TITULO: Phlebovirus and Flavivirus detection in mosquito and sandfly populations and in FTA cards

MASTER OFICIAL EN ECOLOGIA TERRESTRE Y GESTION DE LA BIODIVERSIDAD

ESPECIALIDAD EN GESTION Y DIVERSIDAD DE FAUNA Y FLORA (MARINA Y TERRESTRE)

ESTUDIANTE: Lotty Birnberg Yerovi

DIRECTORAS: Dra. Núria Busquets y Sandra Talavera

TUTOR ACADÉMICO: Dr. Francesc Muñoz

FECHA: 17 de septiembre, 2015

IRTA - CRESA
Title: *Phlebovirus* and *Flavivirus* detection in mosquito and sandfly populations and in FTA cards

Running Title: *Phlebovirus* and *Flavivirus* detection in Spain

**ABSTRACT**

Current *Phlebovirus* (*Bunyaviridae*) and *Flavivirus* (*Flaviviridae*) related diseases are of global public health and animal sanitary interest. Viral and vector dispersion require multidisciplinary targeted strategies to prevent and control arboviral outbreaks in susceptible areas. Locations in various habitats at the Llobregat River Delta were chosen to develop mosquito (Diptera: *Culicidae*) and phlebotomine (Diptera: *Psychodidae*) captures with CO₂ baited traps supplied with sugar baited preservation FTA cards. To test the efficiency of FTA cards in viral detection as a surveillance tool, a standardizing assay was developed evidencing high feeding rates and low mortalities in two species of laboratory mosquito colonies: *Aedes albopictus* and *Culex pipiens*. Generic RT – nPCRs with degenerated primers and sequencing were performed for viral detection in female specimen pools and FTA cards. Species composition of the area showed *Phlebotomus perniciosus* as the dominant phlebotomine sandfly, and *Culex pipiens* as the most abundant mosquito species followed by the tiger mosquito *Aedes albopictus*. *Insect Specific Flaviviruses (ISFV)* were detected in an *Aedes albopictus* and *Culex pipiens* pools, confirming this flavivirus circulation in Catalonia. Further studies in FTA cards are needed to confirm them as useful tool for surveillance programs.

*Key words:* Culicidae, Flavivirus, Phlebotominae, Phlebovirus, FTA cards, sugar – based surveillance.
INTRODUCTION

In the past decades, until now, we have witnessed the appearance of new infectious diseases and a significant expansion in the distribution range of the known ones. Global climatic change, together with human activities such as deforestation, agricultural development, hunting and hasty urban expansion has increased the contact with pathogens (Hollidge et al., 2010; Devaux, 2012) that were confined to sylvatic areas. Environmental changes may also alter vertebrate or vector host composition and create new niches for their potential vectors (Weaver & Reisen, 2010; Devaux, 2012). Population growth, human migration and commerce have contributed to the rapid spread of these emerging or re-emerging diseases (Hollidge et al., 2010; Weaver & Reisen, 2010; Devaux, 2012). Approximately 30% of the emerging and re-emerging infectious diseases were attributed to arboviruses (Pfeffer & Dobler, 2010).

Arthropod borne viruses or, well-known as, arboviruses are transmitted naturally among vertebrate hosts by the bite of a variety of hematophagous arthropod vectors such as mosquitoes, sandflies, biting midges and ticks (Hollidge et al., 2010; Pfeffer & Dobler, 2010; Weaver & Reisen, 2010; Meltzer, 2012). Currently, arboviruses present a worldwide distribution and over than 500 viruses have been catalogued (Blair et al., 2000; Pfeffer & Dobler, 2010). Arboviruses are mainly RNA viruses classified within various families: Bunyaviridae, Flaviviridae, Reoviridae, Togaviridae and Rhabdoviridae (Blair et al., 2000; Hollidge et al., 2010; Meltzer, 2012). Arboviruses, as most RNA viruses exhibit high genetic plasticity, attribute that increases their ability to adapt to changing environments and allows them to accommodate a cycle of alternating replication in different vertebrate and invertebrate hosts (Weaver & Reisen, 2010; Devaux, 2012). Most of the arboviruses are zoonotic, transmissible from animals to humans. Several arboviruses have become important human and veterinary pathogens causing febrile illnesses and encephalitis (Liang et al., 2015). Bunyaviridae is the largest family with approximately 350 viruses clustered according to their morphological, biochemical and serological characteristics into different genera: Orthobunyavirus, Nairovirus, Tospovirus, Hantavirus and Phlebovirus, from which all may be human pathogens except for Tospovirus that mostly infects plants (Hollidge et al., 2010; Meltzer, 2012; Mc Elroy & Vanlandinghan, 2014). Among the bunyaviruses, the Phlebovirus genus includes 70 viruses grouped in 33 tentative species and nine species divided in two groups according to their arthropod vector: sandfly fever viruses,
transmitted by sandflies and mosquitoes; and tick borne viruses (Elliot & Brennan, 2014; Mc Elroy & Vanlandinghan, 2014). Phleboviruses genome consists of a single stranded, negative sense RNA molecule divided in three segments: small segment (S) encoding for nucleoproteins and non-structural proteins, medium segment (M) encoding for envelope proteins and large segment (L) which may encodes the viral polymerase (Charrel et al., 2009; Elliot & Brennan, 2014; Alkan et al., 2015). The Flaviviridae family is represented by the Flavivirus genus due to its diversity and medical importance (Duque Ferreira et al., 2013). Flaviviruses are classified into four clades based on their host range: i) tick-borne, ii) mosquito-borne, iii) unknown vector viruses that are transmitted between vertebrate hosts and iv) mosquito only flaviviruses that are transmitted between arthropods without an intermediate host (Beck et al., 2013; Coffey et al., 2013; Hanley et al., 2013; Grisenti et al., 2014). Members of this group are enveloped, icosahedral, single stranded and positive sense RNA viruses. Their genome encodes for three structural proteins (capsid, envelope and membrane) and seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5) (Cook et al., 2009; Hollidge et al., 2010; Coffey et al., 2013; Hanley et al., 2013; Grisenti et al., 2014; Huang et al., 2014).

