
**Universidade Nova de Lisboa**

# FLAVIVIRUSES IN MOSQUITOES FROM SOUTHERN PORTUGAL, 2009-2010

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DISSERTAÇÃO PARA A OBTENÇÃO DO GRAU DE MESTRE EM PARASITOLOGIA MÉDICA

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**Sónia Cristina Fernandes Da Costa**

Dissertação apresentada para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Parasitologia Médica realizada sob a orientação científica de:

**Orientador: Professor Doutor Paulo Almeida**

**Co-Orientador: Professor Doutor Ricardo Parreira**

I dedicate this thesis to my family, especially

to João and Daniela who have given up

so much for me to achieve my goals.

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## ABSTRACT

Flaviviruses are viruses belonging to the *Flaviviridae* family, genus *Flavivirus*. They comprise a large group of widely spread and genetically diverse arthropod-borne viruses including human and animal pathogens that can potentially cause large-scale epidemics and high mortality and morbidity. In the past few years, flaviviruses have largely expanded their geographical distribution and host range. West Nile virus has been continuously detected throughout Europe lately and has been isolated from mosquitoes in Southern Portugal, where human and animal cases have been reported.

The main aim of this work was to search for flaviviruses in mosquitoes collected from two areas in Southern Portugal where West Nile virus and other flaviviruses have previously been detected.

Mosquito surveys were carried out in 24 locations in the wetlands of the Faro and Setúbal districts, by CDC-$CO_2$ light-traps and indoors resting collections. Pools containing approximately 50 mosquitoes were screened for flaviviruses by heminested RT-PCR, directed at the amplification of a small fragment of the viral NS5 gene, using degenerated flavivirus-specific primers.

A total of 36273 mosquitoes were collected during 2009 and 2010 from April through October, from the following species: *Anopheles algeriensis, An.atroparvus, Aedes berlandi*, *Ae. caspius*, *Ae. detritus*, *Coquillettidia richiardii*, *Culex laticinctus*, *Cx. pipiens*, *Cx. theileri*, *Cx. univittatus*, *Culiseta annulata*, *Cs. longiareolata*, *Cs. subochrea,* and *Uranotaenia unguiculata.* Most abundant species were *Ae. caspius Cx. theileri* and *Cx. pipiens*, respectively. However, mosquito densities varied according to collection method and sampling area. A fourfold increase in mosquito density was registered in 2010 compared to 2009. A total of 745 pools were analysed of which 31% tested positive for flaviviral sequences.

The species with higher positivity rates were *An. algeriensis* with Minimum infection rate (MIR) of 56/1000 in the Algarve 2009, *Cs. annulata* MIR =22/1000 in the Algarve 2010, *Cx.theileri* and *Cx.pipiens* in Setúbal 2010, MIR =20/1000. *An. atroparvus*, *Ae. caspius*, *Ae. detritus* and *Cx. univittatus* also yielded positive pools. Overall, positivity was higher in the Algarve.

Viral sequences obtained from positive pools showed homology with insect-specific flavivirus (ISF) sequences deposited in free access public databases. Phylogenetic analysis reflected the genetic variability of flaviviruses and revealed the relatedness of our sequences with other known flaviviruses, especially the insect-specific.

In view of previous WNV isolations and assessing from the four-fold increase in mosquito density, the increasing temperatures, the recent cases throughout Europe and the unknown and unpredictable pattern of flaviviruses outbreaks, continuous epidemiological surveillance programmes are quickly becoming indispensable tools for Public Health.

## RESUMO

Os flavivírus são vírus pertencentes à família *Flaviviridae*, género *Flavivirus*. Estes formam um grande grupo caraterizado pela sua ampla distribuição e diversidade genética. Os flavivírus são, na sua maioria, transmitidos por artrópodes vectores incluíndo agentes patogénicos para humanos e animais que podem potencialmente provocar grandes epidemias e causar elevadas taxas de mortalidade e morbidade. Nos últimos anos, tem-se registado uma grande expansão a nível da distribuição geográfica dos flavivírus e diversidade dos seus hospedeiros. O vírus do Nilo Ocidental tem sido continuamente detectado em toda a Europa recentemente, e também isolado de mosquitos colhidos no Sul de Portugal, onde já foram registados casos humanos e animais.

O principal objectivo deste trabalho é o rastreio de flavivírus em mosquitos colhidos em duas regiões do Sul de Portugal, onde os mesmos foram anteriormente detectados.

As colheitas de mosquitos foram realizadas em 24 locais em zonas húmidas nos districtos de Faro e Setúbal, através de armadilhas luminosas tipo CDC com $CO_2$ e aspiradores mecânicos manuais para colheita de mosquitos em repouso em abrigos de animais.

Os mosquitos colhidos foram agrupados por lotes contendo aproximadamente 50 espécimens cada, e rastreados para a presença de flavivírus por *heminested* RT-PCR, direccionado à amplificação de um pequeno fragmento do gene NS5 usando oligonucleótidos degenerados específicos para flavivírus.

Entre Abril e Outubro de 2009 e 2010 foram colhidos no total 36273 mosquitos pertencentes às seguintes espécies: *Anopheles algeriensis, An.atroparvus, Aedes berlandi, Ae.caspius, Ae.detritus, Coquillettidia richiardii, Culex laticinctus, Cx.pipiens, Cx.theileri, Cx.univittatus, Culiseta annulata, Cs.longiareolata, Cs.subochrea,* e *Uranotaenia unguiculata.* As espécies mais abundantes foram *Ae.caspius*, *Cx.theileri* e *Cx.pipiens*, respectivamente. Contudo, as densidades de mosquitos foram variáveis de acordo com o método de colheita e área de amostragem. As densidades de mosquitos colhidos em 2010 foram quatro vezes superior às registadas no ano anterior. No total foram analisados 745 lotes dos quais 31% testaram positivos para a presença de sequências de flavivírus.

As espécies que apresentaram taxas de positividade mais elevadas foram: *An.algeriensis* com uma Taxa Mínima de Infecção (TMI) de 56/1000 no Algarve em 2009, *Cs.annulata* TMI =22/1000 no Algarve em 2010, *Cx.theileri* e *Cx.pipiens* em Setúbal em 2010, TMI =20/1000. *An. atroparvus*, *Ae. caspius*, *Ae. detritus* e *Cx. univittatus* também produziram lotes positives. No geral, a positividade foi maior no Algarve.

Análise das sequências virais obtidas revelou homologia das nossas sequências virais com sequências de referência de flavivírus específicos de mosquitos depositadas em bases de dados de acesso livre. A análise filogenética reflectiu a variabilidade genética dos flavivírus e revelou a relação genética das nossas sequências com as de outros flavivírus, especialmente os específicos de insectos.

Tendo em consideração os anteriores isolamentos do vírus do Nilo Ocidental, o aumento acentuado nas densidades de mosquitos, o aumento de temperaturas que se tem vindo a registar, os casos recentes de transmissão de flavivírus por toda a Europa e o padrão desconhecido e imprevisível dos surtos destes vírus, os programas contínuos de vigilância epidemiológica têm-se revelado uma ferramenta indispensável para a Saúde Pública.

**FLAVIVIRUSES IN MOSQUITOES FROM SOUTHERN PORTUGAL,**

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**Keywords:** West Nile virus, flaviviruses, arboviruses, Algarve, flavivirus detection, insect-specific flaviviruses.

**Palavras Chave:** Vírus do Nilo Ocidental, flavivírus, arbovírus, Algarve, detecção de flavivírus, flavivírus específicos de insectos.

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| **µl** | Micro litre |
| **AeFv** | *Aedes* flavivirus |
| **bp** | Base pair |
| **BSA** | Bovine serum albumin |
| **C** | Capsid |
| **CDC** | Centres for Disease Control and Prevention |
| **cDNA** | Complementary deoxyribonucleic acid |
| **CFAV** | Cell-fusing agent virus |
| **$CO_2$** | Carbon dioxide |
| **CPE** | Cytopathic effect |
| **CSA** | Cell-silent agent |
| **CSAV** | Cell silent agent virus |
| **CxFv** | *Culex* flavivirus |
| **DENV** | Dengue virus |
| **DEPC** | Diethylpyrocarbonate |
| **DF** | Degrees of freedom |
| **DHF** | Dengue haemorrhagic fever |
| **DNA** | Deoxyribonucleic acid |
| **DSS** | Dengue shock syndrome |
| **E** | Envelope |
| **ER** | Endoplasmic reticulum |
| **g** | Grams |
| **IR** | Indoor resting |

| | |
|---|---|
| **ISF** | Insect-specific flavivirus |
| **JEV** | Japanese encephalitis virus |
| **KDFV** | Kyasanur Forest Disease virus |
| **KRV** | Kamiti River virus |
| **LAMV** | Lammi virus |
| **LIV** | Louping ill virus |
| **M** | Membrane |
| **MBV** | Mosquito-borne viruses |
| **MIR** | Minimum infection rate |
| **ml** | Millilitre |
| **MVEV** | Murray Valley encephalitis virus |
| **NAKV** | Nakiwogo virus |
| **NCR** | Non-coding region |
| **NKV** | No-known vector |
| **nm** | Nano metre |
| **NOUV** | Nounané virus |
| **NS** | Non-structural |
| **nt** | Nucleotide |
| **ºC** | Degrees Celsius |
| **OHFV** | Omsk haemorrhagic fever virus |
| **ORF** | Open reading frame |
| **PBS** | Phosphate buffered saline |
| **PCR** | Polymerase Chain Reaction |
| **pmol** | Pico moles |
| **POWV** | Powassan virus |

| | |
|---|---|
| **prM** | Precursor M protein |
| **QBV** | Quang Binh virus |
| **RBV** | Rio Bravo virus |
| **RFV** | Royal Farm virus |
| **RNA** | Ribonucleic acid |
| ***spp*** | Species |
| **TABV** | Tamana bat virus |
| **TBEV** | Tick-borne encephalitis virus |
| **TBV** | Tick-borne viruses |
| **TGN** | Trans-Golgi network |
| **USA** | United States of America |
| **UTR** | Untranslated region |
| **UV** | Ultraviolet |
| **WHO** | World Health Organisation |
| **WNV** | West Nile virus |
| **YFV** | Yellow fever virus |

# 1.

# INTRODUCTION

# 1. Introduction

## 1.1. Arboviruses

The term arbovirus originated in the 1940's as the result of the abbreviations made to describe the viruses transmitted by arthropods (arthropod-borne viruses) (Kuno and Chang, 2005). Arboviruses are recognised as an extremely diverse group that harbours many medically important viruses (table 1), which can cause serious disease such as yellow fever, dengue and several encephalitis (Pabbaraju *et al*., 2009). They are included mainly into three viral families: *Flaviviridae*, *Togaviridae* and *Bunyaviridae* (Pabbaraju *et al*., 2009).

**Table 1:** Medically important arboviruses belonging to families *Flaviviridae*, *Togaviridae* and *Bunyaviridae* (adapted from Gubler, 2002).

| Virus | Human disease | Reservoir host | Arthropod vector | Geographic distribution |
|---|---|---|---|---|
| ***Flaviviridae*** | | | | |
| Yellow fever virus (YFV) | Yellow fever – haemorrhagic fever | Primates, humans | Mosquito | Africa, America |
| Dengue virus 1-4 (DENV) | Dengue haemorrhagic fever/ shock syndrome | Humans, primates | Mosquito | Africa, America, Asia |
| Japanese encephalitis virus (JEV) | Encephalitis | Birds | Mosquito | Asia |
| Saint Louis encephalitis virus (SLEV) | Encephalitis | Birds | Mosquito | North America |
| West Nile virus (WNV) | FAR syndrome, encephalitis | Birds | Mosquito | Worldwide |
| Murray Valley encephalitis virus (MVEV) | Encephalitis | Birds | Mosquito | Australia |
| Tick-borne encephalitis virus (TBEV) | Encephalitis | Small mammals, rodents, birds | Ticks | Europe, Asia |
| ***Togaviridae*** | | | | |
| Chikungunya virus | FAR syndrome | Primates, humans | Mosquito | Africa, Asia, Europe |
| Ross River virus | FAR syndrome | Marsupials | Mosquito | Australasia |
| Sindbis virus | Fever/Rash | Birds | Mosquito | Africa, Asia, Australia, Europe |
| O'Nyong nyong | Fever | Unknown | Mosquito | Africa |
| Equine encephalitis viruses (EEV,WEV) | encephalitis | Passerine birds | Mosquito | America |
| ***Bunyaviridae*** | | | | |
| Bunyamwera | Fever | Rodents | Mosquito | Global |
| California encephalitis virus | Encephalitis | Mammals | Mosquito | North America |
| La Cross virus | Encephalitis | Mammals | Mosquito | North America |
| Tahyna virus | Fever, respiratory disease, encephalitis | Mammals | Mosquito | Asia, Europe |

FAR – fever/arthralgia/rash

These viruses are, thus, transmitted by arthropod vectors via a biological process, which can occur vertically or horizontally (Kuno and Chang, 2005; Weaver and Reisen, 2010). For viruses to be biologically transmitted they must replicate in the arthropod vector prior to transmission (Kuno and Chang, 2005; Goddard, 2008; Weaver and Reisen, 2010). In vertical transmission, the virus is passed on from the female to both male and female offsprings by trans-ovarial or trans-stadial transmission (Kuno and Chang, 2005; Weaver and Reisen, 2010). In turn, horizontal transmission can occur venereally (where the virus is passed on from infected males directly onto females, when mating), or orally. This is the most typical arboviral transmission mode, which involves the infection of a susceptible arthropod vector after ingestion of viruses during feeding or, from maternal origin. Viruses are subsequently disseminated within the arthropod, replicate in the salivary glands, ensuring that transmission might occur during the following bloodmeal, through injection of contaminated saliva in a susceptible host (Kuno and Chang, 2005; Weaver and Riesen, 2010).

Furthermore, not all infected arthropods are capable of pathogen transmission. For it to occur it must be competent for transmission, that is, it must be susceptible to infection by the pathogen, allowing the above mentioned replication and dissemination thus becoming infective, and able to transmit the pathogen via an infective bite when blood-feeding (Goddard, 2008; Weaver and Reisen, 2010). Arboviral transmission can only take place where the three principal elements are present: the virus, the vector and vertebrate hosts (figure 1).

