IX CONGRESSO dell'ASSOCIAZIONE TERIOLOGICA ITALIANA Onlus
Civitella Alfedena (AQ), 7-10 Maggio 2014

FLAVIVIRUSES IDENTIFIED IN MOSQUITOES COLLECTED IN VENETO AND TRENTINO ALTO-ADIGE REGIONS (NORTH-EAST ITALY).

GRISENTI M.1,2§, VAZQUEZ A.3, HERRERO L.3, CUEVAS L.3, PEREZ E.3, ARNOLDI D.1, SCREMIN M.1,4, SANCHEZ SECO MP3, CAPELLI G.4, TENORIO A.3, RIZZOLI A.1
1Department of Biodiversity and Molecular Ecology, Research and Innovation Centre, Fondazione Edmund Mach, Via E. Mach 1, 38010 San Michele all'Adige, Italy. 2Department of Veterinary Sciences - University of Turin, Grugliasco (Torino), Italy. 3Laboratory of Arboviruses and Viral Imported Diseases - National Center of Microbiology, Institute of Health "Carlos III", Majadahonda (Madrid), Spain. 4Laboratory of Parasitology - Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro (Padova), Italy. §Corresponding author: Michela Grisenti DMV.

Introduction

The genus Flavivirus (family Flaviviridae) comprises more than 70 viruses, divided according to their ecological and epidemiological characteristics, and disease associations in three groups: 1) those infecting a range of vertebrate hosts through mosquito or tick bites, called "arthropod-borne viruses", 2) those spread by an unknown vector, presumed to be limited to infect vertebrates only, and 3) apparently limited to insects alone, called "insect-specific flaviviruses" ("ISFs") (Ishikawa and Konishi, 2011; Huhtamo et al., 2012). The first group includes some important or emerging human pathogens, as West Nile virus (WNV) and Usutu virus (USUV), which are actively circulating in North Italy. ISFs replicate only in mosquito-derived cells and after the first of them to be discovered in 1975, many others have been isolated and identified, in different geographic regions, from field collected mosquitoes belonging to different species (Cook et al., 2012; Haddow et al., 2013). Despite their non-pathogenicity to humans and animals, ISFs have recently gained attention with respect to their ecological and evolutionary relationships with other flaviviruses, in particular for their possible interaction within the same vector leading to different results as "superinfection exclusion" or ehnanced transmission or replication (Bolling et al., 2012; Hobson-Peters et al., 2013). In Trentino the only evidence of WNV and USUV has been seroconversion in sentinel chickens in 2005, but ISFs have been detected since 2007 (Rizzoli et al., 2007; Roiz et al., 2009, 2012; Grisenti et al., 2013). A completely different ecoepidemiological situation is occurring in Veneto since WNV and USUV have been detected in several occasions during the last decades, but there is only one report regarding the detection of ISFs in this region (Roiz et al., 2012; Barzon et al., 2013; Gaibani et al., 2013; Gobbi et al., 2014). The scope of the present study was to gain a better understanding regarding the occurrence and distribution of flaviviruses in mosquitoes in these two regions.

Materials and methods

Mosquitoes were collected from May to October 2012, using 20 and 10 BG-Sentinel™ traps (BioQuip products, CA, USA) in Veneto and Trentino, respectively, located half in a rural and half in an urban environment, with BG-Lure® attractant and dry ice, checked weekly. Mosquitoes captured were killed at -80°C, identified to species level and pooled according to date, location, species and gender and stored in EMEM (SafcBiosciences, Hampshire, United Kingdom) supplemented with FBS (Thermo Scientific Hyclone Inc., South Logan, Utah, USA) and a mixture of antibiotics (Penicilin, Streptomicine; Euroclone, Milan, Italy) at -80°C until molecular analysis. After RNA extraction with QIAamp® Viral RNA kit (Qiagen, Hilden, Germany), we used a generic RT-nested-PCR targeted on a region of the NS5 gene for the screening of flaviviruses (Sanchez-Seco et al., 2005). The phylogenetic analysis were realized on a fragment of 1000bp (Vazquez et al., 2012). For samples positive at the generic NS5 RT-nested-PCR, virus isolation was attempted in C6/36 cell lines (from *Aedes albopictus* mosquito). Fresh supernatants and cells from cell cultures with evident cytopathic effect, were used for electron microscopy studies.