In Europe, many arboviruses are endemic or occasionally imported. Phleboviruses associated illnesses, such as Sandfly Fever, first were reported after World War II causing outbreaks in troops that visited Mediterranean countries. Currently, Croatia, Italy, France, Spain and Portugal report the presence of various sandfly fevers. Sandfly Fever Sicilian Virus (SFSV), Sandfly Fever Naples Virus (SFNV) and Toscana Virus (TOSV) are human pathogens with actual wide distribution at the Mediterranean basin (Kocak Tufan et al., 2013; Elliot & Brennan, 2014). In Spain, Granada Virus (GRV) and in France Massilia virus has been recently described and several cases are reported from travelers in other European countries (Kocak Tufan et al., 2013). Rift Valley Fever Virus (RVFV), another medically important phlebovirus that may cause severe encephalitic/hemorrhagic diseases in humans and abortion and death in livestock, was identified in 1930 in Kenya and actually represents a threat among European countries for its introduction risk (Pepin et al., 2010; Chevalier, 2013). As well as Phleboviruses, Flaviviruses, for example West Nile Virus (WNV), has been circulating in Central and South – Eastern Europe since 1960s and has increased its virulence, infection rates and geographic distribution. Significant outbreaks have been reported between 2008 and
2010 where the number of human cases surpassed the thousand and more than 50 deaths were produced (Beck et al., 2013; Engler et al., 2013). In the same years, the first autochthonous Dengue Virus (DENV) cases were reported in Southern France, Croatia, Italy (Meltzer, 2012; Beck et al., 2013) and Spain (Bueno & Jimenez, 2010). In 2014 hundreds of suspected DENV cases were also reported (Liang et al., 2015). According to the European Surveillance Net of Imported Diseases “TropNetEuro”, dengue represents approximately 10% of all the imported diseases declared in Europe (Bueno & Jimenez, 2010). Another member of the flaviviruses, Usutu Virus (USUV) was introduced in Central Europe and the Mediterranean basin by migrant birds (Bueno & Jimenez, 2010; Pfeffer & Dobler, 2010). It presents similar distribution as WNV and has been recognized as animal and human pathogen. Even though, no human severe cases have been recorded it may cause considerable bird mortality (Beck et al., 2013; Grisenti et al., 2014).

Arboviruses may be transmitted by hematophagous arthropods within Diptera order. They need blood intake to complete their gonotrophic cycle and may participate in infectious diseases spread (Hubalek and Rudolf, 2011). Phlebotominae, a subfamily in the Psychodidae family, is represented by moth flies and sandflies. There are over than 700 species with worldwide distribution. Phlebotominae is represented by two genera: Phlebotomus in the Old World and Lutzomyia in the New World. These small hematophagous dipterans are implicated in parasitic (Leishmania), bacterial (Bartonella) and several viral (phleboviruses, orbiviruses and vesiculovirus) diseases transmission (Depaquit et al., 2010; Hubalek & Rudolf, 2011; Ready, 2013). Culicidae is a numerous (more than 3400 species) and cosmopolitan family distributed all over the tropical and temperate regions (Herbach, 2007; Collao et al., 2015). Taxonomy of this family remains unclear, but mainly it is classified in two subfamilies: Anophelinae and Culicinae, both of medical importance. Anophelinae contains the genus Anopheles implicated mainly in Malaria transmission and Culicinae comprises the main genera involved in arboviral transmission: Culex, Aedes, Ochlerotatus, Culiseta and Coquilletidia (Herbach, 2007; Hubalek & Rudolf, 2011).