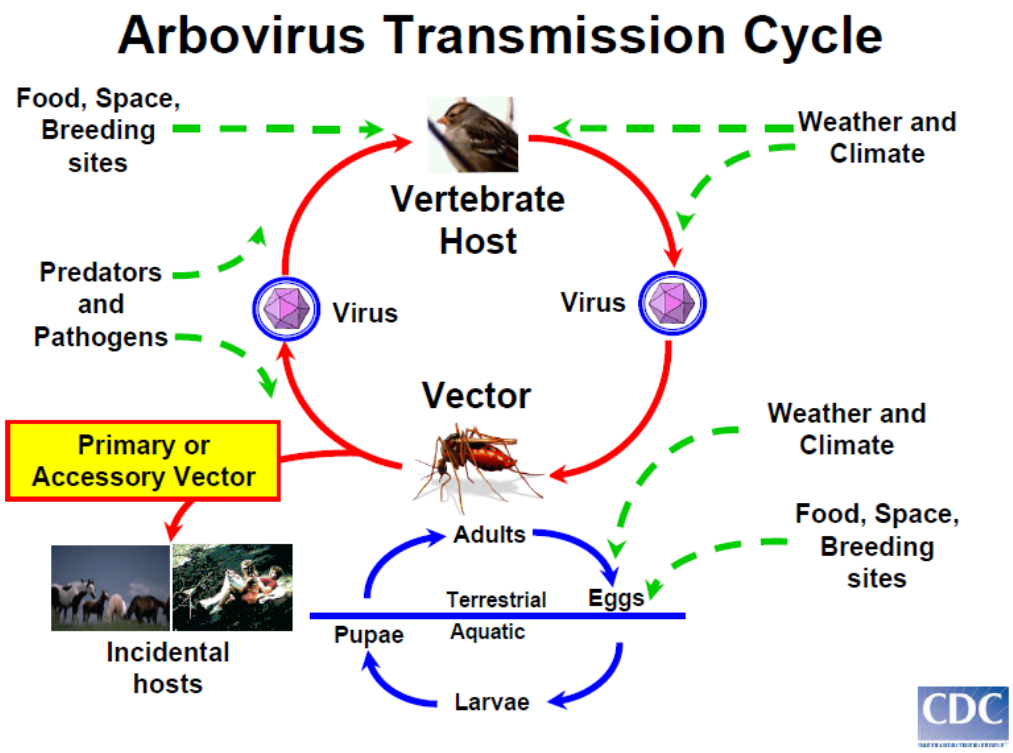


**Figure 1:** Arbovirus transmission cycle **(http://www.cdc.gov/ncidod/dvbid/arbor/schemat.pdf - accessed on the 4th of November 2011).**

Transmission of arboviruses appears to be seasonal, depending on vector and reservoir densities, as well as on climatic variables that affect the former (Hollidge *et al*., 2010). For example, despite the fact that in tropical or endemic regions the transmission occurs all year round, there are still seasonal peaks of activity that frequently coincide with the rainy seasons, and leads to high mosquito densities (Hollidge *et al*., 2010).

In temperate regions, a higher arboviral activity is usually observed during the summer months, since most vectors are not able to survive the cold winter months or do so in hibernation (Hollidge *et al*., 2010). However, due to the raise in temperatures resultant from climate change and other risk factors such as flooding, these arboviruses are conquering new geographical areas, placing many new regions at risk to the introduction of emerging and re-emerging diseases (Aranda *et al*., 2009).

### 1.1.2. Medical importance of mosquitoes (Diptera: Culicidae)

The *Culicidae* family is very important from a human and veterinary medical perspective since it harbours a large number of species, including some of the most important hematophagous arthropods capable of transmitting infectious agents (Eiras, 2004; Eldridge, 2005). This family comprises over 3,500 mosquito species and subspecies, belonging to two medical important subfamilies: the *Anophelinae* and *Culicinae*. (Eldridge, 2005). Mosquitoes included in these subfamilies are capable of transmitting arboviruses such as the dengue and yellow fever viruses as is the case of *Aedes aegypti* and *Ae. albopictus*; moreover, *Culex* species mosquitoes that can transmit West Nile virus, Saint Louis encephalitis and Rift Valley fever viruses are also part of the *Culicinae* subfamily (Manson-Bahr and Bell, 1987; Eldridge, 2005). Apart from viruses, mosquitoes can also transmit nematode worms and protozoa (Rutledge, 2008).

The mosquito life cycle comprises four distinct stages: the egg, the larvae, pupae and adult (figure 2), as they go through complete metamorphosis (Eldridge, 2005; Rutledge, 2008)

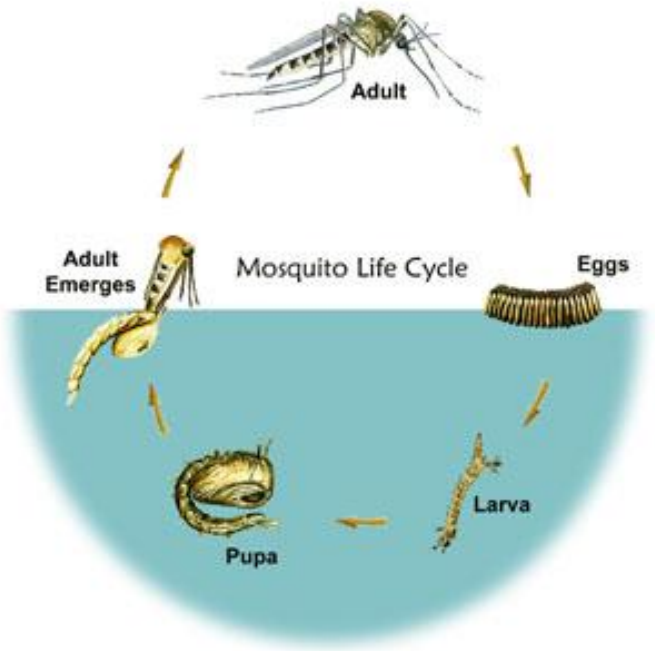


**Figure 2:** Mosquito life cycle (Diptera: Culicidae) **(http://www.osceola.org/mosquitocontrol/129-6426-0/mosquito_life_cycle.cfm – accessed on the 12th of October 2011).**

The immature stages of mosquitoes, egg, larvae and pupae, are aquatic while the adult stage is the only that is terrestrial (Rutledge, 2008). Mosquito biology is highly dependent on climatic conditions, particularly temperature variations, and in warmer temperatures, the life cycle may take 10 days or less to complete (Eldridge, 2005). Eggs may be deposited in water surface or moist ground, in groups or individually, and hatching occurs either in a day or so or when flooding occurs (Eldridge, 2005; Rutledge, 2008). A larva must undergo four molts to become a pupa; though their time of development has no direct relation to water temperatures (Eldridge, 2005). Adult emergence usually happens 1-3 days after pupa formation (Eldridge, 2005; Rutledge, 2008). Mosquito distribution is determined by the climatic conditions, hence their permanent existence in tropical, warm and humid climates, temperate countries and in cooler climate countries (e.g. northern Europe) (Rutledge, 2008). Therefore, changes in these climatic conditions may forcibly change their bioecology.

Mosquito vectors of flaviviruses, including arboviruses, are autochthonous in many European countries, including Portugal, where due to beneficial climatic and ecological conditions there is production of very high-density populations in some areas, especially in the Mediterranean Basin (Almeida *et al*., 2008). This is the case of *Aedes* and *Culex* species mosquitoes, in wetlands, estuarine regions and manmade and natural farming lands all across Europe. Many mosquitoes established in Portugal include potential vectors of WNV (e.g. *Aedes caspius*, *Cx.theileri*, *Cx. pipiens* and *Cx. univitattus*), Sindbis and Rift Valley fever viruses (e.g. *Cx.theileri* and *Aedes caspius*), Chikungunya and Tahyna viruses (*Aedes caspius*), and it is only a matter of time and viral pathogen seasonality, until arboviral activity is detected (Jupp *et al*., 1972; McIntosh *et al*., 1980; Jupp *et al*., 1985; Turell *et al*., 1996; Hubalek and Halouzka, 1999; Lundstrom, 1999; Vazeille *et al*., 2008).

### 1.2. The *Flaviviridae* family

Flaviviruses are viruses belonging to the *Flaviviridae* family, genus *Flavivirus*. The *Flaviviridae* is a large, widely spread and genetically diverse family of viral agents, that includes human and animal pathogens that can potentially cause large-scale epidemics and tens of thousands of deaths annually (Cook *et al*., 2003; Mukhopadhyay *et al*., 2005). This family comprises four genera:

- The *Pestivirus* genus (derives from the Latin word "pestis" which means "plague") that includes four viruses, namely border disease virus, bovine viral diarrhoea viruses 1 and 2, and classical swine fever virus (Lindenbach *et al*., 2007);

- The *Hepacivirus* genus (derives from the Greek words "hepar" and "hepatos", which mean "liver") the sole member of which is the Hepatitis C virus (Lindenbach *et al*., 2007);

- The *Flavivirus* genus (derives from the Latin word "flavus", that means "yellow") which is the largest genus and contains more than 70 RNA viruses (Mukhopadhyay *et al*., 2005; Cook and Holmes, 2006; Lindenbach *et al*., 2007; Cook *et al*., 2009);

- The *Pegivirus* genus ("pe" from the word persistent and "g" from GB or G) which includes the GB viruses (variants A, B, C and D) and Hepatitis G virus (HGV) (Lindenbach *et al*., 2007; Stapleton *et al*., 2011).

The worldwide spread flaviviruses comprises over seventy recognised RNA viruses including many that are responsible for epidemics and high mortality rates among humans (Cook *et al*., 2006; Cook *et al*., 2009; Junglen *et al*., 2009; Huhtamo *et al*., 2009; Monini *et al*., 2010). At least 30 members of the genus *Flavivirus* are regarded as medically important since they can cause serious human disease, including haemorrhagic fever and encephalitis (Sanchéz-Seco *et al*., 2005; Hoshino *et al*., 2009). Nonetheless, the clinical condition of the infected individual is not always life threatening since it may present itself as a mild febrile illness or be completely asymptomatic, as most infections are (Sanchéz-Seco *et al*., 2005; Hoshino *et al*., 2009). Approximately 30% of flaviviruses are not known to have vertebrate hosts and are,

therefore, considered to be insect-specific (Morales-Betoulle *et al*., 2008; Blitvich *et al*., 2009). Furthermore, there are also some members of the genus *Flavivirus* for which no vector is known, designated by no-known vector (NKV) viruses (Cook *et al*., 2006; Blitvich *et al*., 2009).

### 1.3. The importance of Flaviviruses

Viruses belonging to genus *Flavivirus* represent some of the most important emerging or re-emerging pathogenic agents that cause disease in humans (Solomon and Mallewa, 2001). The expanding distribution of these viruses is directly related to the spread and extension of vector distribution within an environment that provides beneficial conditions for vector maintenance and establishment, as well as the presence of vertebrate hosts (Weaver and Reisen, 2010).

The major factors that contribute to geographical dispersal of arboviral diseases include human activity, genetic and environmental changes. These include (Petersen and Marfin, 2005; Gould and Solomon, 2008; Weaver and Reisen, 2010):

- The ability of the RNA viruses to undergo rapid genetic alterations that allow them to adapt more easily to virtually any hosts, vertebrate or invertebrate, under changing climate conditions;
- Population growth and urbanisation;
- Increased travel and commercial transportation around the world;
- The receptivity of an area to viral emergence;
- Invasion of vector natural habitats;
- Lack of vaccination and/or effective vector control programmes in endemic areas possibly due to economic or political issues.

#### 1.3.1. *Flavivirus*: genome structure and morphology of the viral particle

The flavivirus genome, of approximately 11 kilobases in length, is a single stranded, positive polarity RNA molecule that encodes three structural (capsid [C], membrane

[M] and envelope [E]) and seven non-structural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5), as shown on figure 3 (Sanchéz-Seco *et al*., 2005; Harris *et al*., 2006; Hoshino *et al*., 2009).

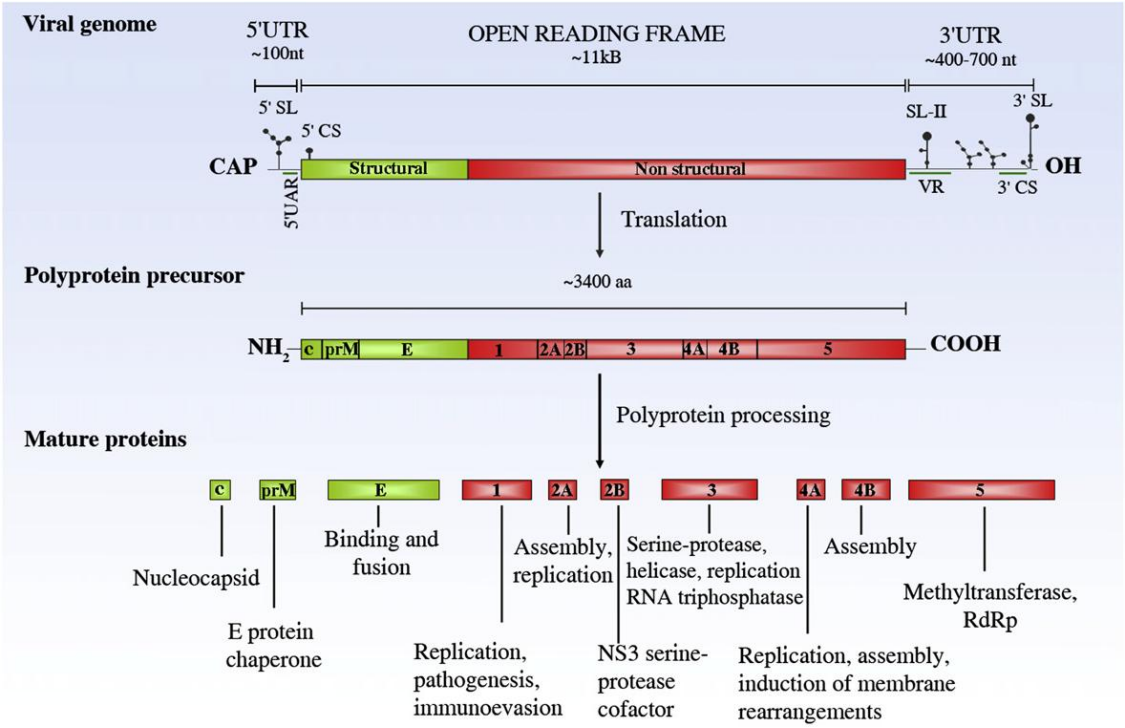


**Figure 3:** *Flavivirus* genome structure and functions of viral proteins

**(Fernandez-Garcia *et al*., 2009)**.

Flavivirus genomes encode a large polyprotein and contain a single open reading frame (ORF) flanked by two non-coding regions (NCR) - the NCR of 5' end is approximately 100 nucleotides (nt) in length, whereas the NCR at the 3' end is between 400 and 700 nucleotides long (Lindenbach *et al*., 2007). Viruses within this genus are small (around 50 nm), spherical particles that contain an electron dense core (approximately 30 nm) surrounded by a lipid envelope formed from membranes (derived from the endoplasmic reticulum) of host cells (Barrett, 2001; Lindenbach *et al*., 2007). The virions present a

complex structure that contains a nucleocapsid, which is hexahedral/icosahedral in symmetry as shown in figure 4 (Carter *et al*., 2008).