Results

In Veneto we collected a total of 52096 female and 1190 male mosquitoes, belonging to *Oc. geniculatus*, *Oc. caspius*, *Cx. pipiens*, *Cx. territans*, *Cx. modestus*, *Cs. annulata*, *An. plumbeus*, *An. maculipennis*, *Ae. cinereus/geminus*, *Ae. albopictus*, *Ae. vexans* and *Ae. koreicus* species. We detected USUV in *Cx. pipiens*, and AeFV in *Cx. pipiens* and in *Ae. albopictus*. In another pool of *Cx. pipiens* we also found sequences of an ISF never reported in literature before, that could be considered as a *new* ISF (table 1). We successfully isolated in cell culture AeFV from one pool of *Ae. albopictus*, with evident cytopathic effect (CPE) (cell aggregation). Electron microscopy study performed on C6/36 cells infected by AeFV confirmed the presence of flaviviruses. The phylogenetic tree of the sequences detected in this work and their relationships with the other flaviviruses previously detected is showed in figure 1. In Trentino we collected a total of 1622 female and 464 male mosquitoes, belonging to *Oc. geniculatus*, *Cx. pipiens*, *Cx. hortensis*, *An. plumbeus*, *An. maculipennis* and *Ae. albopictus* species. We detected only AeFV in pools of *Ae. albopictus* and *Cx. pipiens* that was also successfully isolated in cell culture (table 2). In 1 pool we obtained evident CPE and by electron microscopy we detected viral particles with the tipical morphological characteristics of *Rhabdovirus*. The sequences detected in this work were grouped in three clusters belonging two of them to ISFs and one to mosquito-borne flavivirus groups (figure 1). The AeFV prevalence found in *Ae. albopictus* in Veneto (52,3%) was lower than in Trentino (92,3%). AeFV prevalence in *Ae. albopictus* with respect to the type of environment (urban or rural) did not differ nor in Trentino neither in Veneto region.

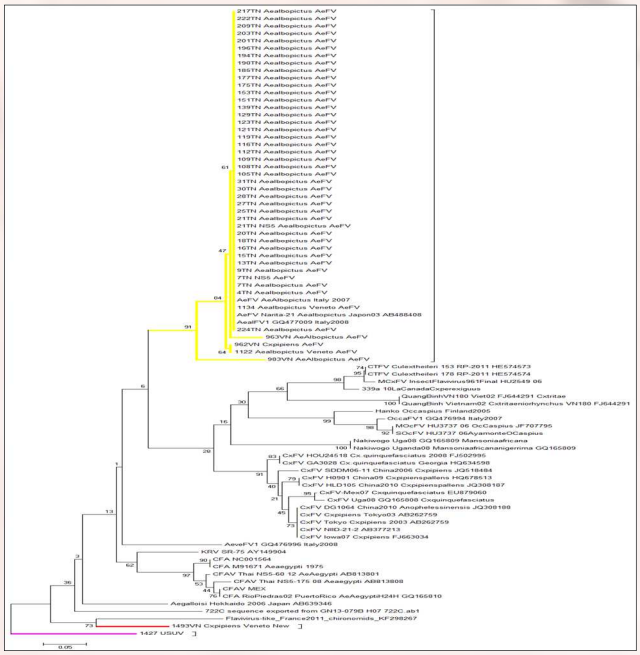
Table 1: Mosquitoes analysed in Veneto.

Mosquito species	N. pools analysed	N. pools Flavivirus positive (AeFV/USUV/New ISF)
	Female	
Oc. geniculatus	2	0
Oc. caspius	43	0
Cx. pipiens	237	3 (1 AeFV, 1 USUV, 1 New ISF)
Cx. territans	1	0
Cx. modestus	8	0
Cs. annulata	0	n.a.
An. plumbeus	0	n.a.
An. maculipennis	6	0
Ae. cinereus/geminus	0	n.a.
Ae. albopictus	65	34 (AeFV)
Ae. vexans	9	0
Ae. koreicus	3	0
Total	**374**	**37**

Table 2: Mosquitoes analysed in Trentino.

Mosquito species	N. pools analysed		N. pools Flavivirus positive	
	Female	Male	Female	Male
Oc. geniculatus	1	n.a.	0	n.a
Cx. pipiens	45	11	2 (AeFV)	0
Cx. hortensis	8	3	0	0
An. plumbeus	0	n.a.	n.a.	n.a.
An. maculipennis	5	n.a.	0	n.a.
Ae. albopictus	65	0	60 (AeFV)	n.a.
Total	**124**	**14**	**62**	**0**

Figure 1: Phylogenetic tree of the sequences decected in Veneto and Trentino.



Discussion

In this study we report for the first time the detection of AeFV sequence in *Cx. pipiens* mosquitoes from Veneto and Trentino. The AeFV sequences found in Veneto showed a high percentage of identity to those detected in this research in Trentino and to those previously detected in Italy. Our results confirm the different eco-epidemiological situation present in North-east Italy underlined by previous studies, but more work is necessary to understand the ecology of these viruses. No co-infections with different flavivirus was detected in this study. The hypothesis under evaluation is that the high prevalence of ISFs could damper the transmission of other important pathogenic zoonotic viruses, but more experimental laboratory work is now needed.

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Acknowledgements

This work was funded by the 7th Framework Programme: European West Nile collaborative research project (Grant agreement n. 261391; www.eurowestnile.org).