The emergence and re-emergence of arboviruses give rise to develop targeted strategies. Traditional arboviral surveillance is based on: entomological surveillance, molecular viral detection, virus or virus – specific antibodies detection in symptomatic patients, as well as, sero – surveys in sentinel animals (Alba et al., 2013; Van der Hurk et al., 2014).
A variety of carbon dioxide (CO₂) baited traps are used to capture and identify the potential arthropod vector diversity of the area and for further viral detection with molecular techniques (Alba et al., 2013; Van der Hurk et al., 2014). Recent approaches for infectious diseases monitoring, employ preservation filter paper cards as a complement for pathogen detection (Hall – Mendelin et al., 2010; Van der Hurk et al., 2014). Preservation cards sensitivity and specificity have been tested for many bacterial, parasitic and viral pathogens (Smit et al., 2014; Van der Hurk et al., 2014). Subsequent contrast with conventional “gold standard” laboratory techniques evidenced the usefulness of the cards in infectious diseases detection.

The main objectives of the present study were: i) to detect phleboviruses and flaviviruses in phlebotomine and culicid mosquito wild populations respectively, and ii) to test the efficiency of FTA cards as a useful tool for arboviral surveillance at the Llobregat River Delta.

**METHODOLOGY**

**FTA Standardizing Assay**

To accomplish an effective sugar baited substrate that attracts and contributes to the survival of the captured specimens, as well as limits viral RNA degradation, a standardizing assay was developed. Individualized FTA™ classic cards (Watham™, GE Healthcare UK limited, Buckinghamshire, UK) were soaked in Manuka’s health honey (MOG®250®, Cheshire, UK), colored with blue food dye (Icing Colour Royal Blue, Wilton Industries, Woodridge, IL, USA), in two different concentrations (low: 1gr/FTA and mild: 2 gr/FTA). FTAs were sealed in plastic bags and left inside thermal chambers (24°C, 86% humidity) for 24, 48 and 72 hours soaking periods. Two species of mosquito laboratory colonies were selected for the assay: *Aedes albopictus* and *Culex pipiens*. Six study groups per species were formed with ten mature female specimens per group. Each group corresponded to a specific concentration and soaking period. FTA cards were maintained at each group per two consecutive days under laboratory conditions (temperature: 24°C, humidity: 86%, daylight/night 14/10 hours) provided by a thermal chamber with humidity (Telewig, Spain). Mortality and feeding ratio were registered per group per day.
To test if the honey concentration used to prepare the FTA cards could cause PCR inhibition while viral detection, individualized FTA cards were soaked in different dyed Manuka’s honey concentrations: low (1 gr/FTA), mild (2 gr/FTA) and high (3 gr/FTA). A honey free FTA card was used as control. Assuming that the amount of saliva expectorated by a mosquito during a blood intake is approximately 4.7µL (Van der Hurk et al., 2014), 5 µL of inactivated viral suspension WNV (2.98 log TCID50/mL) and Phlebo (4.02 log TCID50/mL) were used. Soaked and dipped with the virus FTAs were deposited inside 1.5mL tubes containing 500 µL of DMEM cell culture (BioWhittacker®, Lonza, Belgium) supplemented with Penicillin – streptomycin (PNS) 2% (SIGMA – ALDRICH, Madrid, Spain) and incubated for 20 minutes at 4ºC. FTAs were squeezed with plastic pistils (one per each sample to avoid contamination) and viral detection process was performed.

**Entomological surveillance and viral detection**

**Study Area and Sampling Design**

The present study was conducted at the Llobregat River Delta in Catalonia – Spain. This Delta is located in the Barcelona Province where the Llobregat River encounters with the Mediterranean Sea and forms an extensive flatland between small mountain areas. A variety of habitats, presenting favorable conditions for mosquito/phlebotomine proliferation, coexist with densely populated areas. To obtain a representative sample of the diversity of habitats present in this susceptible ecosystem, areas showing different degrees of human intervention and closeness to human dwellings were considered among: foothills with forested areas, flatlands with crops and wetlands (Table 1). Sampling locations were selected according to previous evidence of arbovirus presence (Alba et al., 2013), and where regular servicing of traps was developed by the Servei de Control de Mosquits del Baix Llobregat (Figure 1). Entomological surveillance was carried out every two weeks from September to October 2014 and from May to August 2015. Due to logistic issues in 2015 “El Remolar” was exchanged for “Les Tanques” at Viladecans and “El Papiol” was eliminated from the trial.

**Sampling Methodology**

To collect host seeking females of different hematophagous species, captures were carried out using CO₂ baited EVS Mosquito Traps (Bioquip, USA) supplied with FTA cards soaked in dyed Manuka’s honey. FTA cards were placed inside the collection bag.
of each trap for further viral molecular detection (Hall – Mendelin et al., 2010; Van der Hurk et al., 2012; Engler et al., 2013). One trap was situated per each location and kept operating approximately 18 hours, from the early evening to the next morning. Specimens from the field were deposited alive in containers with dry ice for transportation and sacrifice. Identification and dissections (phlebotomines) were performed maintaining a cold chain to avoid ARN degradation (Van der Hurk et al., 2012; Alba et al., 2013). FTA cards were removed from trap collection bags, covered with Parafilm® M (Bemis, Neenah, WI) and preserved at 4ºC until molecular processing (Van der Hurk et al., 2014).