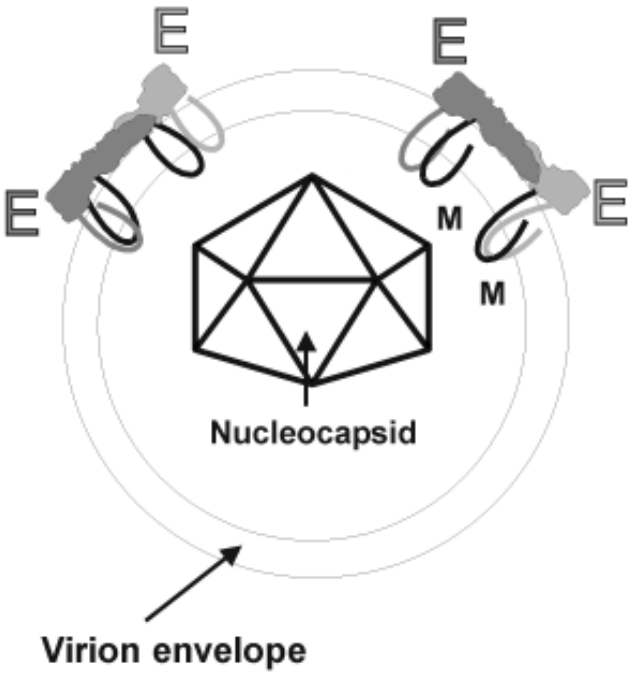


**Figure 4:** The flavivirus virion **(Petersen *et al*., 2001)**.

Flavivirus virions contain two structural proteins at its surface: the E protein (the envelope glycoprotein that surrounds the nucleocapsid) and the M protein (Barrett, 2001; Lindenbach *et al*., 2007). In addition, surrounding the single stranded viral RNA genome is a small capsid protein that has a basic charge in order to interact with the genome (Barrett, 2001).

The envelope glycoprotein E is the major antigenic determinant for the production of neutralizing antibodies that relate to a protective immune response (McMinn, 1997). Furthermore, it mediates receptor binding and membrane fusion, through its cellular receptor binding sites and fusion peptides (Lindenbach *et al*., 2007).

These virions can exhibit two different forms, the mature and the immature virions (figure 5); the mature virions are extracellular and contain the M (membrane) protein, whereas the immature ones are intracellular and contain precursor M protein (prM) that is cleaved by proteolysis to produce the M protein (Barrett, 2001).

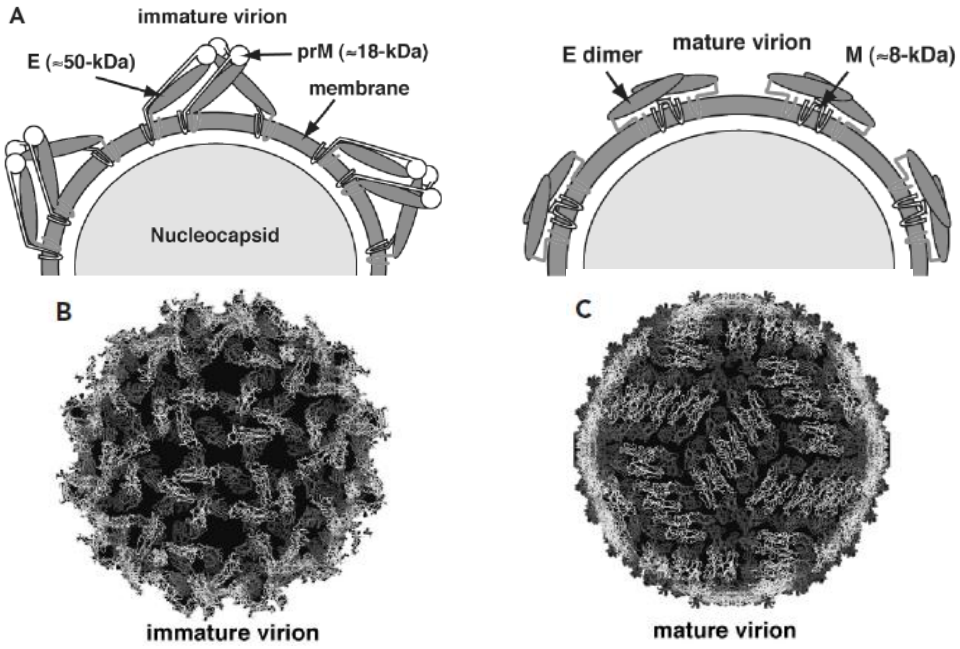


**Figure 5:** Flavivirus particle structure. A- Envelope proteins of immature and mature virions; B- Immature dengue virus type-2 virion; C- Mature dengue virus type-2 virion **(adapted from Lindenbach *et al.*, 2007)**.

### 1.3.2. Replication cycle

The molecular biology of flavivirus has been actively studied, as the *Flaviviridae* family comprises many viruses with major medical importance. A schematic representation of the viral replication cycle (Lindenbach and Rice, 2003) is shown in figure 6.

Flavivirus virions adhere to the surface of the host cell and enter it by means of receptor-mediated endocytosis via attachment to high affinity cellular receptors specific for viral envelope proteins (unknown for most viruses) (Mukhopadhyay *et al*., 2005; Harris *et al*., 2006; Lindenbach *et al*., 2007). The low pH of the endosomal vesicles triggers the particles to undergo conformational changes and induces viral fusion with host cell membranes and virus disassembly, causing the uncoating and release of the virus nucleocapsid into the cytoplasm (Lindenbach and Rice, 2003; Mukhopadhyay *et al*., 2005; Harris *et al*., 2006; Lindenbach *et al*., 2007). It is thought that viral genomes are available for translation immediately after membrane fusion. Thus, once the viral genome is released into the cytoplasm, the RNA molecule is translated into a single

polyprotein that is cleaved by viral and host proteases (Mukhopadhyay *et al*., 2005; Lindenbach *et al*., 2007).

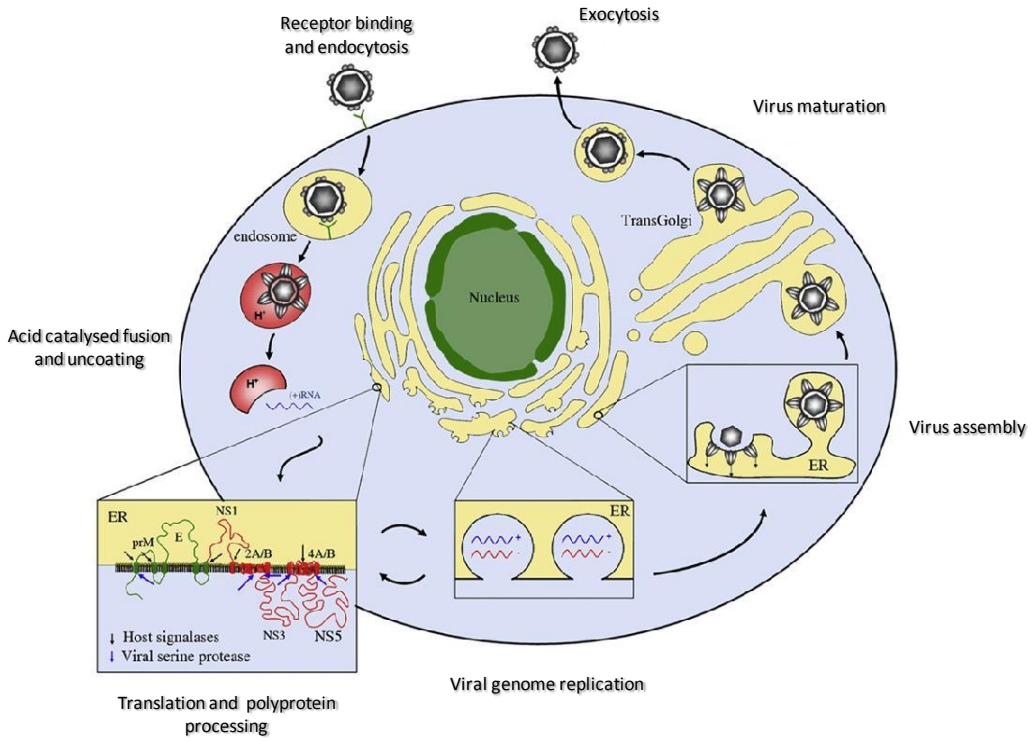


**Figure 6:** *Flavivirus* replication cycle **(adapted from Fernandez-Garcia *et al*., 2009)**.

The translation process yields proteins that play an important role in the replication of the viral genome and the formation of new virus particles (Lindenbach and Rice, 2003). Genome replication takes place on intracellular membranes, more specifically on cytoplasm replication complexes associated with perinuclear membranes (Lindenbach and Rice, 2003; Lindenbach *et al*., 2007). Virions are thought to assemble by budding into an intracellular membrane compartment, in the lumen of the endoplasmic reticulum (ER), resulting in the formation of immature non-infectious viral particles containing E and prM proteins, nucleocapsid and lipid membrane thus making them unable to induce host-cell fusion (Mukhopadhyay *et al*., 2005; Lindenbach *et al*., 2007). These immature non-infectious viral particles are transported through the trans-Golgi network (TGN) where cleavage of the prM protein occurs, by the host protease furin, thus creating

mature infectious virions (Mukhopadhyay *et al*., 2005). Finally, the mature virions are ready to be released from the host cell by exocytosis (Mukhopadhyay *et al*., 2005).

### 1.3.3. Flavivirus classification: phylogeny

The genus *Flavivirus* is unique in the *Flaviviridae* family since, contrarily to genus *Hepacivirus* and *Pestivirus*, its members present the ability to infect and replicate in vertebrate and invertebrate host cells and display genetic, epidemiological and ecological characteristics that are distinct from the other two genera (Gould *et al*., 2003). Even though flaviviruses are known to be related, based on phylogenetic and antigenic analysis, the members of this genus characteristically present high genetic divergence thus emphasizing that these correlations are not simple, nor are they always clear (Kuno *et al*., 1998; Gaunt *et al*., 2001; Gould *et al*., 2003; Cook and Holmes, 2006). The evolutionary process may have contributed greatly to the genetic diversity and divergence among these viruses, through factors such as the gradual adaptation to new hosts, new geographical areas and genetic alterations, which might still be occurring these days (Cook and Holmes, 2006).

The genus *Flavivirus* is considered to be an invaluable model for the evolutionary investigation of vector-borne diseases and their modes of transmission, since it has been confirmed by many publications that the viral transmission mode is strongly related to virus phylogeny (Gaunt *et al*., 2001; Cook and Holmes, 2006). In order to understand the origin, and spread patterns of emerging and re-emerging diseases, it is essential to gather valuable knowledge by the analysis of the evolutionary history of flaviviruses (Kuno *et al*., 1998; Gaunt *et al*., 2001). Genetic characteristics were typically investigated based on antigenic cross reactivity, haemagglutination and complement fixation tests (Gaunt *et al*., 2001). More recently, investigators have implemented the use of other tools such as molecular sequencing, and phylogenetic data analysis, in order to understand and solve taxonomic issues, thus allowing viruses to be correctly assigned positions within their respective genus (Gaunt *et al*., 2001).

Phylogenetic analysis of flaviviruses commonly separates the genus into three different groups, namely the mosquito-borne viruses, the tick-borne viruses and the no-known

vector viruses (NKV) (figure 7). Moreover, these groups of viruses can be subdivided into several clades determined by their epidemiology, clinical condition caused, type of preferred host and their geographic distribution (Kuno *et al*., 1998; Gaunt *et al*., 2001; Gould *et al*., 2003; Cook and Holmes, 2006).

