerotatus caspius* in Spain, named Marisma Mosquito virus (MMV) (Vázquez et al., 2012).

Minimum infection rates (MIRs) and MLEs for the inferred prevalence of infection in mosquitoes are reported in Table 4.

Discussion

This study highlights the importance of entomological surveillance in northwestern Italy, where no cases of MBDs in humans or animals have so far been reported, despite recent outbreaks in nearby regions (Angelini et al., 2010; Jourdain et al., 2007). The knowledge of mosquito populations and their vector competence in the study area may provide important information for the early detection of MBDs. Our aim was to gain a better understanding of mosquito-population dynamics by

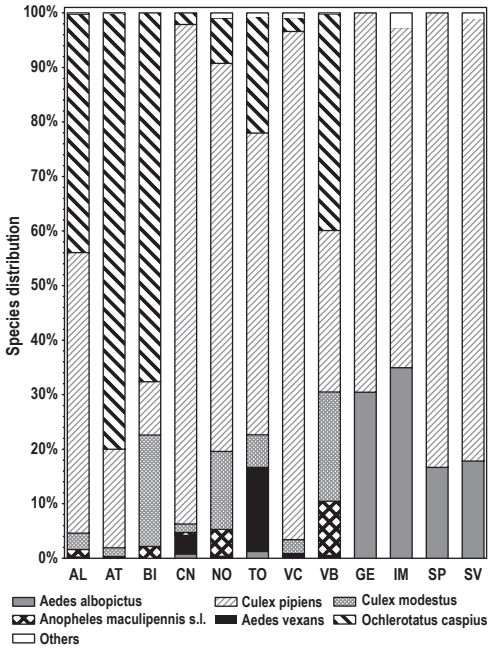

Fig. 2. Relative mosquito species distribution according to province. Province abbreviations: AL Alessandria; AT Asti; BI Biella; CN Cuneo; NO Novara; TO Torino; VB Verbania; VC Vercelli; GE Genoa; IM Imperia; SP La Spezia; SV Savona.

studying their spatial distribution and arbovirus infection in the study area.

The use of an accurate mosquito-trapping method is crucial in vector surveillance owing to the significant differences in capture efficiencies between methods. (Williams and Gingrich, 2007; Almeida et al., 2008). For this reason, in this survey, we used CO_2 CDC dry ice-baited traps, the most efficient and common sampling method for adult mosquito collection, routinely used in surveillance programmes in many regions around the world.

This type of trap allowed the collection of a great number and variety of questing mosquitoes (mainly females): production of CO_2 simulates animal breath which attracts mosquitoes. According to some authors, mosquito trapping is very intensive and expensive but remains an important tool for the early detection of virus activity in a territory (CDC guidelines, and Braks et al., 2011). In Italy, for example, WNV was detected in mosquitoes before cases were reported in birds and humans in Emilia Romagna (Angelini et al., 2010) and USUV-positive mosquitoes were detected in 2009 before cases of human disease occurred (Cavrini et al., 2009; Pecorari et al., 2009). Moreover, in the course of small-scale preliminary screening in 2012, Japanese encephalitis virus (JEV) was detected for the first time in Europe in a mosquito pool collected in Emilia Romagna: no cases of disease were reported, but the event raised important health issues nonetheless (Ravanini et al., 2012).

Mosquito species collected in the study area accounted for 14 (21.8%) of the 64 known Italian species (Romi et al., 1997), consistent with European chart distribution (Snow and Ramsdale, 1999). *Culex pipiens*, *Ochlerotatus caspius*, *Culex modestus* and *Anopheles maculipennis* s.l. were the most common species trapped.