**Specimen Identification**

For phlebotomine female identification, genitalia dissections were performed and Botet 1991 classification key was used. Female mosquitoes were classified to species level according to their morphological characteristics (Schaffner et al., 2001). Females were pooled inside 1.5mL tubes according to species, location and sampling date. Each tube contained 2mm diameter glass spheres suspended in DMEM cell culture (BioWhittacker®, Lonza, Belgium) supplemented with PNS 2% (SIGMA – ALDRICH, Madrid, Spain).

**Viral Detection**

Pools were mechanically homogenized using QIAGEN® Tissuelyser II (Spain). RNA extraction was performed using NucleoSpin® Viral RNA isolation kit (Macherey – Nagel, Düren, Germany) according to manufacturer´s instructions protocol and RNA was eluted in 50 µL of DEPC water. Viral dilutions, corresponding to Phlebovirus and West Nile Fever Virus (WNV) were used as positive extraction controls. Amplification of the Phlebovirus L gene (244bp) (Sanchez-Seco, 2003) and amplification of a 143 bp region of Flavivirus NS5 gene (Sanchez-Seco, 2005) were performed from phlebotomine and mosquito samples respectively. Initial RT-PCR was carried out using Promega Access RT-PCR System (Madison, WI, USA) adding 2.5µL isolated RNA. Degenerated primers: Phlebo 1+ (10µM) (5’ - ATGGARGGITTTGTTIWSICIICCC) and Phlebo 1- (10µM) (5’ - ATGGARGGITTTGTTIWSICIICCC) (ROCHE Applied Science, Spain; TIB MOLBIOL Syntheselabor GmbH, Germany) were used for phleboviruses detection in phlebotomines and Flavi 1+ (10µM) (5’ - GAYYTGITITYGGIIIGIGGIRTGG) and Flavi 1- (10µM) (5’ -
TCCCAICCICIRTRTCTCIGC) (ROCHE Applied Science, Spain) for flaviviruses detection in mosquitoes. Following nPCR was accomplished using a master mix conformed by degenerated primers: Phlebo 2+ (10µM) (5’ - WTICCIAICCIYMSAARATG) and Phlebo 2- (10µM) (5’ - TCYTCYTTRTTYTTRARRTARCC) (ROCHE Applied Science, Spain; TIB MOLBIOL Syntheselabor GmbH, Germany) and Flavi 2+ (10µM) (5’ - YGYRTIYAYAWCAYSATGGG) and Flavi 2- (10µM) (5’ - CCARTGITYKYRTTIAIRAAICC) (ROCHE Applied Science, Spain) respectively, an in-house Buffer 5X (1M TRIS HCl pH=8.5, MgCl2 (25 mM), 1 M (NH4)2 SO4), dNTPs (10mM), DEPC water and AmpliTaq® (5 u/µL) (Applied Biosystems, California, USA) adding 0.5 µL cDNA previously obtained. For phlebovirus nPCR master mix MgCl2 (25mM) was added to complement the reaction. Amplification cycles were developed in a GeneAmp® PCR System 2700 (Applied Biosystems, California, USA). RT- nPCR results were evidenced with 2.5% agarose (AMRESCO®, Ohio, USA) gel electrophoresis in Tris Acetate EDTA Buffer (TBE) 1X (iNtRON Biotechnology INC., Korea) and visualized with V:GENUS® Gel Image Documentation System (Syngene, Cambridge, UK).

Purification and Sequencing

Preparation 2.5% agarose gel electrophoresis was performed for positive Phlebovirus and Flavivirus RT – nPCR band recovery. Purification was developed with Nucleospin® Gel and PCR Clean – up kit (Macherey – Nagel, Düren, Germany) according to manufacturer’s instructions protocol.

Purified PCR products were sequenced at the Servei de Genòmica i Bioinformàtica – UAB and Macrogen – Netherlands. Sequences were edited using Bioedit v7.2.5 (Tom Hall Ibis Biosciences, Carlsbad, CA) to cut – off primer sequences and submitted to the NCBI Nucleotide Local Basic Alignment Search Tool (BLASTn) to be compared with the GenBank database.
RESULTS

FTA standardizing assay

After the trial to set up honey concentration in FTA cards, less mortality and higher feeding ratios were observed in the groups corresponding to longer soaking periods and higher honey concentrations. Higher mortality proportions were observed in *Aedes albopictus*, meanwhile no evident differences were observed in the feeding behavior between both species (Table 2).

*Phlebovirus* and *Flavivirus* RT-nPCR inhibition assay showed that no inhibition may be caused by the honey’s concentration present at the FTA cards (Figure 2).

