Entomological surveillance of Zika virus in Sardinia, Italy, 2016

Cipriano Foxi[1,3], Giantonella Puggioni[1,3], Giorgio Meloni[1,3*], Renata Rossi[1], Angela Maria Rocchigiani[1], Luigi Vento[1], Antonio Collovà[2] and Giuseppe Satta[1]

[1] *Istituto Zooprofilattico Sperimentale della Sardegna, via Duca degli Abruzzi 8, 07100 Sassari, Italy.*
[2] *Ufficio Sanità Marittima e di Frontiera (USMAF), Molo teleferica, Porto Torres, Italy.*
[3] *These authors contributed equally to this work.*

[*] *Corresponding author at: Istituto Zooprofilattico Sperimentale della Sardegna, via Duca degli Abruzzi 8, 07100 Sassari, Italy. Tel.: +39 0792892303, e-mail: giorgiomeloni82@gmail.com.*

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Summary

Zika Virus (ZIKV) is a RNA virus belonging to the genus *Flavivirus*, family *Flaviviridae*. This virus is transmitted through bite of *Aedes mosquitoes*, in particular *Ae. aegypti*. On February 1st 2016, the World Health Organization (WHO) has declared ZIKV a Public Health Emergency of International Concern. Successively, considering the establishment of *Ae. albopictus*, WHO has classified Italy as having a moderate likelihood of local transmission of ZIKV, preceded in Europe only by France. For this reason an entomological surveillance plan was been activated in Sardinia in 2016. BG Sentinel Mosquito Traps have been positioned in 29 sites, comprising urban areas and points of entry, as ports and airports. Mosquitoes were collected fortnightly from April to December. A total of 3,089 mosquitoes were collected belonging to 10 species. The most numerous species have been *Cx. pipiens s.l.* and *Ae. albopictus*. All mosquitoes sampled have been assayed by real time reverse transcriptase PCR for detection of ZIKV RNA. A total of 584 pool have been analyzed and have been reported no evidence of ZIKV. A permanent entomological surveillance should be implemented principally in the urban areas and points of entry, as ports and airports, because *Ae. albopictus*, susceptible to ZIKV, is established in Sardinia and also know the recent introduction of invasive mosquitoes species *Ae. koericus* and *Ae. japonicus* in Italy.

Sorveglianza entomologica per il virus Zika in Sardegna, Italia, 2016

Parole chiave
Aedes albopictus,
Culex pipiens,
Sorveglianza entomologica,
Italia,
Sardegna,
Zika virus

Riassunto

Virus zika (ZIKV) è un virus a RNA appartenente al genere *Flavivirus*, famiglia *Flaviviridae*, e si trasmette con la puntura delle zanzare *Aedes*, in particolare *Aedes aegypti*. Il 1 febbraio 2016 la World Health Organization (WHO) ha dichiarato il virus Zika un'emergenza per la salute pubblica di interesse internazionale e, per la presenza ormai diffusa di *Aedes albopictus*, ha classificato l'Italia a moderato rischio di trasmissione, preceduta in Europa solo dalla Francia. Per questo motivo nel 2016 si è avviata in Sardegna una sorveglianza entomologica. Sono state posizionate trappole BG Sentinel in 29 siti, inclusi aree urbane e punti di accesso come porti e aeroporti. Le zanzare sono state catturate con cadenza bisettimanale da aprile a dicembre 2016. È stato catturato un totale di 3.089 zanzare appartenenti a 10 specie. Le più abbondanti sono state *Culex pipiens s.l.* e *Ae. albopictus*. L'analisi con real time RT-PCR eseguita su tutti i campioni, raggruppati in 584 pool, non ha evidenziato la presenza del virus. Una sorveglianza entomologica dovrebbe essere attivata e resa permanente in prossimità di aree urbane e punti di accesso anche in considerazione della recente introduzione di specie esotiche come *Aedes koericus* e *Aedes japonicus* in Italia e dell'ampia diffusione di *Ae. albopictus*.

Introduction

Zika virus (ZIKV) is a RNA virus belonging to the genus *Flavivirus*, family *Flaviviridae*. ZIKV is related to the other arboviruses of public health importance, including Dengue (DENV) and Chikungunya (CHIKV) viruses. ZIKV is transmitted through infected mosquito bites and it causes an infection characterized by non-specific symptoms including fever, skin rash, conjunctivitis, joint inflammation and pain (Calvo *et al.* 2016, Pabbaraju *et al.* 2016).