Other species detected in lower concentrations had more scattered distributions. It is worth noting that because the collection method used allowed the collection of mainly nocturnal or crepuscular mosquito species, the possibility to collect diurnal mosquitoes, such as *Aedes albopictus*, was reduced. Nevertheless, *Aedes albopictus* was the second most frequent species trapped in Liguria. This finding may be related to the recently shown behavioural changes *Aedes albopictus* has developed to promote human biting, such as endophagy and night-time biting (Drago, 2003). In addition, the environmental features of the Ligurian trap sites favoured the establishment of the vector: mild winter tem-

Table 3. Virological findings in mosquitoes collected in Piedmont from June to September 2011

Mosquito species	No. pools tested	No. individuals tested	No. pools WNV positive	No. pools USUV positive	No. pools Mo- Flavivirus positive
Culex pipiens	99	4866	0	1	1
Ochlerotatus caspius	57	1503	0	0	2
Culex modestus	29	685	0	0	0
Anopheles maculipennis	22	475	0	0	0
Aedes vexans	36	241	0	0	0
Culex theileri	10	34	0	0	0
Aedes albopictus	10	17	0	0	0
Culiseta subochrea	1	9	0	0	0
Ochlerotatus geniculatus	1	2	0	0	0
Culiseta longiareolata	1	1	0	0	0
Total	**266**	**7833**	**0**	**1**	**3**

Total values in bold characters.

Table 4. Maximum likelihood estimation and minimum infection rate of mosquito-borne flavivirus and USUV in *Culex pipiens* and *Ochlerotatus caspius*

Virus	Mosquito species	Individual tested	Pools tested	Positive pools	MIR (95% CI)	MLE (95% CI)
Mo-flavivirus	*Culex pipiens*	4866	99	1	0.21 (0.00–0.61)	0.21 (0.00–1.01)
Mo-flavivirus	*Ochlerotatus caspius*	1503	57	2	1.33 (0.0–3.17)	1.48 (0.26–5.15)
USUV	*Culex pipiens*	4866	99	1	0.21 (0.00–0.61)	0.21 (0.00–1.01)

peratures that permit egg survival; sufficient amounts of water to fill appropriate aquatic breeding sites, and summer temperature optimal for rapid development from immature stages to adult mosquitoes.

In this study, most mosquitoes (60.9% of total – *Culex pipiens*, *Culex modestus* and *Aedes albopictus*) showed high vector competence and so might be able to acquire and transmit the most common mosquito-borne viruses.

Culex pipiens was the most frequently collected species: its presence was confirmed in all provinces. It is a rural mosquito, mostly bird-biting but with a considerable degree of anthropophily. It is considered a high competent vector for WNV and USUV (Balenghien et al., 2008), highlighting its importance as threat to public health. Biomolecular analysis was performed on 22% of *Culex pipiens* trapped in Piedmont: flavivirus RNA was detected in two pools (1 confirmed as USUV).

One of the most competent species for WNV transmission reported by various authors (Balenghien et al., 2008; Bisanzio et al., 2011) is *Culex modestus*. Feeding mainly on birds, it is found mostly in wetland areas and plays an important role in the transmission cycle as both a maintenance (bird-to-bird transmission) and a bridge (bird-to-mammal transmission) vector. In this study, *Culex modestus* was collected only in Piedmont: 13.1% of *Culex modestus* trapped were analysed and no flavivirus RNA was detected.

The high vector competence of *Aedes albopictus* is chiefly related to its role in the transmission cycle of such human pathogens as Dengue and Chikungunya virus (Gratz, 2004) and it can be considered a moderate vector for flaviviruses. USUV RNA has been found in pools of *Aedes albopictus* in Italy (Calzolari et al., 2010; Savini et al., 2011), and further experimental studies are ongoing to confirm its competence and role in the spread of the virus.

Finally, in this survey, mosquito flavivirus RNA was detected in pools of *Ochlerotatus caspius*, one of the most common species in Piedmont. This species proliferates in brackish waters, but it may also be found in freshwater environments such as flooded meadows and rice fields and in abundant vegetation, which furnishes an important habitat for residential and migratory birds, providing a possible entry door for WNV. It is an aggressive biter and opportunistic feeder. On the basis of laboratory experiments, *Ochlerotatus caspius* is considered moderate competent for WNV. The hypothesis for its potential role in zoonotic flavivirus transmission has been strengthened by the recent detection of positive pools in Europe (Orshan et al., 2008; Monaco et al., 2010) and by its known capacity to feed on both birds and mammals.

The virological risk-based surveillance allowed to detect mosquito flavivirus and USUV circulation in new areas of Piedmont in addition to those where previous studies had shown the viruses to be circulating, even in the absence of clinical signs in animals. Previously, in Piedmont, USUV RNA was identified in *Culex pipens* pools only in Alessandria province (Cerutti et al., 2012), whereas our study allowed the detection of USUV also in Novara province, near the Ticino river. USUV strains isolated in Piedmont showed a high homology with strains isolated in Austria from dead blackbirds, supporting the hypothesis for a possible role of migratory birds in the transmission cycle.