Species composition

A total of 127 phlebotominae sandflies were collected. Two species were identified: *Phlebotomus perniciosus* as the dominant species (98.43%), found in all locations except for “Les Tanques” and “Remolar”, and *Phlebotomus ariasi* (1.57%) only recorded at Can Rigol.

On the other hand, 222 mosquitoes were collected. Six species were identified of which *Culex pipiens* is the most abundant species (41.44%), followed by the tiger mosquito *Aedes albopictus* (18.47%) and *Ochlerotatus caspius* (18.02%), the least proportions correspond to *Coquillettidia richiardii* (7.66%), *Aedes vexans* (7.21%) and *Culiseta longiaerolata* (7.21%). *Culex pipiens* and *Aedes albopictus* as the most abundant species are present in all the locations. *Culiseta longiaerolata* is recorded at “Hospital Martorell”, “Les Tanques” and “El Papiol”. *Ochlerotatus caspius* is found in “Can Comas” and “Remolar”. Meanwhile, *Aedes vexans* is only recorded at “Camps Blancs” and *Coquillettidia richiardii* only at “Can Comas” (Table 3).

*Phlebovirus* and *Flavivirus* Molecular Detection: Infection Status

From 65 culicid pools analyzed, two (2/65) yielded positive results for *Flavivirus* RT-nPCR: *Culex pipiens* (M030) captured in “Can Comas”, located at the Llobregat Delta flatlands surrounded by crops and livestock; and *Aedes albopictus* (M063) captured in “Camps Blancs” located at an urban foothill area surrounded by a deforested patch. All 127 phlebotomine samples were analyzed and no phleboviral presence was evidenced (Table 3) (Figure 3 a, b).
After *Phlebovirus* and *Flavivirus* molecular screening, two of 56 FTA cards resulted positive for *Phlebovirus*, (2/56). Positive RT – nPCR FTA cards belong to: “El Papiol”, (T007) an urban area located at the Delta flatlands, and to “Hospital Martorell”, (T015) an urban area located at a foothill characterized by the proximity of forested areas and the river. No *Flaviviruses* were detected in FTA cards (Table 3) (Figure 3c).

*Ae. albopictus* flavivirus sequence BLASTn analysis showed an 82% similarity (67/82 identities, 2 e^{-12} e-value) with *Kamiti River Virus* accession number EU074051.1, an insect-only flavivirus. Due to sequence quality, *Culex pipiens* pool and one of the FTA cards positive were not able to be performed. The other FTA card positive resulted to be an unspecific amplification and no similarities were obtained in BLASTn analysis.

**DISCUSSION**

FTA standardizing assay

FTA cards with greater amount of honey exposed to longer soaking periods (48 – 72 hours) remain moist throughout the testing period being a permanent sugar source for the specimens. Meanwhile, the ones exposed to 24 hours period, no matter the honey’s concentration, showed desiccating and hardening. Higher mortalities probably would be explained by the fact that specimens weren’t able to feed from the dry FTA cards. For the field component of the study FTA cards were prepared with two grams of dyed Manuka’s honey and soaked for 48 hours to guarantee the availability of an adequate sugar source while the traps were functioning.

Entomological surveillance and viral detection

The emergence and re-emergence of arboviral diseases depend on the interactions among different factors: i) viral presence/circulation, ii) competent vector populations to transmit the virus, iii) permissive environment with susceptible vertebrate host populations to allow viral replication (Pffefer & Dobler, 2010).

a) **Viral presence/circulation**: The present study yielded RT – nPCR positive *Phlebovirus* FTA cards and positive *Flavivirus* wild caught *Culex pipiens* and *Aedes albopictus* mosquito pools. An Insect specific flavivirus (ISFV), similar to *Kamiti River Virus* (KRV) genome was identified in an *Ae. albopictus* pool. The presence of ISFV in *Aedes* populations has been reported previously by other authors (Crabtree, *et al*).
al., 2003; Lutomiah et al., 2007; Junglen et al., 2009; Alba et al 2013; Collao et al., 2015). ISFV present the same structure as the other members of the Flaviviridae family but they would be unable to infect vertebrate cells. They replicate exclusively in insect cells and sometimes their genomes may be integrated in the host´s (Liang et al., 2015). The lack of vertebrate host would indicate venereal or vertical transmission from infected males to females and from parents to offspring respectively (Cook et al., 2009; Calzolari et al., 2012; Duque Ferreira et al., 2013; Grisenti et al., 2014; Collao et al., 2015). The structural similarities and the impossibility to infect vertebrate cells suggest their ancestral status within the group (Cook et al., 2009; Junglen et al., 2009; Liang et al., 2015). The first ISFV characterized was the Cell Fusing Cell Aedes Flavivirus (CFAV) in 1974, since then, it has been detected in different countries all over the globe. After its discovery, several ISFV have been detected in wild caught mosquitoes (Cook et al., 2009; Junglen et al., 2009; Liang et al., 2015). The results from the present study would confirm the current circulation of arboviruses in Catalonia as reported by other authors (Busquets et al., 2008; Bueno & Jimenez, 2010; Alba et al., 2013). Negative Flavivirus RT – nPCR results in FTA screening may be explained by the fact that ISFV would not be transmissible or may be integrated in the vector´s genome, therefore not expectorated with saliva while sugar feeding from the honey soaked FTA cards. If the Culex pipiens flavivirus detected was not an ISFV, the absence of flavivirus detection in FTA cards could be explained for the limit of the technic. Further studies on this field are needed. Mosquito intrathoracical inoculation of flavivirus and in vivo assays with FTA cards could be performed to elucidate this issue.