ZIKV was first isolated in Uganda in 1947. Only human sporadic mild cases were successively reported in East and West Africa (MacCrae and Kirya 1982). Recent studies conducted in South America have shown a link between ZIKV infection and congenital malformations and neurological disorders (Fauci and Morens 2016, Oliveira Melo *et al.* 2016). For the first time, an arbovirus has been associated with severe human congenital complications. At this regard on February 1st 2016 the World Health Organization (WHO) has declared ZIKV a Public Health Emergency of International Concern (WHO 2016a). During the last year, many imported cases have been notified in Europe. Just in Italy, a total of 94 imported cases have been confirmed (ECDC 2017). In Sardinia, one case has been notified in July. No autochthonous case of Zika transmission has been reported in Europe, but a secondary autochthonous case has been notified in Italy due to a possible sexual transmission (Venturi *et al.* 2016).

Mosquitoes belonging to the *Aedes* genus are considered as main vectors of the Zika virus (Diallo *et al.* 2014, Musso and Gubler 2016). *Aedes aegypti* is the main mosquito species implicated in outbreaks in the Americas (Chouin-Carneiro *et al.* 2016). Nowadays, the presence of *Ae. aegypti* is confirmed in Europe in Island of Madeira (Portugal), in parts of Georgia, in South Western Russia and a new recent introduction has been reported in Holland (ECDC 2016). Numerous studies indicate that *Aedes albopictus* can be equally competent to transmit ZIKV (Chouin-Carneiro *et al.* 2016). This species is established in many countries of Mediterranean basin (ECDC 2016). In Italy, *Ae. albopictus* was reported for the first time in 1990 and in Sardinia it was detected in 1994, where is currently widely established (Sabatini *et al.* 1990, Romi 1995). More recently, new introductions of exotic mosquito species were observed in Italy as *Ae. koreicus* and *Ae. japonicus* (Capelli *et al.* 2011, Seidel *et al.* 2016).

Since 2014, in Italy ZIKV has been included within a control strategy for arboviruses, combining direct and indirect control measures and providing guidelines for an appropriate management of ZIKV infection (Ministero della Salute 2014). Considering established populations of *Ae. albopictus*, WHO has classified Italy with a moderate likelihood of local transmission of ZIKV, preceded in Europe only by France (WHO 2016b). According to these reports it is essential to assess a possible new introduction of different *Aedes* species and to examine every autochthonous mosquito species able to transmit viruses. The aim of this work is to evaluate in Sardinia: i) new introductions of invasive mosquito species, with particular attention to *Ae. aegypti*; ii) abundance of *Ae. albopictus* and to define his seasonality; iii) presence of ZIKV in all mosquito species captured, using a specific real time RT-PCR.

Materials and methods

Study area and mosquito sampling

The study was conducted in Sardinia island, located in the centre of Mediterranean Basin. This region is characterized by a mild winter and a hot and dry summer. During the winter, sometimes, are recorded temperatures below zero degrees, while in the summer days, temperatures often exceeding 30 °C. The period with the highest rainfall, on an average 400-600 mm along the coast and 500-800 mm inland, is from November to April (Chessa and Delitala 1997). The monitoring of the insects was carried out in urban areas of the major cities (12 sites) and in the most important border areas, ports and airports (17 sites). In details a total of 29 sites were included in this survey: 8 traps were positioned in Cagliari, 4 in Oristano, 2 in Tortolì, 2 in Nuoro, 6 in Olbia, 4 in Alghero, 1 in Sassari, 1 in Porto Torres and 1 in Santa Teresa di Gallura (Figure 1). Mosquitoes were collected fortnightly from April to December 2016. In the maritime and commercial ports the traps were placed close to moored ships principally represented by cruise and container ships. In the airports traps were positioned in the proximity of the airport apron, at arrivals of domestic and international flights. In urban areas traps were positioned in public services facilities, such as schools, hospitals and/or cemeteries, places with availability of larval breeding sites. A supplementary trap worked for 3 days, inside a pilothouse of a coal ship, moored in Porto Torres port and coming from Russia. Mosquitoes were collected using a BG Sentinel Mosquito Traps, originally developed to monitor *Aedes* genus mosquitoes. Traps, baited with a chemical lure mimicking human odor, were placed on the ground and in locations sheltered from rainfall, wind, direct sunlight and close to mosquito breeding sites. The cartridges of BG-lure were replaced every two months. Each trapping period was of 24 hours and the collected mosquitoes were taken to the laboratory as soon as possible for species identification.