The evidence that the mosquito flavivirus variants identified in Piedmont in 2011 (81618.1-3) are phylogenetically closely related to the MMV Spanish isolate needs further investigation. The genetic characterization carried out by Vasquez et al., based on a 860-bp fragment of the NS5 gene, revealed that MMV clusters within the antigenic group defined as MBV, which, among the *Flaviviridae* family, comprises several important human pathogens including Yellow fever virus, Dengue virus and the Japanese encephalitis complex, as well as WNV and USUV. Mosquito-borne viruses are generally transmitted by mosquitoes and associated with vertebrate hosts where they can cause disease (Huhtamo et al., 2009). It is remarkable that MMV isolation from insect cell lines (C6/36) was successful, whereas attempts on mammalian lines (Vero and BHK21) have failed so far; despite this, sequence homology for the MBV group could indicate a zoonotic potential for this virus. More investigations to confirm this hypothesis are needed. Currently, ongoing studies on Piedmont variants are trying to achieve isolation on both insect and mammalian cell lines to obtain stronger genetic characterization based on wider genome fragments necessary for correct strain identification.

In conclusion, the availability of continuous data on mosquito populations provides important information to be used in case of an epidemic emergency. The maintenance of this surveillance system for the next years, inte-

grated with human and animal surveillance, will provide stronger data which may inform the design of a risk-based surveillance for the early detection of the occurrence of outbreaks of tropical MBDs.

Acknowledgements

This work was funded by Italian Ministry of Health in the framework of Ricerca Sanitaria Corrente and by the 'Programme de Coopération Transfrontalière Italie–France Maritime', project REDLAV.

Conflicts of interest

The authors have no conflicts of interest to declare.

References

Corine Land Cover Classification. Available at http://stweb.sister.it/itaCorine/corine/corine.htm; http://www.centrointerregionale-gis.it/script/corinedownload.asp (accessed June 2013).

Piano di sorveglianza nazionale WNV Istituto Zooprofilattico Sperimentale di Teramo. Available at http://sorveglianza.izs.it/emergenze/west_nile/emergenze.html (accessed June 2013).

Almeida, P. G., R. P. Galão, C. A. Sousa, M. T. Novo, R. Parreira, J. Pinto, J. Piedade, and A. Esteves, 2008: Potential mosquito vectors of arboviruses in Portugal: species, distribution, abundance and West Nile infection. *Trans. R. Soc. Trop. Med. Hyg.* 102, 823–832.

Angelini, P., M. Tamba, A. C. Finarelli, R. Bellini, A. Albieri, P. Bonilauri, F. Cavrini, M. Dottori, P. Gaibani, E. Martini, A. Mattivi, A.M. Pierro, G. Rugna, V. Sambri, G. Squintani and P. Macini, 2010: West Nile virus circulation un Emilia-Romagna, Italy: the integrated surveillance system 2009. *Euro. Surveill.* 16, 11–15.

Balenghien, T., M. Vazeille, M. Grandadam, F. Schaffner, H. Zeller, P. Reiter, P. Sabatier, F. Fouque, and D. J. Bicout, 2008: Vector competence of some French Culex and Aedes mosquitoes for West Nile virus. *Vector Borne Zoonotic Dis.* 8, 589–595.

Bellini, R., R. Veronesi, G. Gentile, and N. Pandolfi, 2002: Optimization of carbon dioxin traps for mosquito monitoring in Italy. 68th Annual meeting of American Mosquito Control Association. Denver, CO.

Biggerstaff, B. J., 2006: PooledInfRate, Version 3.0: a MicrosoftH ExcelH Add-In to compute prevalence estimates from pooled samples. CDC, Fort Collins, CO, USA.

Bisanzio, D., M. Giacobini, L. Bertolotti, A. Mosca, L. Balbo, U. Kitron, and G. M. Vazquez-Prokopec, 2011: Spatio-temporal patterns of distribution of West Nile virus vectors in Eastern Piedmont Region, Italy. *Parasit. Vectors* 4, 230.