b) Competent vector populations: Species recorded at the sampling locations are considered medically important. The genus Phlebotomus, and more specifically Phlebotomus perniciosus is the main vector of endemic phleboviruses (TOSV, SFSV, SFNV, GRV) at the Mediterranean basin (Depaquit et al., 2010; Kocak Tufan et al., 2013; Elliot & Brennan, 2014) and may spread them among other European countries within its distribution range. The abovementioned phleboviruses have been isolated from sandflies in indistinct Mediterranean and Asian countries demonstrating their role in phlebovirus transmission. The presence of Ph. perniciosus also implies a transmission threat of imported RVFV (Depaquit et al., 2010; Mc Elroy & Vanlandinghan, 2014).
The common mosquito, *Culex pipiens* is one of the most abundant culicids around the world. *Cx. pipiens* is an ornithophilic (marked preference to feed with bird blood) and opportunistic biter. It has been incriminated as a competent vector of some flaviviruses: WNV (*Flavivirus*) in the American continent (Pffefer & Dobler, 2010; Collao *et al.*, 2015), central and southern Europe (Bueno & Jimenez, 2010; Pffefer & Dobler, 2010; Devaux, 2012); USUV in Austria (Devaux, 2012) and in Spain (Busquets *et al.*, 2008; Bueno & Jimenez, 2010). It has also been incriminated as potential vector of other flaviviruses within the Japanese Encephalitis Group not yet found in Europe, as well as RVFV (*Phlebovirus*), due to animal trade, commerce and migration (Hollidge *et al.*, 2010; Weaver & Reisen, 2010; Mc Elroy & Vanlandinghan, 2014; Liang *et al.*, 2015).

As reported in several studies (Gratz, 2004; Hollidge *et al.*, 2010; Weaver & Reisen, 2010; Waldock *et al.*, 2013), the Asian tiger mosquito, *Ae. albopictus* is a highly invasive and aggressive biting species that exhibits high zoophilic/anthropophilic host seeking preferences (Gratz, 2004). In Europe, the tiger mosquito has colonized all the Mediterranean basin and Central countries threatening human and animal health all over its distribution range. *Ae. albopictus* is recognized as a competent vector implicated in rural DENV transmission in South – East Asia, China, Japan and Hawaii in absence of *Aedes aegypti* (Gratz, 2004; Hollidge *et al.*, 2010; Weaver & Reisen, 2010; Tuiskunen Bäck & Lundkvist, 2013). In Europe, the New World and Australia it has also been implicated in WNV transmission between bird populations and possibly to humans (Gratz, 2004; Liang *et al.*, 2015).

The least abundant species, but not the least important, *Ochlerotatus caspius*, *Culiseta longiaerolata* and *Coquillettidia richiardii* have been involved as a bridge vector of pathogenic viruses such as WNV and RVFV (Engler *et al.*, 2013).

The species documented in this work have also been associated with other Insect Specific Flaviviruses (ISFVs) in previous studies throughout the world (Engler *et al.*, 2013; Calzolari *et al.*, 2015; Liang *et al.*, 2015). *Culex* Flavivirus (CxFV), *Aedes* Flavivirus (AeFV), *Ochlerotatus caspius* Flavivirus (OcF) (Calzolari *et al.*, 2015). The evidence of ISFV existence in wild mosquito and some phlebotomine populations is of great epidemiological value. It has been hypothesized that competition between arboviruses may occur within the vertebrate host and invertebrate vector by superinfection, cross – neutralization or concurrent infections (Calzolari *et al.*, 2012; Hanley *et al.*, 2013). As viral infection in arthropod vectors is lifetime and may be
venereal or vertically transmitted, competitive exclusion of a virus may occur in particular geographic areas where its competitor is established (Cook et al., 2009; Hanley et al., 2013; Huang et al., 2014), for instance Yellow Fever and Dengue viruses (Hanley et al., 2013). The same situation may happen, or not, with ISFV that co-circulate with pathogenic flaviviruses in a given region. The influence of ISFV in the bionomics of vector populations should also be analyzed to clarify if it affects negatively or enhances the arthropod adaptation to an ecosystem (Calzolari et al., 2012).