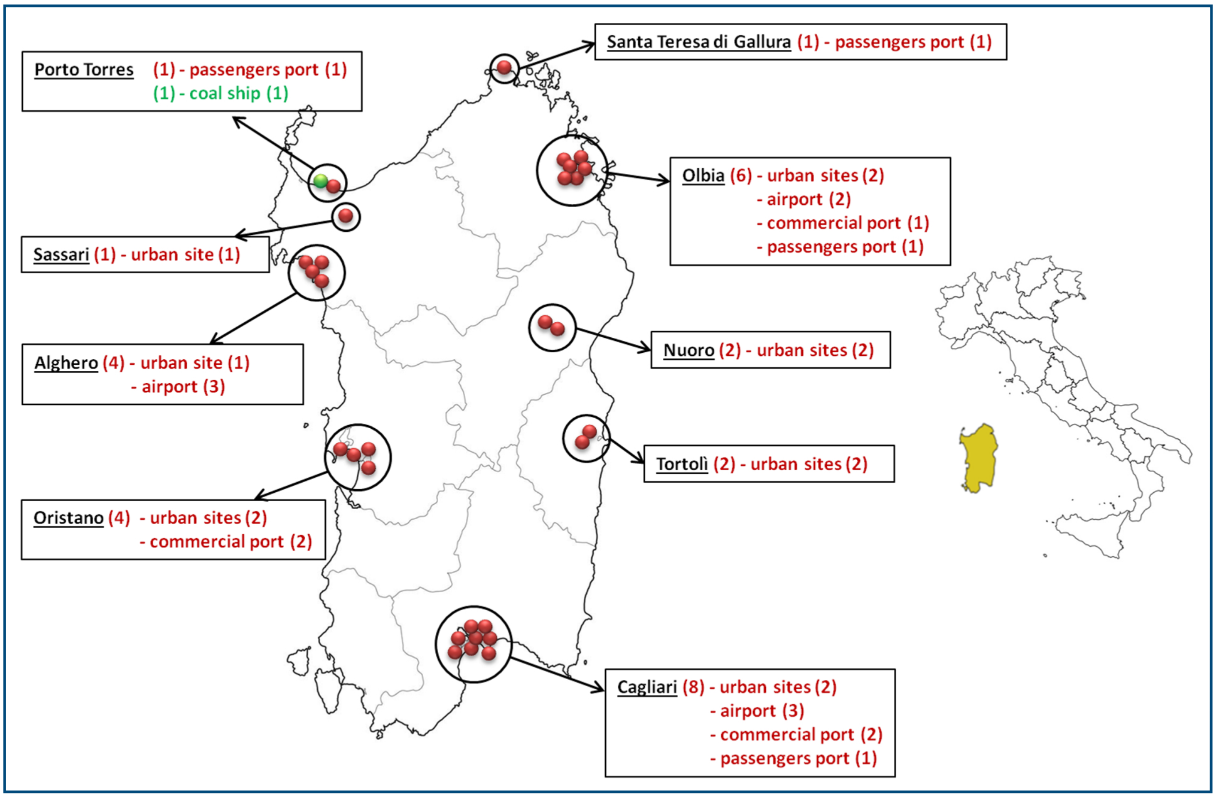

Figure 1. *Map of Sardinia (Italy) showing the collection sites.*

Two additional BG Mosquito Traps were used in July when a human imported case was notified, in according to the Ministerial guidelines. Traps were positioned in the house garden of the infected patient and 3 catches were carried out, one day a week.

Mosquitoes identification

Sampled mosquitoes were morphologically identified at the species level under a stereo microscope using taxonomic keys (Severini *et al.* 2009). Mosquitoes were then pooled according to species, sex, presence (engorged) or absence of blood (not engorged) in the abdomen of females, collection site and date of sampling, with a maximum of 25 individuals per pool. To avoid cross-contamination, due to lose mosquito parts, the pools were obtained by handling specimens individually with tweezers. The pooled mosquitoes were stored in 2 ml polypropylene cryotubes (Eppendorf®) and frozen at - 80 °C until biomolecular analysis for virus detection.

Detection of Zika virus

Pools of mosquitoes were re-suspended in 500 µl of phosphate buffered saline pH 7.3 ± 0.1 and homogenized using the Tissuelyzer II (QIAGEN, Valancia, CA) with two 3 mm tungsten beads. Samples were shaking at frequency of 17/sec for 90 sec and then centrifuged in a refrigerated centrifuge at 10,000 g for 10 minutes at 4 °C. Viral RNA was extracted from 100 µl supernatant using the commercial kit Biosprint 96 One-for-all Vet Kit (QIAGEN®) according to the manufacturer's instructions. All samples were assayed by a specific real time RT-PCR for detection of ZIKA virus RNA (Lanciotti *et al.* 2008). The real-time assay was performed by using QuantiTect Probe RT-PCR Kit (QIAGEN®) following the manufacturer's protocol, with amplification in the 7900 HT Fast Real-Time PCR System (Applied Biosystems).