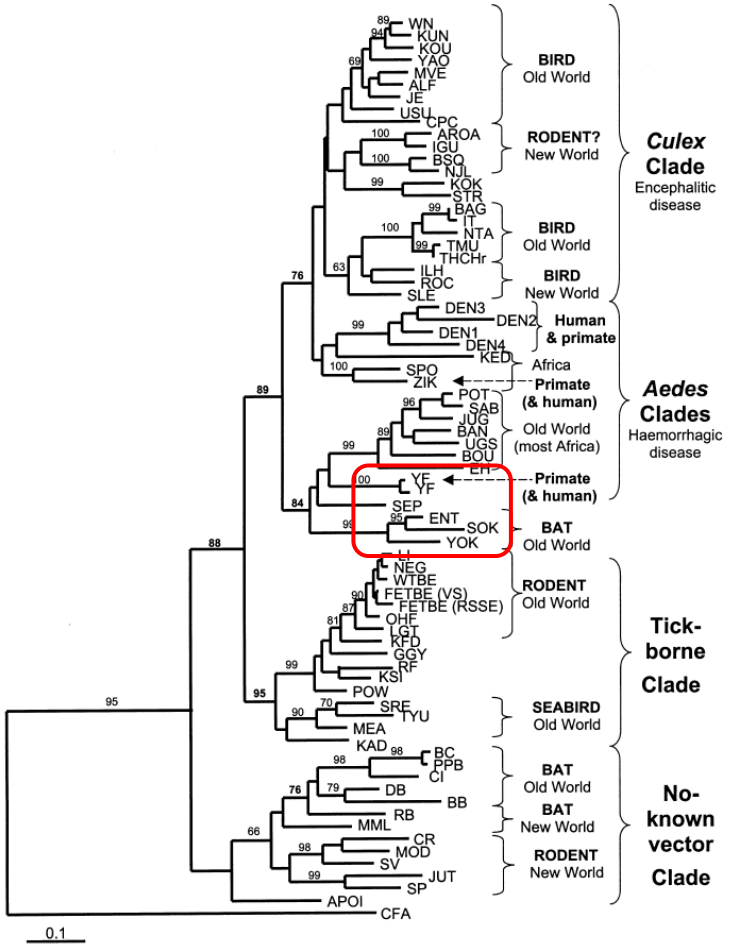


**Figure 7:** Phylogeny of the Flavivirus genus based on analysis of the NS5 gene

**(adapted from Gaunt *et al*., 2001)**.

Many previous studies have found their phylogenetic results to be questionable due to inconsistency in the positions occupied by the three main transmission groups; this is thought to originate mainly from taxonomic and virus nomenclature errors (Kuno *et al*., 1998; Cook and Holmes, 2006). The taxonomy of flavivirus is misunderstood and unclear as can be perceived by the confusion regarding misplacements of some viruses that clearly present flavivirus-type characteristics. Such are the cases of the yellow fever

virus and many others that are not incorporated into any of the serological complexes that flaviviruses are divided into (Kuno *et al*., 1998). For instance, in the study carried out by Kuno *et al* (1998), phylogenetic analysis of sequences obtained from the E and NS5 genes yielded conflicting results. Despite the similarities in the overall division and positioning of viruses, there were some exceptions, as was the case for some bat-related viruses that were included in the mosquito-transmitted clade, grouped together with yellow fever and Sepik viruses (figure 7, red box) (Kuno *et al*., 1998; Gaunt *et al*., 2001).

To the contrary, there are also viruses that do not display clear relationships to other members and are placed within this genus (Kuno *et al*., 1998). Tamana bat virus and cell-fusing agent are good examples of "candidate" viruses occupying uncertain positions within the genus *Flavivirus* (Gould *et al*., 2003). Despite many attempts, the doubts and uncertainty surrounding the flaviviruses phylogenetic relationships still linger on (Cook and Holmes, 2006).

Flavivirus classification through molecular genetics has been undertaken using different methods and targeting different viral genes (Gould *et al*., 2003; Cook and Holmes, 2006). Viral sequences obtained from the envelope (E) gene, NS3, NS4, and NS5 have been used for phylogenetic inference. However, recent work has been mainly directed at the analysis of the most conserved region of the flavivirus genome (the NS5 coding sequence), as well as their complete sequence (Cook and Holmes, 2006).

The analysis of sequences gathered from distinct genes indicates an important separation between the mosquito-borne viruses from those transmitted by ticks (Cook and Holmes, 2006). According to the study carried out by Kuno *et al* (1998), the non-vectored and vector-borne virus groups were the first to have emerged, assuming cell-fusing agent virus (CFAV) as sharing a common ancestor with which other members of the genus *Flavivirus*. Following these events, the vector-borne group was split into tick-borne and mosquito-borne clades (Kuno *et al*., 1998; Cook and Holmes, 2006). Based on phylogenetic analysis of the envelope gene, it is hypothesized that CFAV may have diverged from flavivirus prior to the separation between mosquito and tick-borne groups, thus forming a basal lineage (Sang *et al*., 2003).

Furthermore, other associations have resulted from this type of analysis, as is the case of the mosquito-borne clade that was subdivided into a further two groups according to the mosquito vector species, their vertebrate hosts and the nature of the disease caused (Kuno *et al*., 1998; Gaunt *et al*., 2001). As shown on figure 7, the mosquito-borne viruses were clearly separated into two clades: the viruses isolated from *Aedes* species and those from *Culex* spp. (Gaunt *et al*., 2001). Besides, when other characteristics such as the sort of disease caused were included in the analysis, the generated tree revealed a solid association between the *Culex* clade and the neurotropic viruses causing encephalitic disease; and between the *Aedes* clade and the non-neurotropic viruses that tend to produce hemorrhagic disease (Gaunt *et al*., 2001; Gould *et al*., 2003). In addition, correlations were established based on host preference, linking *Culex* species mosquitoes with birds, and *Aedes* mosquitoes with primates (Gaunt *et al*, 2001; Gould *et al*., 2003).

Although no-known vector (NKV) viruses have not been thoroughly examined, the results from many studies tend to support the hypothesis that they have evolved away from the vector-borne group of viruses (Kuno *et al*., 1998; Gaunt *et al*., 2001; Gould *et al*., 2003; Cook and Holmes, 2006). Sequence analysis of the NS5 gene produces a phylogenetic tree that separates the NKV group into three, the rodent and bat clades and a "basal lineage" composed of an individual virus – the APOI virus that is linked to rodents and therefore would later be added to the rodent-borne clade (Kuno *et al*., 1998; Gaunt *et al*., 2001). However, as mentioned before, some members of this group were included into the mosquito-borne clade, which suggests that during the flavivirus evolution, and following the divergence of the NKV viruses from the vector-borne viruses, there was a consequent loss of vector-borne transmission (Gould *et al*., 2003; Cook and Holmes, 2006). Phylogenetic analysis was not conclusive as to which group was the most divergent (Gould *et al*., 2003). Contrarily, more recently it was concluded that the NKV viruses group is indeed the most divergent of the three main flavivirus groups (Cook and Holmes, 2006).

The tick-borne group of viruses is composed of two distinct clades (figure 8), of which one includes flaviviruses that infect seabirds and the other contains the mammalian tick-borne viruses (Kuno *et al*., 1998; Gaunt *et al*., 2001; Gould *et al*., 2003; Cook and

Holmes, 2006). The latter is said to be linked to rodent hosts and *Ixodes* spp. ticks in woodland areas (Gaunt *et al*., 2001; Gould *et al*., 2003). With the exception of Omsk haemorrhagic fever virus (OHFV) and Kyasanur Forest disease virus (KFDV) that tend to cause haemorrhagic disease, infections by flaviviruses within this group primarily produces encephalitic disease (Gaunt *et al*., 2001).

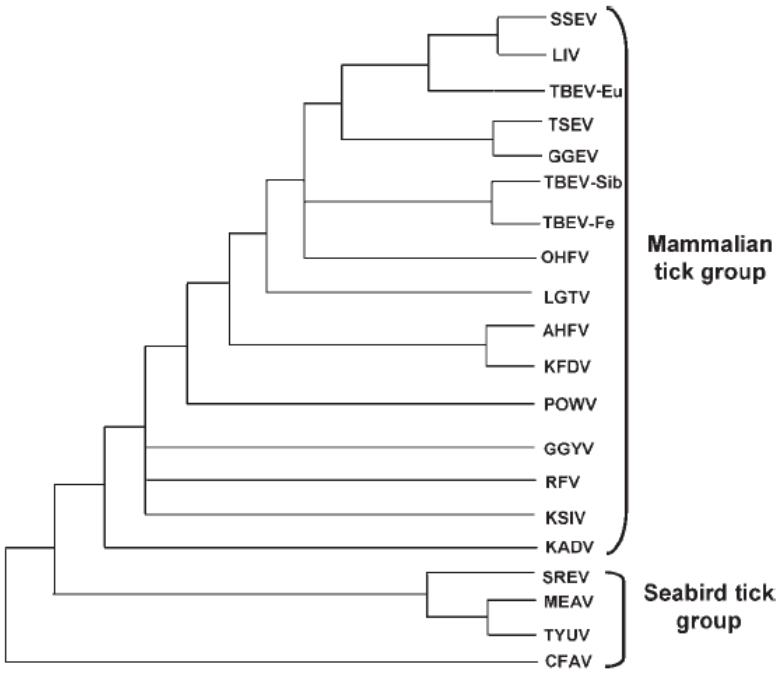


**Figure 8:** Phlylogenetic tree of tick-borne flaviviruses based on the analysis of NS3 gene **(Mansfield *et al*., 2009)**.

### 1.4. Medically Important Pathogenic Flaviviruses

Included into the *Flavivirus* genus are some of the most virulent and medically important viruses known to medical science including, amongst others, the Dengue viruses (DENV- 1, DENV- 2, DENV- 3 and DENV- 4), Yellow Fever virus (YFV) and West Nile virus (WNV) (Harris *et al*., 2006; Junglen *et al*. 2009; Calzolari *et al*., 2010). The distribution of these viruses has been increasing geographically and, over the years, new host populations have been targeted (Junglen *et al*., 2009).

Hypothetically, in case of continued *Ae. albopictus* geographic expansion (especially northwards) and *Aedes aegypti* reestablishment, combined with changes to ecological conditions as a consequence of climate change, there may be a risk of eventual pathogenic flaviviruses transmission in Europe (Reiter, 2010). This may be the case of DENV and YFV since the historical records of these diseases in the continent confirm that the conditions existent are suitable for their transmission (Reiter, 2010).

### 1.4.1. Dengue and Yellow Fever

#### 1.4.1.1. Epidemiology

Viruses of the genus *Flavivirus* are the causative agents of both dengue and yellow fever, which are associated with *Aedes spp* mosquitoes and cause haemorrhagic disease in humans and simians (Endy *et al*., 2010; Reiter, 2010). These viruses are considered the most important arboviral pathogens that, for a long time, have been causing disease around the world (Endy *et al*., 2010; Weaver *et al*., 2010). Infection by DENV and YFV can be manifested as a mild or potentially fatal disease, and it can take a mere seven to ten days of disease before death occurs (Gould and Solomon, 2008; Endy *et al*., 2010; Weaver and Reisen, 2010).

The main factors that contributed to the great geographical dispersal of *Aedes aegypti* and *Aedes albopictus*, the principal vectors of dengue and yellow fever, include the commercial transportation of goods and people during the slave trade, particularly by ship (Endy *et al*., 2010). This resulted in the evolution and adaptation of these vectors to urban environments and, consequently, in epidemics affecting particularly port cities (Reiter, 2010; Weaver and Reisen, 2010). Northern cities such as Dublin (Ireland) and Boston (North America) were unexpectedly hit by these epidemics (Reiter, 2010).

The risk of contracting dengue threatens more than 2.5 billion individuals, in tropical and subtropical countries, causing 50-100 million new infections, 500,000 hospitalisations, and 24,000 reported deaths, per year (Samuel and Tyagi, 2006; Gould and Solomon, 2008; Weaver and Reisen, 2010). The World Health Organization (WHO) confirmed that a hundred countries around the globe are endemic for dengue disease (figure 9) (Endy *et al*., 2010).

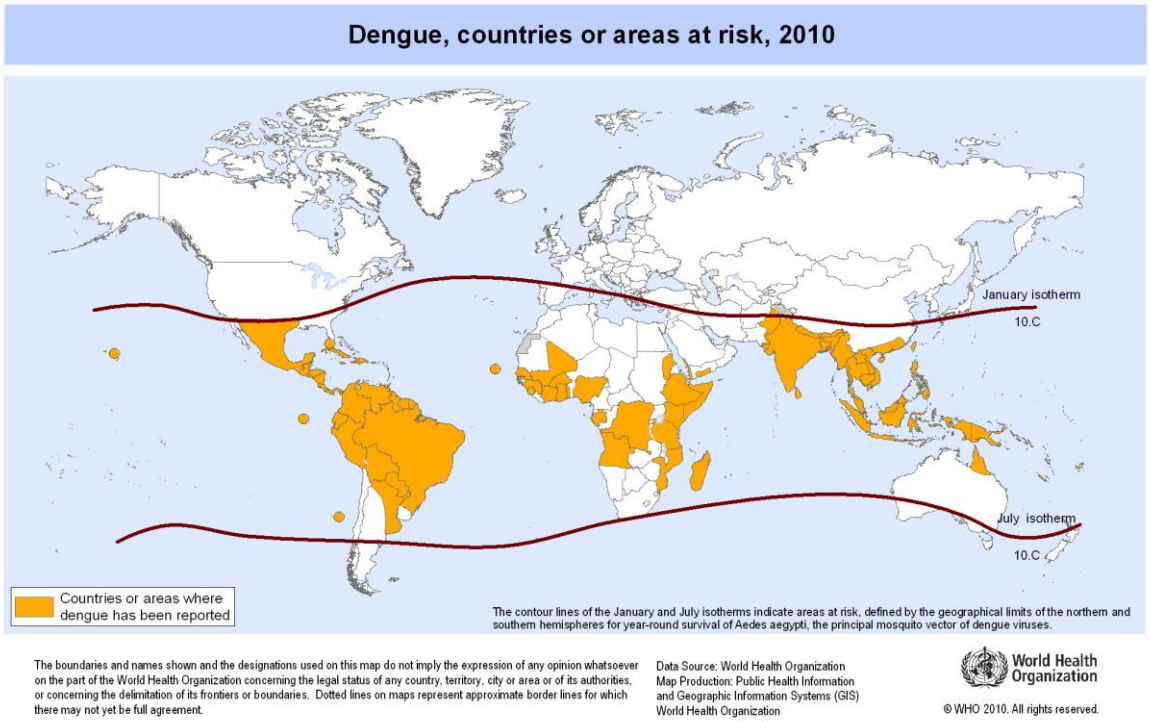


**Figure 9**: Dengue risk areas, 2010 **(**http://www.wpro.who.int/health_topics/dengue/ – **accessed on the 5th July 2011).**

Dengue is an emerging disease that has shown dramatic geographic and incidence increase in the past few decades, and has caused large epidemics in the Americas in 2007 (890,000 cases) and in Greece in 1927-28 (over one million cases) (Endy *et al*., 2010; Reiter, 2010). Recently, an outbreak of dengue was reported in Cape Verde, affecting thousands of people though not causing as many fatalities (DREF, 2009). According to WHO, the yellow fever virus is responsible for approximately 200,000 clinical cases of haemorrhagic fever resulting in around 30,000 deaths per year, occurring mainly in South America and Sub-Saharan Africa (figure 10) (Petersen and Marfin, 2005; Gould and Solomon, 2008).