c) **Permissive environment and susceptible vertebrate host populations:** Habitat diversity at the Llobregat River Delta provides suitable conditions for arboviral transmission. Numerous available breeding sites are distributed along highly populated areas, throughout rural environments and wetlands. Urban and sylvatic cycles are feasible due to domestic animals, livestock and the avian fauna (including migratory birds) of the region (Meltzer, 2012). Epidemiologically speaking, animal and human movement will be involved in the introduction of new viruses into this geographic area. Global warming may also enhance epidemic spread in this susceptible area. High temperatures and humidity fastens larval development, extends adult survival and shortens extrinsic incubation periods leading to rapid viral replication (Shope, 1991; Calzolari & Albieri, 2013). In drought situations breeding sites are reduced, early stages and adult vector survival are compromised but an enhancing effect on spread and circulation of arboviruses has been reported (Calzolari & Albieri, 2013) maybe due to increased host density on a particular area close to water sources, that at the same time, work as breeding sites and increases the contact with vector populations.

**Surveillance Strategies and Challenges**

Knowledge concerning vector diversity and distribution is necessary to identify the potential vector species, how they spread and their environmental preferences. Viral prevalence monitoring in arthropod populations provides valuable information about the circulating viruses at specific areas and will incriminate species as competent vectors or enlightens their role in the transmission cycle (Hall – Mendelin, 2010). Viral surveillance will also contribute to the knowledge of ISFV, how they interact with pathogenic flaviviruses and with their vectors. This information will setup the baseline for future studies concerning the potential use of ISFV in infectious diseases control.

The viral presence in honey baited FTA cards exhibit their value as a helpful tool for arbovirus detection. While FTA card ensures the preservation of the pathogen genome if
expectorated, Manuka’s honey provides a moist, antibacterial/antifungal sugar source that contributes to the survival of the captured specimens, hence the viability of the virus if present (Hall – Mendelin et al., 2010; Van der Hurk et al., 2014) until molecularly processed. Moreover, FTA cards inactivate the virus expectorated in it avoiding direct contact with potential pathogenic agents. Timing between trap placing and servicing, specimen manipulation during processing may end up in nucleic acid degradation making imperative the maintenance of a cold chain throughout the complete process (not logistically feasible during field work) to guarantee the sample’s integrity (Van der Hurk et al., 2012). Besides the preservation and biosafety advantages, FTA cards require less time consuming and more economic expenses than mosquito pools for their analysis. Entomological and sentinel animal surveys mostly would require specialized logistics and equipment to provide similar information as the arbovirus detection in FTA card.

Phlebovirus and Flavivirus molecular detection based on degenerated primers allows to confirm the distribution and presence of previously described viruses and detect new viruses or new distribution ranges/vector species of the known ones (Sánchez – Seco et al., 2003; Sánchez – Seco et al., 2005; Alba et al., 2013; Collao et al., 2015). Although, some limitations are evident at the time of sequence analysis. Degenerated primers s may cause unspecific band amplifications and limited sequence quality. Further studies complemented with species – specific primers and viral isolation techniques would be required to confirm the identity and characterize the detected virus genomes.

CONCLUSIONS

Findings at the present study corroborated the existence of the main phlebotomine and culicid pathogen vectors at the Llobregat River Delta: Phlebotomus perniciosus, Culex pipiens and Aedes albopictus, as well as yielded phleboviral and flaviviral presence in different habitats within this ecosystem. Susceptibility socio - environmental features and confirmed viral circulation sustain the importance of regular and exhaustive arboviral surveillance/control strategies in this area for opportune pathogen detection and avoid a possible outbreak.

In a globalized world, where dispersal of potential vectors and pathogens is eased by human technological improvements, arthropods develop tolerance to insecticides and
pathogens acquire resistance to drugs, strategic programs should focus in multidisciplinary activities where entomological surveillance was improved. Further studies are needed to elucidate the utility of the sugar baited FTA cards for arbovirus molecular detection.

ACKNOWLEDGEMENTS
We would like to thank the European Vmerge project for their financial support; to Carles Aranda, Roger Eritja and Eva Herreros from the Servei de Control de Mosquits del Baix Llobregat for their technical and logistic support during the field work and specimen identification.

REFERENCES


Table 1: Localities’ Environmental and Socio-economic Features

<table>
<thead>
<tr>
<th>Locality</th>
<th>Environmental / Socio-economic Features</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Urban</td>
</tr>
<tr>
<td>Camps Blancs (Sant Boi de Llobregat)</td>
<td>X</td>
</tr>
<tr>
<td>Can Comas (El Prat de Llobregat)</td>
<td>X</td>
</tr>
<tr>
<td>Can Rigol (Begues)</td>
<td>X</td>
</tr>
<tr>
<td>Hospital (Martorell)</td>
<td>X</td>
</tr>
<tr>
<td>Les Tanques (Viladecans)**</td>
<td>X</td>
</tr>
<tr>
<td>Remolar (Viladecans)*</td>
<td>X</td>
</tr>
<tr>
<td>El Papiol (El Prat de Llobregat)*</td>
<td>X</td>
</tr>
</tbody>
</table>