The baseline and threshold were set using the auto-baseline and threshold feature in SDS Software version 2.4 (Applied Biosystems®). Samples were considered positive if target amplification was recorded within 45 cycles.

Results

During the survey period, 438 catches were performed and a total of 3,089 mosquitoes belonging to 10 species collected (Table I). The most abundant species were *Cx. pipiens* s.l. (n = 2,473) and *Ae. albopictus* (n = 481) representing 80.1 % and 15.6 % of the total catches, respectively. Most of mosquitoes were represented by not engorged females (68.4 %) while engorged females

Table I. *Mosquitoes species collected in Sardinia (Italy) during 2016 and number of pool tested.*

Species	N. positive sites/40 collection sites	Males	Females not engorged	Females engorged	Total (%)	N. of pool tested
Culex pipiens s.l.	37	591	1703	179	2473 (80.05)	354
Aedes albopictus	22	142	336	3	481 (15.59)	146
Culiseta longiareolata	17	42	17	4	63 (2.04)	43
Culex spp.	5	4	35	1	40 (1.30)	11
Ochlerotatus caspius	7	5	6	----	11 (0.36)	10
Culex hortensis	4	3	7	----	10 (0.32)	9
Ochlerotatus detritus	3	----	2	1	3 (0.10)	3
Culex theileri	2	----	2	----	2 (0.06)	2
Culiseta spp.	2	----	2	----	2 (0.06)	1
Culiseta annulata	1	----	1	----	1 (0.03)	2
Aedes spp.	1	----	1	----	1 (0.03)	1
Anopheles algeriensis	1	----	1	----	1 (0.03)	1
Anopheles labranchiae	1	----	1	----	1 (0.03)	1
Total		**787**	**2,114**	**188**	**3,089**	**584**

(25.5 %) and males (6.1 %) were less abundant. *Culex pipiens s.l.* was the most ubiquitous species collected in 26 out of 29 collection sites. In particular 1,414 specimens (57.2 %) were captured in 10 urban sites and 1,059 (42.8 %) in 16 sites in the border areas. *Ae. albopictus* was also captured in all regional territory and its presence was detected in 21 out of 29 trapping sites. This species was captured in all urban areas traps counting a total of 382 specimens (79.4 %), while only 99 adults (20.6 %) were captured in 9 sites situated in border areas. No mosquito was captured in the coal ship. A total of 6 mosquitoes were collected in the garden of infected patient. In particular, 2 females of *Ae. albopictus*, 2 females of *Cs. longiareolata*, 1 female of *An. labranchiae* and 1 male of *Oc. caspius* were recorded.

Culex pipiens s.l. was captured during all study period showing significant seasonal changes with the highest density from May to July (Figure 2). The population density of *Ae. albopictus* increased from April and peaked in September, while no specimens were captured in December (Figure 2).

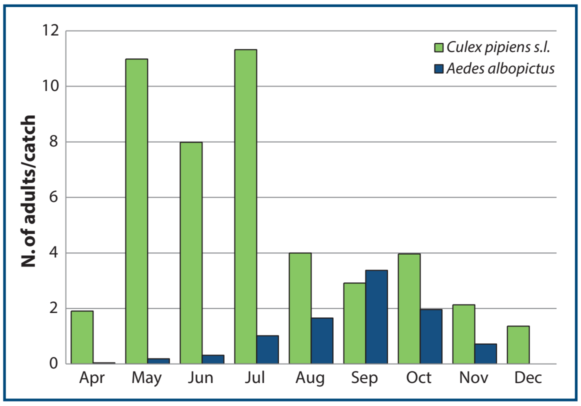

Figure 2. *Seasonal abundance of* Cx. pipiens s.l. *and* Ae. albopictus *during 2016 in Sardinia (Italy).*

All mosquitoes collected during the study period were tested using ZIKV real time RT-PCR assay. Overall a total of 584 pools of mosquitoes were tested and no pool was found positive.

Discussion

The surveillance conducted in Sardinia during 2016 has shown the presence of 10 mosquitoes species indigenous currently established in the Island. Furthermore, no invasive mosquito species has been detected. During our monitoring *Cx. pipiens s.l.* and *Ae. albopictus* have been the main species reported representing over 95% of the total mosquitoes captured. Both species are widely distributed in Europe and represent potential vectors of arboviruses. Their distribution is ubiquitously, largely diffuse in urban, sub urban and rural areas.