Braks, M., J. van der Giessen, M. Kretzschmar, W. van Pelt, E. J. Scholte, C. Reusken, H. Zeller, W. van Bortel, and H. Sprong, 2011: Towards an integrated approach in surveillance of vector-borne diseases in Europe. *Parasit. Vectors* 4, 192.

Calistri, P., A. Giovannini, Z. Hubalek, A. Ionescu, F. Monaco, G. Savini, and R. Lelli, 2010: Epidemiology of West Nile in Europe and in the Mediterranean Basin. *Open Virol. J.* 4, 29–37.

Calzolari, M., P. Bonilauri, R. Bellini, A. Albieri, F. Defilippo, G. Maioli, G. Galletti, A. Gelati, I. Barbieri, M. Tamba, D. Lelli, E. Carra, P. Cordioli, P. Angelini, and M. Dottori, 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.

Calzolari, M., L. Zé-Zé, D. Růžek, A. Vázquez, C. Jeffries, F. Defilippo, H. C. Osório, P. Kilian, S. Ruíz, A. R. Fooks, G. Maioli, F. Amaro, M. Tlusty, J. Figuerola, J. M. Medlock, P. Bonilauri, M. J. Alves, O. Šebesta, A. Tenorio, A. G. Vaux, R. Bellini, I. Gelbič, M. P. Sánchez-Seco, N. Johnson, and M. Dottori, 2012: Detection of mosquito-only flaviviruses in Europe. *J. Gen. Virol.* 93, 1215–1225.

Cavrini, F., P. Gaibani, G. Longo, A. M. Pierro, G. Rossini, P. Bonilauri, G. E. Gerundi, F. Di Benedetto, A. Pasetto, M. Girardis, M. Dottori, M. P. Landini, and V. Sambri, 2009: Usutu virus infection in a patient who underwent orthotropic liver transplantation, Italy, August -September 2009. *Euro. Surveill.* 14, pii: 19448.

CDC Guidelines, 2003: CDC Guidelines for Surveillance, Prevention, & Control. 3th revision. FortCollins, CO. Available at http://www.cdc.gov/ncidod/dvbid/westnile/resources/wnv-guidelines-aug-2003.pdf (accessed June 2013).

Cerutti, F., M. Giacobini, A. Mosca, I. Grasso, L. Rambozzi, L. Rossi, and L. Bertolotti, 2012: Evidence of mosquito-transmitted flavivirus circulation in Piedmont, north-western Italy. *Parasit. Vectors* 5, 99.

Drago, A., (2003): Education, information and public awareness in vector control. 14th European conference of the Society of Vector Ecology. Bellinzona, Switzerland.

Gould, E. A., and S. Higg, 2009: Impact of climate change and other factors on emerging arbovirus diseases. *Trans. R. Soc. Trop. Med. Hyg.* 103, 109–121.

Gratz, N. G., 2004: Critical review of the vector status of Aedes albopictus. *Med. Vet. Entomol.* 18, 215–227.

Hubalek, Z., 2008: Mosquito-borne viruses in Europe. *Parasitol. Res.* 103, S29–S43.

Huhtamo, E., N. Putkuri, S. Kurkela, T. Manni, A. Vaheri, O. Vapalahti, and Y. Uzcategui, 2009: Characterization of a novel Flavivirus from mosquitoes in Northern Europe that is related to mosquito-borne Flaviviruses of the tropics. *J. Virol.* 83, 9532–9540.

Jourdain, E., I. Schuffenecker, J. Korimbocus, S. Reynard, S. Murri, Y. Kayser, M. Gauthier-Clerc, P. Sabatier, and H. G. Zeller, 2007: West Nile virus in wild resident birds, Southern France, 2004. *Vector Borne Zoonotic Dis.* 7, 448–452.

Lanciotti, R. S., A. J. Kerst, R. S. Nasci, M. S. Godsey, C. J. Mitchell, H. M. Savage, N. Komar, N. A. Panella, B. C. Allen, K. E. Volpe, B. S. Davis, and J. T. Roehrig, 2000: 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.* 38, 4066–4071.