* Locations included only from September to October 2014

** Location included from May 2015
Table 2: Feeding and Mortality Ratios observed in the FTA Standardizing Assay

<table>
<thead>
<tr>
<th>Species</th>
<th>Low 24 hours</th>
<th>Mild 24 hours</th>
<th>Low 48 hours</th>
<th>Mild 48 hours</th>
<th>Low 72 hours</th>
<th>Mild 72 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mortality</td>
<td>Feeding</td>
<td>Mortality</td>
<td>Feeding</td>
<td>Mortality</td>
<td>Feeding</td>
</tr>
<tr>
<td>Culex pipiens</td>
<td>(%)*</td>
<td>(%)*</td>
<td>(%)*</td>
<td>(%)*</td>
<td>(%)*</td>
<td>(%)*</td>
</tr>
<tr>
<td>GAVA</td>
<td>30</td>
<td>20</td>
<td>10</td>
<td>60</td>
<td>0</td>
<td>60</td>
</tr>
<tr>
<td>Aedes albopictus</td>
<td>100</td>
<td>10</td>
<td>40</td>
<td>60</td>
<td>80</td>
<td>20</td>
</tr>
</tbody>
</table>

*n=10
Table 3: Species Composition and Infection Status per Sampling Locality

<table>
<thead>
<tr>
<th>SPECIES</th>
<th>Camps Blancs</th>
<th>Can Comas</th>
<th>Can Rigol</th>
<th>Hospital Martorell</th>
<th>Les Tanques</th>
<th>Remolar</th>
<th>El Papiol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td># Pools n</td>
<td># Pools n</td>
<td># Pools n</td>
<td># Pools N</td>
<td># Pools n</td>
<td># Pools n</td>
<td># Pools n</td>
</tr>
<tr>
<td>Aedes albopictus**</td>
<td>7 (1)* 15</td>
<td>1 4</td>
<td>2 3</td>
<td>3 4</td>
<td>1 1</td>
<td>3 11</td>
<td>3 3</td>
</tr>
<tr>
<td>Aedes vexans</td>
<td>3 16</td>
<td>- -</td>
<td>- -</td>
<td>- -</td>
<td>- -</td>
<td>- -</td>
<td>- -</td>
</tr>
<tr>
<td>Coquillettidia richiardii</td>
<td>- - 3 17</td>
<td>- -</td>
<td>- -</td>
<td>- -</td>
<td>- -</td>
<td>- -</td>
<td>- -</td>
</tr>
<tr>
<td>Culex pipiens**</td>
<td>4 6 9 (1)* 21</td>
<td>3 3</td>
<td>3 4</td>
<td>5 41</td>
<td>3 16</td>
<td>1 1</td>
<td></td>
</tr>
<tr>
<td>Culiseta longiaerolata</td>
<td>- - - - -</td>
<td>- -</td>
<td>- -</td>
<td>4 14</td>
<td>1 1</td>
<td>- -</td>
<td>1 1</td>
</tr>
<tr>
<td>Ochlerotatus caspius</td>
<td>- - 1 1</td>
<td>- -</td>
<td>- -</td>
<td>- -</td>
<td>- -</td>
<td>3 38</td>
<td>- -</td>
</tr>
<tr>
<td>Phlebotomus perniciosus°</td>
<td>- 15 - 61</td>
<td>- 6</td>
<td>- 36 (1)**</td>
<td>- -</td>
<td>- -</td>
<td>- -</td>
<td>7 (1)**</td>
</tr>
<tr>
<td>Phlebotomus ariasi</td>
<td>- - - - -</td>
<td>2 -</td>
<td>- -</td>
<td>- -</td>
<td>- -</td>
<td>- -</td>
<td>- -</td>
</tr>
</tbody>
</table>

** Species with positive pools
* Number of positive pools
° Species with positive FTA card
** Number of positive FTA cards
Figure 1: Sampling Locations at the Llobregat River Delta
Barcelona Province showing sampling locations according to field capture dates. Yellow marks depict locations sampled only from September to October 2014, Orange mark depicts location sampled only from May to August 2015 and Red marks depicts locations sampled along the whole trial.

Figure 2: RT – nPCR Inhibition Assay
Aaamplification results of a) Phlebovirus RT – nPCR and b) Flavivirus RT – nPCR performed with different Manuka’s Honey concentrations.
Figure 3: RT – nPCR Plebovirus and Flavivirus Detection

Amplification results of a) M030 Culex pipiens mosquito pool with Flavivirus positive band, b) M063 Aedes albopictus mosquito pool with Flavivirus positive band and c) FTA cards T7 and T15 positive bands for Phlebovirus

M = Molecular Weight (1114 – 19bp)
CN = Negative Control
CWNV = Flavivirus Positive control
M030 = Positive mosquito pool
M063 = Positive mosquito pool
CPb = Phlebovirus Positive Control
T7 = Positive FTA card
T15 = Positive FTA card