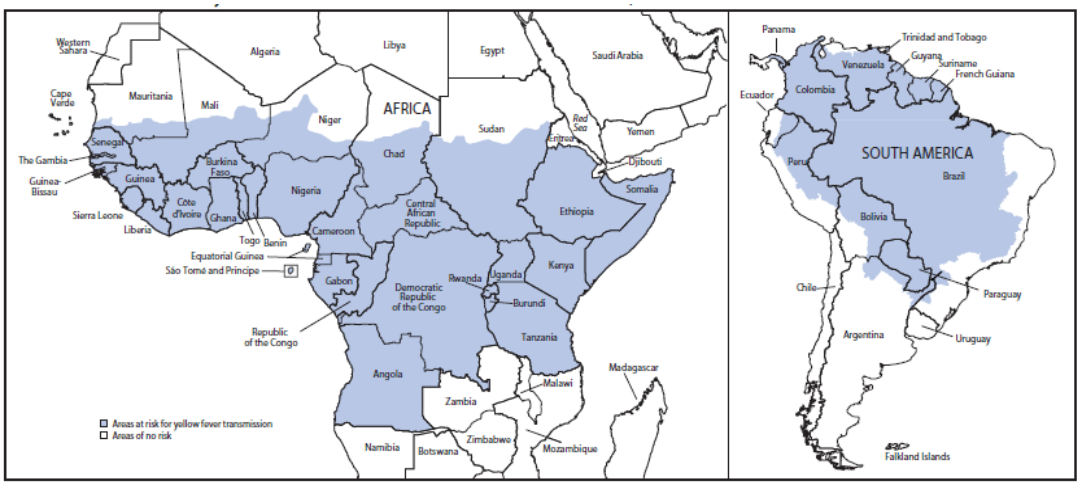


**Figure 10:** Geographic distribution of yellow fever**.**
**(http://www.cdc.gov/mmwr/preview/mmwrhtml/rr5907a1.htm – accessed on the 7th of July 2011)**.

Reports state that the true incidence of this haemorrhagic disease is unknown, and the real figure is thought to be far higher (Petersen and Marfin, 2005). Despite the number of people affected, this virus is the only flavivirus for which a vaccine is available internationally, and nevertheless, it may be of too high a cost for endemic countries to create a standard vaccination programme against the disease (Reiter, 2010).

### 1.4.1.2. Transmission cycle

Dengue and yellow fever viruses are both transmitted by *Aedes spp* mosquitoes that are characterised as anthropophilic[1] and live in close association with domestic and peridomestic environments (Reiter, 2010). Originally, dengue and yellow fever shared an enzootic/sylvatic cycle that consisted in the transmission of viruses between sylvatic *Aedes* mosquitoes and primates (Endy *et al*., 2010; Reiter, 2010). Nowadays, dengue is mainly transmitted via the direct/urban transmission cycle between mosquitoes and humans (figure 11), perhaps because of the adaptation of *Aedes aegypti* to urban environments (Endy *et al*., 2010; Reiter, 2010).

[1] Feed on humans.

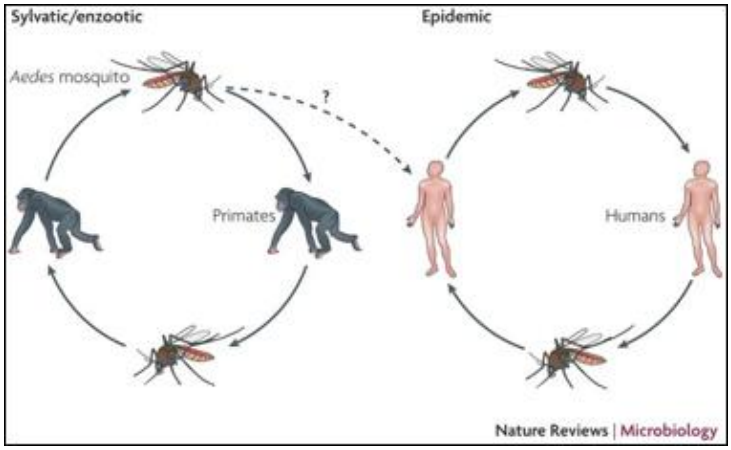


**Figure 11:** Dengue and yellow fever transmission cycle.
**(http://www.stanford.edu/group/parasites/ParaSites2008/Nkem_Cristina%20Valdoinos/ugonabon_valdovinosc_denguepropposal.htm – accessed on the 6th July 2011)**.

Yellow fever can be transmitted via three different cycles (figure 12). In the sylvatic cycle YFV is transmitted between primates and forest-dwelling mosquitoes, humans become infected when in contact with the sylvatic cycle through forest invasion and the virus can then be transmitted via the urban cycle between infected humans and *Ae. aegypti* (CDC, 2010b). The intermediate cycle which occurs only in Africa, is maintained between primates/humans and *Aedes spp* mosquitoes which bite humans who live or work in the forest periphery (CDC, 2010b).

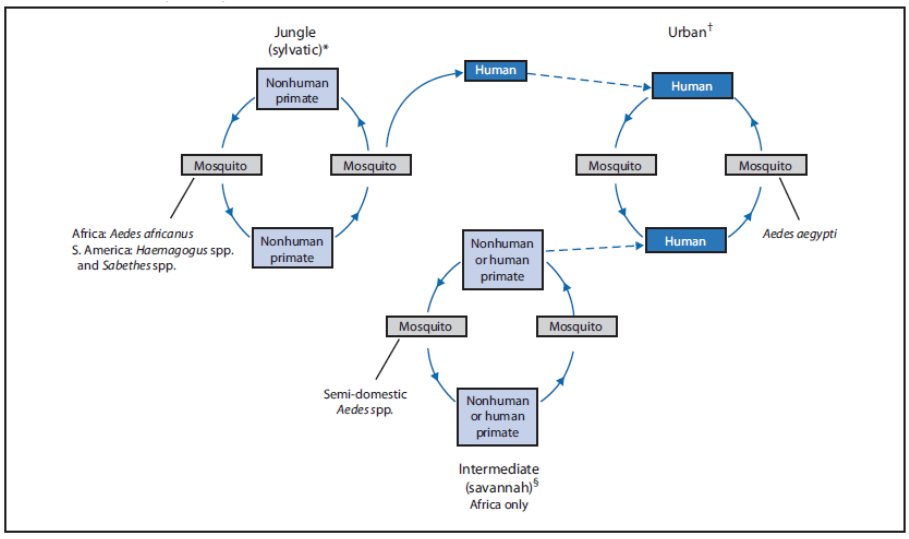


**Figure 12:** Yellow fever transmission cycles.
**(http://www.cdc.gov/mmwr/preview/mmwrhtml/rr5907a1.htm - accessed on the 24th November 2011)**

### 1.4.2. West Nile Virus

#### 1.4.2.1. Epidemiology

West Nile virus (WNV) is a mosquito-transmitted flavivirus that belongs to the Japanese encephalitis antigenic complex (Hubalek *et al*., 1999; Petersen and Roehrig, 2001). This virus was isolated in 1937 for the first time in the West Nile Province in Uganda, from the blood of a woman presenting with classical systemic febrile illness (Hubalek *et al*., 1999; Monini *et al*., 2010). The virus was later recognized and isolated from mosquitoes, birds and humans in the 1950's, in Egypt, and also in France (1962-63). During the 1970s-1990s WNV caused isolated outbreaks in countries such as South Africa (1974), India (1980-81) and Romania (1996) (Hubalek *et al*., 1999; Zeller and Schuffenecker, 2004; Monini *et al*., 2010). Since its first isolation, WNV has been considered one of the most widely spread flaviviruses thus becoming an increasing public health and veterinarian problem all over the globe (Zeller and Schuffenecker, 2004; Monini *et al*., 2010). The virus already affects several European countries, predominantly in the Mediterranean region, whereas in the Americas its transmission has been recorded from Canada to Argentina, after its recent introduction in the United States (figure 13) (Zeller and Schuffenecker, 2004; Monini *et al*., 2010).

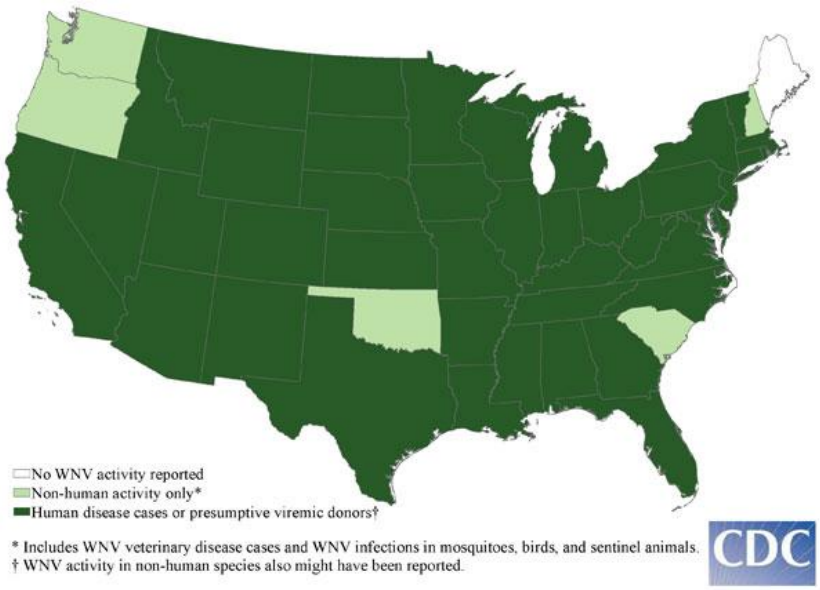


**Figure 13:** West Nile distribution in North America, as of 15th of November 2011.

**(http://www.cdc.gov/ncidod/dvbid/westnile/Mapsactivity/surv&control11MapsAnybyState.htm – accessed on the 22nd November 2011)**.

The virus is thought to have entered the United States through viraemic migratory birds or imported domestic birds, however the exact means of entry are unclear (Epstein and Defilippo, 2001; Solomon and Mallewa, 2001). WNV is the causative agent of West Nile fever and has been spreading globally (figure 14) using migratory birds as dispersal vehicles since it was first documented in Africa in the 1930's (Zeller and Schuffenecker, 2004; Gould and Solomon, 2008).

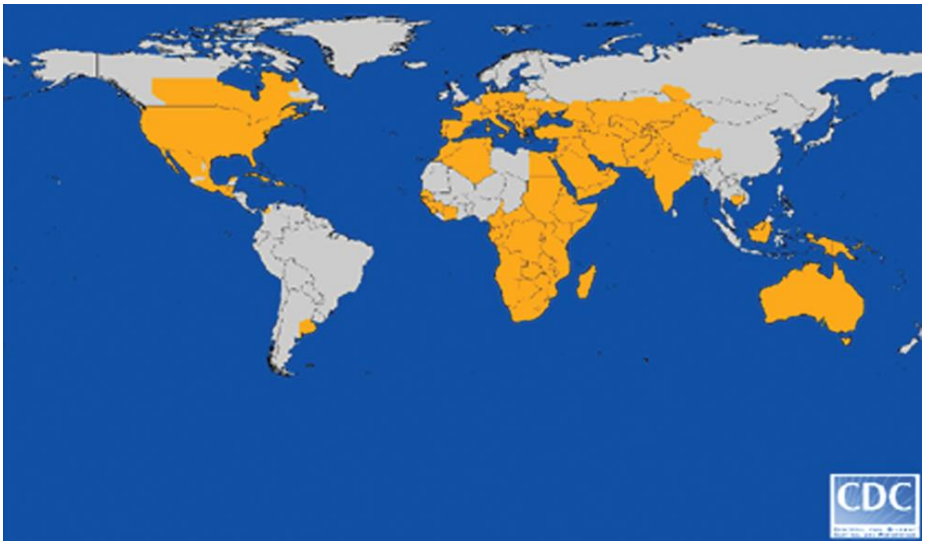


**Figure 14:** Approximate West Nile virus distribution map, 2006 **(Gubler, 2007)**.

Most known viral strains have been distributed into two main genetic lineages (figure 15), both clearly correlating to the virus geographic distribution (Zeller and Schuffnecker, 2004; Monini *et al*., 2010).

Viruses in lineage 1 are classified as more virulent and broadly distributed through Africa, Australia, India, Asia and Europe, mainly in Mediterranean countries (Monini *et al*., 2010) In the other hand, WNV strains composing lineage 2 have kept a more strict distribution in Sub-Saharan Africa (Petersen and Roehrig, 2001; Zeller and Schuffenecker, 2004; Petersen and Marfin, 2005; Weaver and Reisen, 2010). However, they have recently been found circulating in Hungary and Greece (Zeller *et al*., 2010; Chaskopoulou *et al*., 2011). Until very recently, only lineage 1 virus strains had been

associated with severe human disease, including cases of clinical human encephalitis (Petersen and Marfin, 2005; Petersen and Roehrig, 2001).