Although ZIKV has been detected in *Culex* mosquito species as *Culex perfuscus* in Senegal (Diallo *et al.* 2014), several authors have reported that *Cx. pipiens* is not a competent vector to ZIKV (Boccolini *et al.* 2016, Heitmann *et al.* 2017). In Europe *Cx. pipiens* is considered a competent vector of several zoonotic viruses including West Nile virus (WNV), Usutu virus (USUV), Rift Valley Fever virus (RVFV) and Japanese Encephalitis virus (JEV) (Lundström 1999, Busquets *et al.* 2008, Moutailler *et al.* 2008, Ravanini *et al.* 2012). In Sardinia, WNV and USUV have been found in *Cx. pipiens* mosquitoes through real time

RT-PCR (Rossi *et al.* 2016). *Culex pipiens s.l.* include two biotypes named *Culex pipiens pipiens* and *Culex pipiens molestus* (Amara Korba *et al.* 2016). These forms are morphologically indistinguishable but exhibit different characteristics in both behavioral and physiology. In addition, exist a hybrid form that present biologic characteristics of both forms. In this study, no biomolecular analyses have been performed to discriminate these 3 forms. Considering a previous study conducted in Italy (Di Luca *et al.* 2016b), we can suppose the presence of all three forms of *Cx. pipiens*, included hybrid form.

Aedes aegypti is considered as the main vector of ZIKV infection in Urban areas, but the viral RNA has been detected also in several *Aedes* species (Diallo *et al.* 2014). In Sardinia are reported three species of the *Aedes* genus: *Ae. albopictus*, *Ae. vittatus* and *Ae. vexans* (Severini *et al.* 2009). *Aedes albopictus*, highly correlated to *Ae. aegypti*, is considered a potential vector. In fact, field collected *Ae. albopictus* was found ZIKV RNA positive in Gabon during CHIKV and DENV outbreaks in 2007 (Grard *et al.* 2014). Moreover recent experimental studies highlights that *Ae. albopictus* is susceptible to ZIKV infection (Chouin-Carneiro *et al.* 2016, Di Luca *et al.* 2016a, Heitmann *et al.* 2017). *Aedes albopictus* feeds primarily on wild and domestic animals included humans, and it has exophagic and exophilic behavior. Currently, Italy is the most greatly-infested country in Europe with well established populations in several regions including Sardinia (Cristo *et al.* 2006). During our study, *Ae. albopictus* has resulted more abundant in the urban areas as reported by other authors (Medlock *et al.* 2012). *Aedes vittatus* has been found naturally infected with ZIKV in Senegal and Ivory Cost (Diallo *et al.* 2014). This species is heavily anthropophilic and bites during the daytime and night. *Aedes vexans*, though it is not considered susceptible to ZIKV infection, is able to transmit other arboviruses, as RVFV and WNV (Ndiaye *et al.* 2016). This mosquito has a behavior resembling to *Ae. vittatus*. Other species found in low numbers during our study as *Culiseta longiareolata*, *Ochlerotatus caspius* and *Cx. theileri* are capable of transmitting arboviruses (Maslov 1967, Moussieget 1988, Lundström 1999) .

Similarly to *Ae. albopictus*, other species as *Ae. koericus* and *Ae. japonicus* were introduced in Italy via passive transport of eggs from heavily infested areas (Capelli *et al.* 2011, Seidel *et al.* 2016). These mosquitoes have found favorable ecological niches and nowadays are established in Italy. *Aedes aegypti* was reported in Italy up to 1972 (Callot and Delecolle 1972) therefore, it is worth expecting its potential reintroduction in the future, considering also its actual presence in some European regions, as Madeira Island, Black Sea coastal areas of Georgia and Russia and Holland (WHO 2016b).

The increase of connectivity, commercial trades and climatic changes represent a real risk of spread of exotic pathogens and disease vectors. The outbreaks of WNV, CHIKV and DENV in Europe represent an important threat to consider. Epidemiological surveillance of imported cases is a critical point to evaluate the potential risk of arboviruses introduction. An imported case is a potential source of infection that in presence of suitable vector populations could determine a spread of the disease. Sardinia represents a territory at high likelihood of introduction of arboviruses, because is located in the middle of Mediterranean basin with high intensity of touristic and trade flows and with a well-established *Ae. albopictus* population.

Although ZIKV presence has not been detected during our study, a permanent entomological surveillance in urban areas and at the points of entry, as ports and airports, should be implemented to provide epidemiological information to evaluate the risk of introduction of ZIKV and other arboviruses in Italy.

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