Manarolla, G., T. Bakonyi, D. Gallazzi, L. Crosta, H. Weiss-enböck, G. M. Dorrestein, and N. Nowotny, 2010: Usutu virus in wild birds in northern Italy. *Vet. Microbiol.* 141, 159–163.

Monaco, F., R. Lelli, L. Teodori, C. Pinoni, A. Di Gennaro, A. Polci, P. Calistri, and G. Savini, 2010: Re-emergence of West Nile virus in Italy. *Zoonoses Public Health* 57, 476–86. doi: 10.1111/j.1863-2378.2009.01245.x.

Orshan, L., H. Bin, H. Schnur, A. Kaufman, A. Valinsky, L. Shulman, L. Weiss, E. Mendelson, and H. Pener, 2008: Mosquito vectors of West Nile Fever in Israel. *J. Med. Entomol.* 45, 939–47.

Pecorari, M., G. Longo, W. Gennari, A. Grottola, A. Sabbatini, S. Tagliazucchi, G. Savini, F. Monaco, M. Simone, R. Lelli, and F. Rumpianesi, 2009: First human case of Usutu virus neuroinvasive infection, Italy, August–September 2009. *Euro. Surveill.* 14, pii: 19446.

Ravanini, P., E. Huhtamo, V. Ilaria, M. G. Crobu, A. M. Nicosia, L. Servino, F. Rivasi, S. Allegrini, U. Miglio, A. Magri, R. Minisini, O. Vapalahti, and R. Boldorini, 2012: Japanese encephalitis virus RNA detected in Culex pipiens mosquitoes in Italy. *Euro. Surveill.* 17, pii: 20221.

Savini, G., F. Monaco, C. Terregino, A. Di Gennaro, L. Bano, C. Pinoni, R. De Nardi, P. Bonilauri, M. Pecorari, L. Di Gialleonardo, L. Bonfanti, A. Polci, P. Calistri, and R. Lelli, 2011: Usutu virus in Italy: an emergence or a silent infection? *Vet. Microbiol.* 151, 264–274.

Scaramozzino, N., J. M. Crance, A. Jouan, D. A. DeBriel, F. Stoll, and D. Garin, 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.

Scientific Report submitted to EFSA, 2009: Scientific review on mosquitoes and mosquito-borne diseases EFSA (Prepared by Pagesa, N., K. Huberb, M. Ciprianic, V. Chevallier, F. J. Conraths, M. Goffredo, and T. Balenghien), 1–96.

Severini, F., L. Toma, M. Di Luca, and R. Romi, 2009: Le zanzare italiane: classificazione e identificazione degli adulti.Diptera (Culicidae). *Fragm. Entomol.*, 41, 213–372.

Snow, K., and C. Ramsdale, 1999: Distribution chart for European mosquitoes. *Eur. Mosq. Bull.* 3, 14–31.

Stojanovic, J., and H. G. Scott, 1997: Mosquitoes of the Italian Biogeographical area which includes the Republic of Malta, the French Island of Corsica and all of Italy except the far Northern Provinces.

Sutherland, G. L., and R. S. Nasci, 2007: Detection of West Nile virus in large pools of mosquitoes. *J. Am. Mosq. Control. Assoc.* 23, 389–395.

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

Vázquez, A., M. Jimenez-Clavero, L. Franco, and A. Donoso-Mantke Tenorio, 2011: Usutu virus: potential risk of human disease in Europe. *Euro. Surveill.* 16, pii: 19935.

Vázquez, A., M. P. Sánchez-Seco, G. Palacios, F. Molero, N. Reyes, S. Ruiz, C. Aranda, E. Marqués, R. Escosa, J. Moreno, J. Figuerola, and A. Tenorio, 2012: Novel flaviviruses detected in different species of mosquitoes in Spain. *Vector Borne Zoonotic Dis.* 12, 223–229.

Williams, G. M., and J. B. Gingrich, 2007: Comparison of light traps, gravid traps and resting boxes for West Nile virus surveillance. *J. Vector Ecol.* 32, 285–291.

Williams, M. C., D. I. Simpson, A. J. Haddow, and E. M. Knight, 1964: The isolation of west Nile virus from man and of Usutu virus from bird biting mosquito Ansonia aurites (Theobald) in the Entebbe area of Uganda. *Ann. Trop. Med. Parassitol.* 58, 367–374.