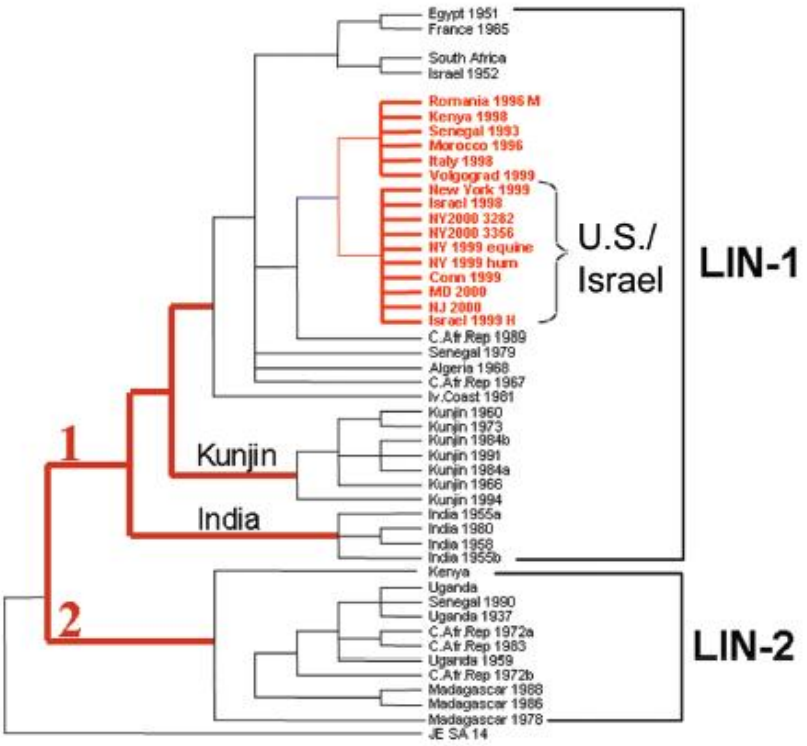


**Figure 15:** Phylogenetic tree of West Nile viruses based on the analysis of the envelope (E) gene **(Gubler, 2007)**.

West Nile fever outbreaks affecting humans and/or animals have occurred all over the globe, in both tropical and temperate regions (Almeida *et al*., 2008). In 1996-1997, Romania had the largest outbreak of arboviral disease seen in Europe, where more than 600 people presented neurological complications of which nearly 10% died (Hubalek, 1999; Solomon and Mallewa, 2001).

Other outbreaks have occurred in Israel in the 1950's and in 2000, South Africa in the 1970's, Algeria in 1994, Morocco in 1996, Tunisia in 1997, Italy in 1998, Russia in 1999, France in 2000 (Monini *et al*., 2010). By 1999, the virus had spread to New York

causing 60 clinical cases of encephalitis leading to 7 human deaths with a dramatically higher equine and bird mortality rate (figure 16) (Hubalek, 1999; Zeller and Schuffenecker, 2004; Gould and Solomon, 2008).


**Figure 16:** West Nile virus epidemics between 1937 and 2006 **(Gubler, 2007).**

Red stars represent epidemics associated with severe neurological disease in both humans and animals.

More recently, nine cases of WNV infection were reported in Italy in 2010, six of which were symptomatic (Barzon *et al*., 2011). Three of the patients presenting symptoms were confirmed to have developed neurological disease (Barzon *et al*., 2011). In Greece 35 deaths from 262 human cases were reported in that same year (Chaskopoulou *et al*., 2011). In 2011, WNV activity was detected in sentinel chickens between May and July, and by August 37 cases had been registered, of which 31 evolved into neurological disease (Danis *et al*., 2011).

### 1.4.2.2. Transmission cycle

WNV is mainly transmitted by mosquito species belonging to the *Culex* genus via two possible modes of transmission, the rural and the urban cycles (figure 17) (Hubalek, 1999; Monini *et al*., 2010).

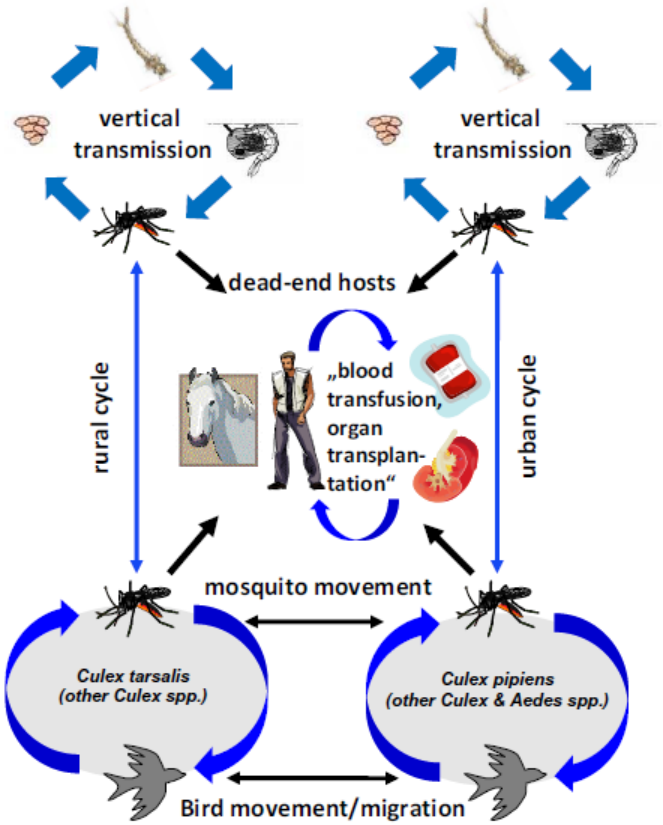


**Figure 17:** West Nile virus transmission cycle and possible modes of dispersal (**Pfeffer and Dobler, 2010**).

In a rural setting, the primary transmission cycle occurs involving competent ornitophilic[2] *Culex* species mosquitoes and migratory and local bird species that act as reservoirs as well as amplifying hosts whilst humans and horses represent accidental "dead-end" hosts, since they do not reach the level of viraemia needed for mosquito

[2] Feed on birds.

infection (Solomon and Mallewa, 2001; Petersen and Marfin, 2005; Monini *et al*., 2010; Weaver and Reisen, 2010).

The urban cycle occurs in a similar way, however it does involve different vector and bird species; for example, in this transmission mode, the principal vectors are *Culex pipiens* and *Cx. molestus* seen as both species feed on synanthropic[3], domestic birds and humans (Hubalek, 1999).

Moreover, the main WNV vectors vary according to their geographic distribution, for example, the predominant vector species in Europe are *Culex pipiens*, *Cx. molestus* and *Coquillettidia richiardii* (Hubalek, 1999). In Africa and The Middle East, the most important vector is *Culex univittatus*, whereas in Asia, *Culex quinquefasciatus*, *Cx. tritaeniorhyncus* and *Cx. vishnui* are the main vectors (Hubalek, 1999).

Although the rural cycle is of utmost importance for WNV transmission, this flavivirus can also be transmitted via organ transplants, blood transfusions and via infectious maternal milk (Zeller and Schuffenecker, 2004; Gould and Solomon, 2008). Additionally, reports show that transovarial transmission occurs in some species, namely *Cx. univittatus*, *Cx. tritaeniorhyncus*, *Aedes aegypti* and *Ae. albopictus* (Hubalek,1999).

#### 1.4.2.3. Clinical features

West Nile disease was at first considered a minor health concern, as the majority of infections were mild or completely asymptomatic; however, after the major outbreaks that occurred in Europe and especially in North America from 1999, WN disease became a public health priority (Zeller and Schuffenecker, 2004; Gould and Solomon, 2008).

After an incubation period of 3 to 14 days, between 15-20% of humans present with a mild febrile illness accompanied by flu-like symptoms, a rapid onset of moderate to high fever, headache, malaise, myalgia, anorexia, nausea, backache and retro-orbital

[3] Ecologically associated with humans.

pain (Hubalek, 1999; Zeller and Schuffenecker, 2004; Gould and Solomon, 2008). Other disease manifestations include lymphadenopathy, conjunctivitis, maculopapular or roseolar rash, which is normally observed in 50% of patients (Hubalek, 1999; Gould and Solomon, 2008). Around 1% of all patients tend to develop neurological signs such as acute aseptic meningitis, encephalitis or myelitis[4] (Hubalek, 1999; Zeller and Schuffenecker, 2004). Additionally, severe infections may also provoke hepato- and splenomegaly, hepatitis, pancreatitis and myocarditis (Zeller and Schuffenecker, 2004; Gould and Solomon, 2008). A mortality rate of 5 to 10% usually results from all patients presenting with neurological symptoms (Gould and Solomon, 2008).

During the outbreaks in North America, Romania, Russia and Israel mortality rates of 5% to 14% among neurologically affected patients were registered, as well as other symptoms such as serious motor complications, skin rash and lymphadenopathy (Petersen and Roehrig, 2001). In the Romania outbreak alone, 17 fatalities resulted from 352 neurological affected patients, from whom 44% presented with meningoencephalitis, a further 40% had meningitis and the remaining 16% suffered from encephalitis (Zeller and Schuffenecker, 2004). In accordance with most studies, fatalities are more likely to arise among patients over 50 years of age (Zeller and Schuffenecker, 2004; Gould and Solomon, 2008).

### 1.5. Insect-specific flaviviruses

This group of flaviviruses have had many designations throughout the years, from "non-classical" to "mosquito-only" flaviviruses. However, as the latter designation is somehow conflicting since insect-specific flaviviruses have been detected in field caught phlebotomine sand flies and ticks it completely invalidates the continued use of that designation, hence in this study it is referred to as insect-specific flaviviruses.

As previously mentioned, most viruses within the genus *Flavivirus* are arthropod-borne, and only a few have no-known vectors (Crabtree *et al*., 2003; Cook *et al*., 2003; Sang *et al*., 2003; Mukhopadhyay *et al*., 2005). However, the increasing surveillance and investigations into these pathogens have lead to a better understanding of their genetics,

[4] Inflammation of the spinal cord.

classification and phylogenetic relationships (Sang *et al*., 2003). It is now common knowledge that pathogenic flaviviruses do not exhaust the *Flaviviridae* family, as it also harbours another type of viruses that are, so far, not known to be medically important (Blitvitch *et al*., 2009). On the contrary, they are thought to be insect-specific since they replicate in mosquitoes but have not yet been detected in a vertebrate host. They are, thus, designated by insect-specific flaviviruses (ISFs) (Blitvitch *et al*., 2009). ISFs are a heterogeneous group of highly diverse and widely geographically dispersed viruses.

Even though most ISFs have only been described and classified over the past decade, it is certain that they have existed for much longer. For example, the first one was found many years ago and was referred to as "an agent in the *Ae. aegypti* cell line that causes fusion of *Ae. albopictus* cells" by Stollar and Thomas (1975), and was, as a result, named cell-fusing agent virus (CFAV). This insect-specific flavivirus causes distinguishable massive syncytia formation, an effect that had only been observed with JEV, WNV and DENV (Stollar and Thomas, 1975; Igarashi *et al*., 1976). However, it was stated that syncytium formation was not observed immediately after infection, instead, it became evident after 60 hours of cell infection and, after 72 hours more than 90% of the cells in culture would be fused having formed large syncytia (Stollar and Thomas, 1975). Infection of mammalian cells with CFAV resulted in no cytopathic effect (CPE), including cell fusion. Indeed, the virus does not seem to replicate at all, thus confirming that it is insect-specific (Stollar and Thomas, 1975; Sang *et al*., 2003). In addition, replication in *Ae. aegypti* cells is not cytopathic (Stollar and Thomas, 1975).

Further analysis revealed that CFAV did not cross react with other known flaviviruses (Igarashi *et al*., 1976). CFAV was described as similar to togaviruses in size and morphology, whereas other characteristics indicated strong similitude to flaviviruses (Igarashi *et al*., 1976). However, despite all evidence and tests, the classification of CFAV remains unresolved.

The isolation of a new flavivirus described as CFA-like from mosquitoes collected in Kenya's Central Province, during a rainy season in 1999 was reported and later genetically and phenotypically characterised (Sang *et al*., 2003).

This virus, named Kamiti River virus (KRV), was isolated from *Ae. macintoshi* immature mosquitoes and was temporarily classified as a flavivirus (Crabtree *et al*., 2003). The fact that KRV was isolated from adult male and female mosquitoes that were collected, in nature, in their immature forms, is indicative of the maintenance of this virus via transovarial transmission between generations. Nonetheless, there also remains the possibility that the virus may have been acquired by larval ingestion (Crabtree *et al*., 2003).

Like CFAV, only replicated in mosquito cells and presented no antigenic cross-reaction with other arboviruses (Sang *et al*., 2003). However, contrarily to CFAV, there was no cell fusion observed in culture, despite the morphologic and genomic similarities (Crabtree *et al*., 2003). The genomic organisation of KRV was found to be similar to that of other flaviviruses, though its nucleotide sequence was considerably longer (11,375 nt) (Crabtree *et al*., 2003). Two viral strains were isolated and their RNA sequences compared which revealed they were virtually identical, thus suggesting they represent the same virus; however, when comparison was extended to other flaviviruses a low sequence identity was observed (Crabtree *et al*., 2003; Sang *et al*., 2003). KRV showed maximum identity to CFAV based on nucleotide and amino acid sequence analysis of the NS3 and NS5 genes (Crabtree *et al*., 2003; Sang *et al*., 2003). Both KRV isolates produced cytopathic effect[5] (CPE) in *Ae. albopictus* cells (C6/36) in culture. However, the same was not observed with Vero cells (monkey kidney cell line) or in baby hamster kidney cells (BHK-21), as expected (Crabtree *et al*., 2003; Sang *et al*., 2003).

Some years later, the discovery of 40 CFA-like viral sequences from adult mosquitoes collected in Puerto Rico after the rainy season of 2002 was reported (Cook *et al*., 2006). These were obtained from male and female mosquitoes of different species, namely *Ae. aegypti*, *Ae. albopictus* and *Culex sp*. The sequences analysed were found to represent different strains of CFAV and, therefore have been referred to as CFAV Culebra stains (Cook *et al*., 2006).

[5] Structural cell changes, such as cell degeneration or detachment, caused by viral infection.

In addition to CFAV and KRV, other insect-specific flaviviruses have been detected and isolated. However, the new isolate seems to be associated only to *Culex sp*. mosquitoes, especially *Cx. pipiens*, hence their name *Culex* flavivirus (CxFV) (Hoshino *et al*., 2007). The genome of this new flavivirus is 10,834 nucleotides long, also flanked by two untranslated (UTR) regions, as described for other flaviviruses (Hoshino *et al*., 2007).

Infection of mosquito C6/36 cells in culture resulted in moderate CPE visible 4 days post-infection. In addition, no viral replication was detected in mammalian cell lines (Hoshino *et al*., 2007). Phylogenetically, CxFV showed greater similarities with CFAV and KRV than with other mosquito-transmitted flaviviruses (Hoshino *et al*., 2007). Alike the CFA-like viruses and KRV, CxFV have been detected in healthy wild-caught mosquitoes, thus suggesting once again that vertical transmission occurs in the wild, and despite causing moderate CPE in culture, it does not seem to negatively affect their hosts. (Hoshino *et al*., 2007).

A virus similar to CxFV found in Japan was identified in Guatemala and was later named CxFV Izabal 2006; it was isolated from *Culex quinquefasciatus* collected from urban and rural areas between March and October 2006 (Morales-Betoulle *et al*., 2008).

Analysis of the envelope and NS5 gene sequences were the basis for characterisation of the detected viral strains which, after phylogenetic assessment were classified as strains of the CxFV species following a result of 89% nucleotide homology with the Japanese virus (Morales-Betoulle *et al*., 2008). The isolates were cultured into C6/36 and Vero cells, with no CPE observed for either (Morales-Betoulle *et al*., 2008).

In addition to the diversity of this heterogeneous group of flaviviruses previously identified, Crabtree *et al* (2009) have also found a new insect-only flavivirus from *Cx. tritaeniorhyncus* captured in Vietnam in August 2002. These virus, denominated Quang Binh virus (QBV) was described as genetically different from pre-existing flaviviruses and though its complete genome had similar size (10,865 nt) and genomic organisation (Crabtree *et al*., 2009). Sequence analysis revealed that Quang Binh virus had over 60% identity with Japanese CxFV and, when in culture, it induced plaque formation and CPE only in C6/36 cells. (Crabtree *et al*., 2009).

Meanwhile, a new ISF of Asian origin was discovered and designated *Aedes* flavivirus (AEFV) (Hoshino *et al*., 2009). Contrarily to most pre-existing flaviviruses, AEFV was isolated from Aedes and not *Culex* mosquitoes, namely *Ae. albopictus* and *Ae. flavopictus* collected from Japan and Indonesia (Hoshino *et al*., 2009). However, other flavivirus-like sequences were also obtained (Hoshino *et al*., 2009). Infection of C6/36 produced moderate CPE after 4 days, whereas, as expected for ISFs, mammalian cells showed no such effects (Hoshino *et al*., 2009). AEFV was therefore classified a flavivirus with similar replication and translation mechanisms; furthermore, it was stated that it showed high virus-host adaptation to its invertebrate host, an *Aedes* mosquito (Hoshino *et al*., 2009).

AEFV's genome mirrors that of flaviviruses and its polyprotein is thought to be divided into 3 structural (C, prM and E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Hoshino *et al*., 2009). AEFV was classified as a new species of insect flavivirus, particularly similar to KRV (Hoshino *et al*., 2009).

Insect-only flaviviruses previously characterised have been isolated from *Aedes* and *Culex* mosquitoes; however, a study carried out by Cook and others (2009) reports the first isolations of a ISF from a *Mansonia* species mosquito and an African strain of CxFV. Both new strains, namely CxFV Uganda and Nakiwogo virus (NAKV) were obtained from mosquitoes collected in the same trap (CDC-type, with $CO_2$) on the 24$^{th}$ of February 2008 in Entebbe, Uganda (Cook *et al*., 2009). The NAKV and CxFV Uganda strain were isolated from *Mansonia africana nigerrima* and *Cx. quinquefasciatus*, respectively (Cook *et al*., 2009). The genomes of both viruses were found to be 10,092 nt long for CxFV, and 10,122 nt long for NAKV (Cook *et al*., 2009). These viruses were morphologically identical to flaviviruses, and phylogenetic analysis revealed that CxFV Uganda to be more closely related to the Mexico strain, while NAKV was inserted into the ISF clade, but as a sister group (figure 18) (Cook *et al*., 2009).

Infection of C6/36 cells with NAKV resulted in moderate CPE with the formation of large syncytia, similarly to what was described for CFA, whereas CxFV induced structural changes, causing them to turn triangular from their normal circular shapes, and reduced density (Cook *et al*., 2009).

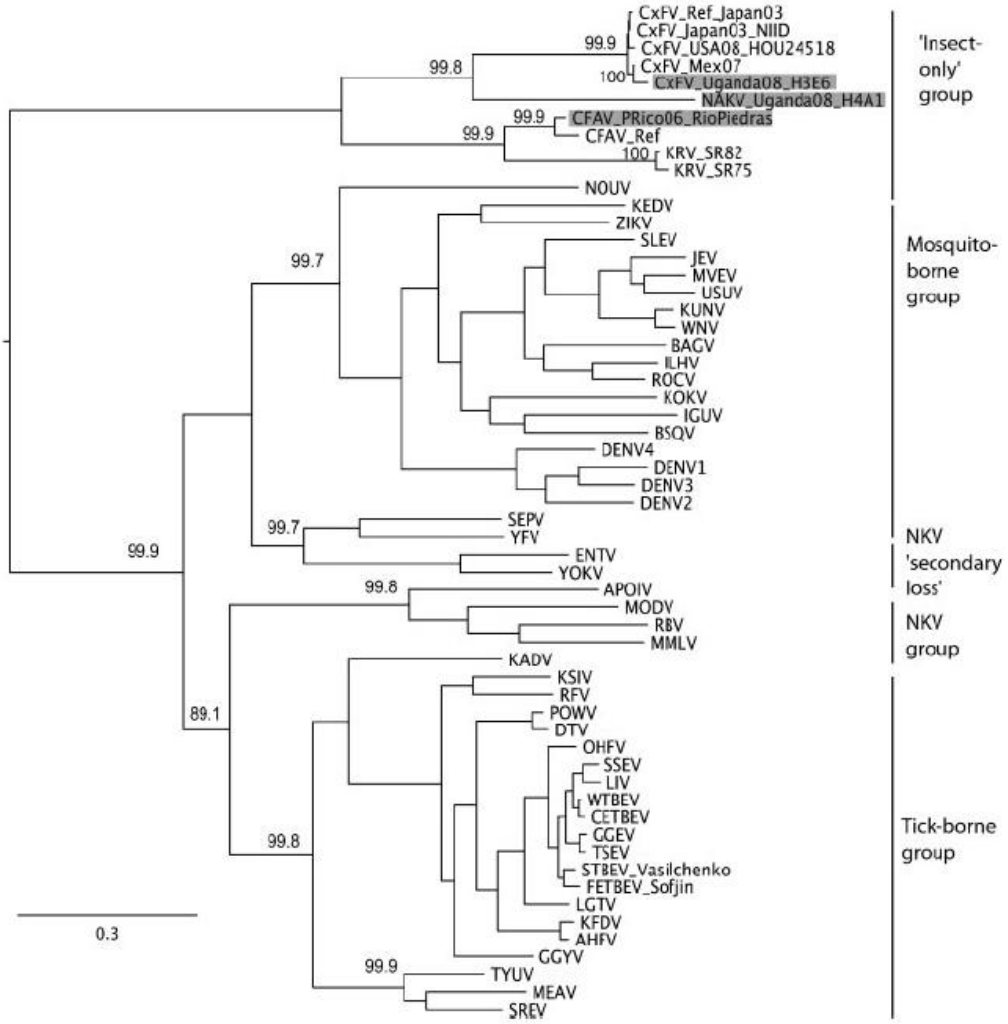


**Figure 18:** Maximum-likelihood tree showing relative positions of CxFV Uganda and NAKV within the *Flavivirus* genus, based on NS5 gene analysis **(Cook *et al.*, 2009)**.

A study conducted by Junglen *et al.* (2009) revealed the discovery of a novel flavivirus particle isolated from *Uranotaenia mashonaensis* from the tropical rain forest, in Côte d'Ivoire, that was named Nounané virus (NOUV). Similarities to other ISFs include structure, morphology and genomic organisation; however, the viral ORF displayed was the longest known to flaviviruses. Surprisingly, phylogenetic analysis showed closer relationship to mosquito-borne flaviviruses (YFV, JEV and WNV) than ISFs such as

CFAV and KRV (Junglen *et al*., 2009). As observed for ISFs, viral replication was observed only in insect-cell culture (Junglen *et al*., 2009).

Further away, in North America many other flavivirus-like sequences have been found. For example, as a result of a surveillance programme in Alberta (Canada), some flaviviruses were detected, the majority were isolated from *Culex tarsalis* mosquitoes while a lower number was obtained from non-*Culex* mosquitoes; these sequences showed higher similarities to KRV (Pabbaraju *et al*., 2009). In Iowa, again as part of the surveillance programme, mosquitoes were collected between May and October 2007, of which *Cx. pipiens* and *Cx. tarsalis* pools turned out positive for flaviviruses (Blitvich *et al*., 2009). Analysis of the sequences obtained revealed that the isolates had 98% homology with CxFV isolated in Japan but also shows close relation to the Texas and Mexico strains (Huhtamo *et al*., 2009). It was characterised as having a 10,089 long genomic sequence and production of moderate CPE and cell clumping were observed in culture with insect cell lines only (Huhtamo *et al*., 2009).

Once again, it was due to arboviral surveillance programmes that flavivirus strains have been found and isolated from mosquitoes collected from January to December 2007, in the Yucatan Peninsula of Mexico (Farfan-Ale *et al*., 2009). Several of the analysed pools tested positive for flaviviruses, the majority of them showing identical sequences to that of CxFV (Farfan-Ale *et al*., 2009). A new strain of CxFV closely related to the Japanese strain (figure 19) was isolated from *Cx. quinquefasciatus,* and as some pools consisted only of male specimens, this suggested, again, vertical transmission in nature (Farfan-Ale *et al*., 2009). However, contrarily to previously isolated strains of CxFV, this virus did not produce CPE on C6/36 cells (Farfan-Ale *et al*., 2009).

It is noteworthy that CxFV presented a high prevalence, as it was found in 145 of 146 pools from which flavivirus RNA was detected (Farfan-Ale *et al*., 2009; Kim *et al*., 2009).

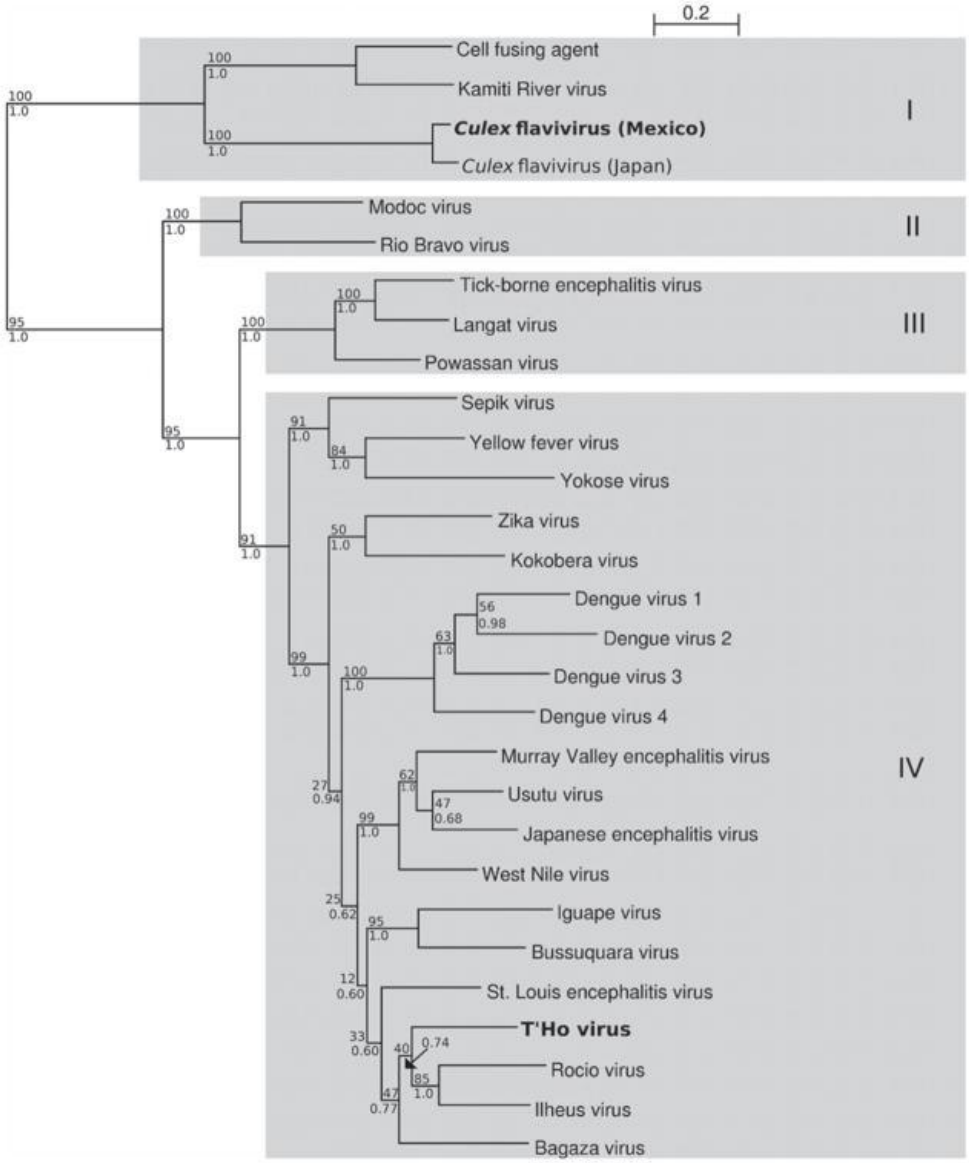


**Figure 19:** Phylogenetic tree showing the position of the newly isolated CxFV and T'Ho virus, based on the analysis of a fragment of the NS5 gene **(Farfan-Ale *et al.*, 2009)**.

Collections made during February and March 2008 in Texas (USA) and Trinidad yielded many pools positive for flaviviruses that had been obtained from *Cx. restuans* and *Cx. quinquefasciatus* females (Kim *et al*., 2009). From these positive pools, it was possible to isolate nine strains from both locations, however only one, designated by TX24518, was selected for further analysis (Kim *et al*., 2009). Inoculation of some of the other strains into Vero cells and intracerebrally in mice produced no signs of

replication or CPE, thus suggesting that they belong to the insect-only flavivirus group (Kim *et al*., 2009).

TX24518 was inoculated into C6/36 cells, which resulted in the formation of large syncytia as observed for CFA (figure 20).

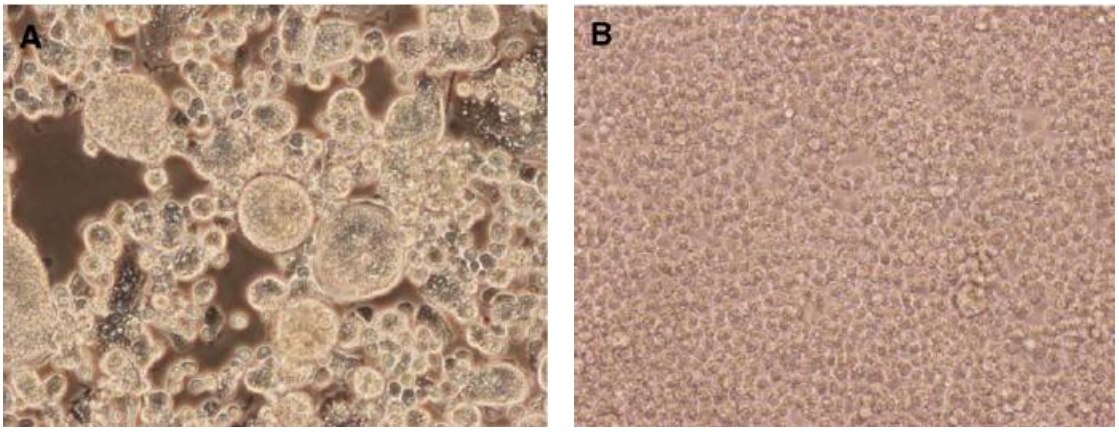


**Figure 20:** A) Cell fusion and large syncytia present in C6/36 cells, 7 days post-infection with TX24518; B) Uninfected C6/36 cells (control) **(Kim *et al*., 2009)**.

A polyprotein similar to those of the *Flavivirus* members was identified, consisting of three structural and seven non-structural proteins, and its genome was found to be 10,089nt long (Kim *et al*., 2009). Sequence identity values revealed TX24518 to have higher similarities to CxFV-Tokyo than to CFAV or KRV, based on protein analysis; the other isolates from Houston were more similar to Japanese CxFV strains, while the Trinidad isolates showed higher identity to the Izabal virus isolated in Guatemala (Kim *et al*., 2009).

Kim and others (2009) concluded that the isolates from both locations represented new genetic variants of CxFV and were genetically distinct from CFA and KRV; moreover, it was suggested that the activity of these viruses in their arthropod vectors is seasonal, as continued testing did not detect any more flavivirus-positive pools.

After the isolation of cell-fusing agent and Kamiti river viruses, many other insect-specific flaviviruses have been described, classified and incorporated into the *Flavivirus* genus. However, in Trinidad in 1972-74, two viruses denominated Tamana bat virus

(TABV) and Rio Bravo virus (RBV) were isolated from insectivorous bats, none of which had ever been detected before (Price, 1978). Both these viruses share characteristics with enveloped arboviruses, however, whereas Rio Bravo virus has been included in the NKV group within the genus *Flavivirus*, TABV's serological and phylogenetic relationships and classification remains unresolved (Lamballerie *et al*., 2002). Despite that, TABV shares some characteristics flaviviruses, such as the identical genomic organisation, similarities related to polyprotein-cleavage sites, hydropathy plots, amino acid domains in enzymes and structural proteins (Lamballerie *et al*., 2002; Cook *et al*., 2006). Furthermore, no link has ever been found between an arthropod-vector and Rio Bravo and Tamana bat viruses (Price, 1978).

### 1.6. Europe: current situation

Since the last decade, and after WNV invasion of America, many arboviruses have been actively spreading and causing outbreaks throughout the world, including Europe (central Spain and Italy) where WNV was recently detected (Aranda *et al*., 2009). However, other "non-classical" flaviviruses have also been detected. For that reason, and due to autochthonous and non-autochthonous arboviral activity detected in Spain, there is an increased risk for introduction of emerging and re-emerging diseases (Aranda *et al*., 2009). Subsequently, a surveillance system was set up with the main purpose to better understand arboviruses, and their vectors (Aranda *et al*., 2009; Sánchez-Seco *et al*., 2010). It is noteworthy that all the areas sampled for arbovirus vectors are migratory routes and resting spots for birds coming from Africa (Aranda *et al*., 2009).

As a result of multidisciplinary surveillance programmes, many flavivirus-like sequences have been detected (Aranda *et al*., 2009). Some of those sequences were found to have similarities with CFAV, KRV and CxFV, while others showed closer relations to arthropod-borne flaviviruses (Sánchez-Seco *et al*., 2010). In addition, the discovery of a European CxFV strain similar to that isolated in Japan was reported (Sánchez-Seco *et al*., 2010).

Recently, Italian investigators discovered yet another insect-specific flavivirus, displaying similarities to KRV. Phylogeny of the NS5 gene showed that these sequences formed a new group within the KRV/CFAV clade (figure 21) (Roiz *et al*., 2009). Identity analysis of sequences, based on nucleotide and amino acid comparison, yielded values between 62% and 86.9% identity with KRV, CFAV, and CxFV thus confirming the new virus was indeed an ISF (Roiz *et al*., 2009). Other sequences detected in Italy, from *Aedes* species mosquitoes, showed similarities to AeFv, CxFv, and KRV (Calzolari *et al*., 2010).

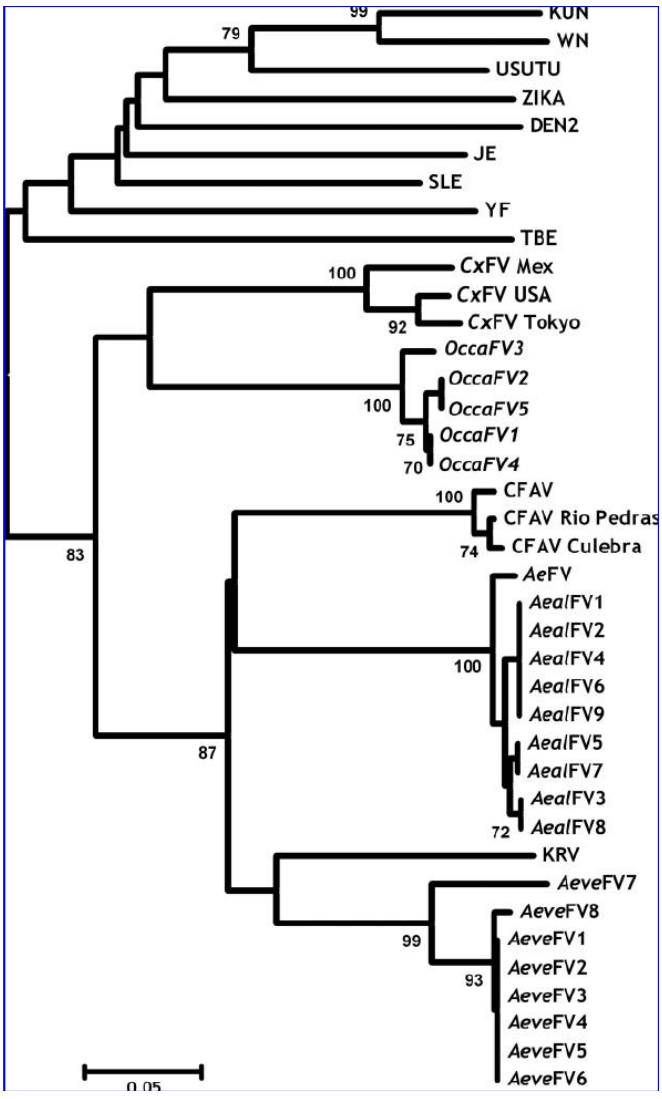


**Figure 21:** Neighbour-joining tree based on the analysis of the NS5 gene of the flavivirus sequences amplified (OccaFV1-FV5, AealFV1-FV9, and AeveFV1-FV8) **(Calzolari *et al*., 2010).**

Finland is, in Europe, the northernmost point at which a mosquito-borne flavivirus has ever been detected. This Finnish isolate, named Lammi virus (LAMV) was found to be highly divergent from other flaviviruses, although it did show typical flavivirus morphology (Huhtamo *et al*., 2009). Like NOUV, LAMV was found to be closely related to human pathogens transmitted by *Aedes* species mosquitoes, which in this case is thought to have been *Ae. cinereus*, a known anthropophilic species (Huhtamo *et al*., 2009). These two related viruses, despite a sequence homology of less than 50%, were grouped together, in what seems to be a different species or lineage within the *Flavivirus* genus (Huhtamo *et al*., 2009).

The geographic distribution of emergent and re-emergent vector-borne diseases, such as Dengue, Malaria, Chikungunya and West Nile fever has been continuously expanding all over the world in the last 30 years (figure 22), thus increasing the global burden of these viral infectious diseases (Lapied *et al*., 2008; Moreira *et al*., 2009; Lambrechts *et al*., 2010).

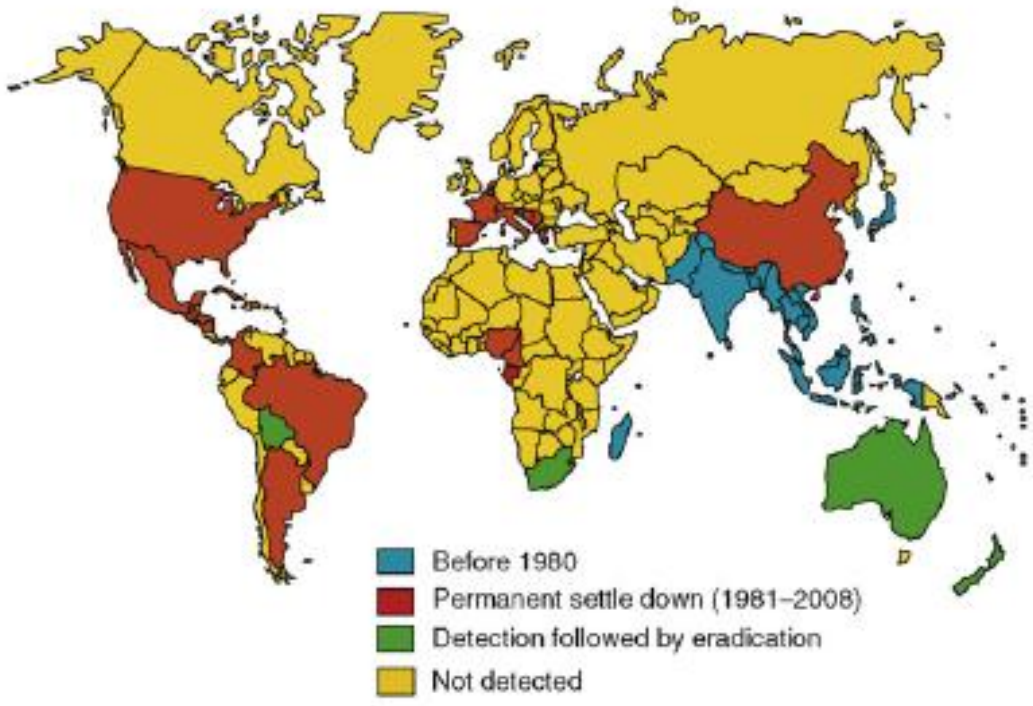


**Figure 22:** Worldwide geographic distribution of *Aedes albopictus* between 1980 and 2008

**(Lapied *et al*., 2008).**

As previously described, many factors have contributed to that range expansion; they include the increasing human movement, demographic growth, ecologic and climatic alterations probably resulting from global warming effects, as well as economic determinants that may restrict vaccination and vector control programmes (Lapied *et al*., 2008; Moreira *et al*., 2009). However, viral evolution has promoted increased virulence and allowed viruses to better adapt to a wider variety of vectors, as is the case for West Nile and Chikungunya viruses (Lapied *et al*., 2008). Additionally, the distribution of these potentially fatal diseases is usually parallel and highly dependent on the presence and range distribution of their vectors, especially *Ae. aegypti* and *Ae. albopictus* (Lambrechts *et al*., 2010).

The past three decades have seen *Ae. albopictus* spread to all continents from its origins in Asia; and the major cause for concern is that this mosquito species is known to be experimentally susceptible to the infection of at least 22 arboviruses (Lapied *et al*., 2008). Moreover, the presence and establishment of *Ae. aegypti* in certain regions is also an important factor in the increasing risk of arboviral disease transmission in Europe and neighbouring countries.

*Aedes aegypti* is the main and most competent vector for the transmission of dengue viruses, but *Ae. albopictus* is also capable of transmitting the viruses even though it is considered a secondary vector mainly because it is considered less competent and presents different host preferences (Lambrechts *et al*., 2010). Moreover, each species is associated with a different degree of disease severity, being *Ae. aegypti* associated with the more serious forms of dengue such as dengue haemorrhagic fever whereas *Ae. albopictus* is usually linked to the classical dengue fever (Lambrechts *et al*., 2010).

*Aedes albopictus* has become established in many European countries, especially those situated around the Mediterranean Sea, and including Albania, Greece, Serbia and Montenegro, Croatia, France, Italy, Spain, but also in the Netherlands, and in Switzerland (figure 23) (Lambrechts *et al*., 2010).

The establishment of *Ae. albopictus* in Europe was first reported in Albania in 1979, and has since spread to Italy where, in 1991, a breeding population was found followed by the subsequent invasion across the country; and in 1997, the vector finally reached

Rome where colonies were found throughout the entire city (Aranda *et al*., 2006; Lapied *et al*., 2008).

In 1999, populations of *Ae. albopictus* were discovered in tyre dumps in France, followed by their appearance in Belgium in 2000; after that and more recently countries such as Switzerland, Greece, Serbia and Montenegro have reported the establishment of this mosquito species (Aranda *et al*., 2006; Lapied *et al*., 2008). However, other regions have been invaded by populations of *Ae. albopictus* and their range expansion is still ongoing (figure 23) (Aranda *et al*., 2006).

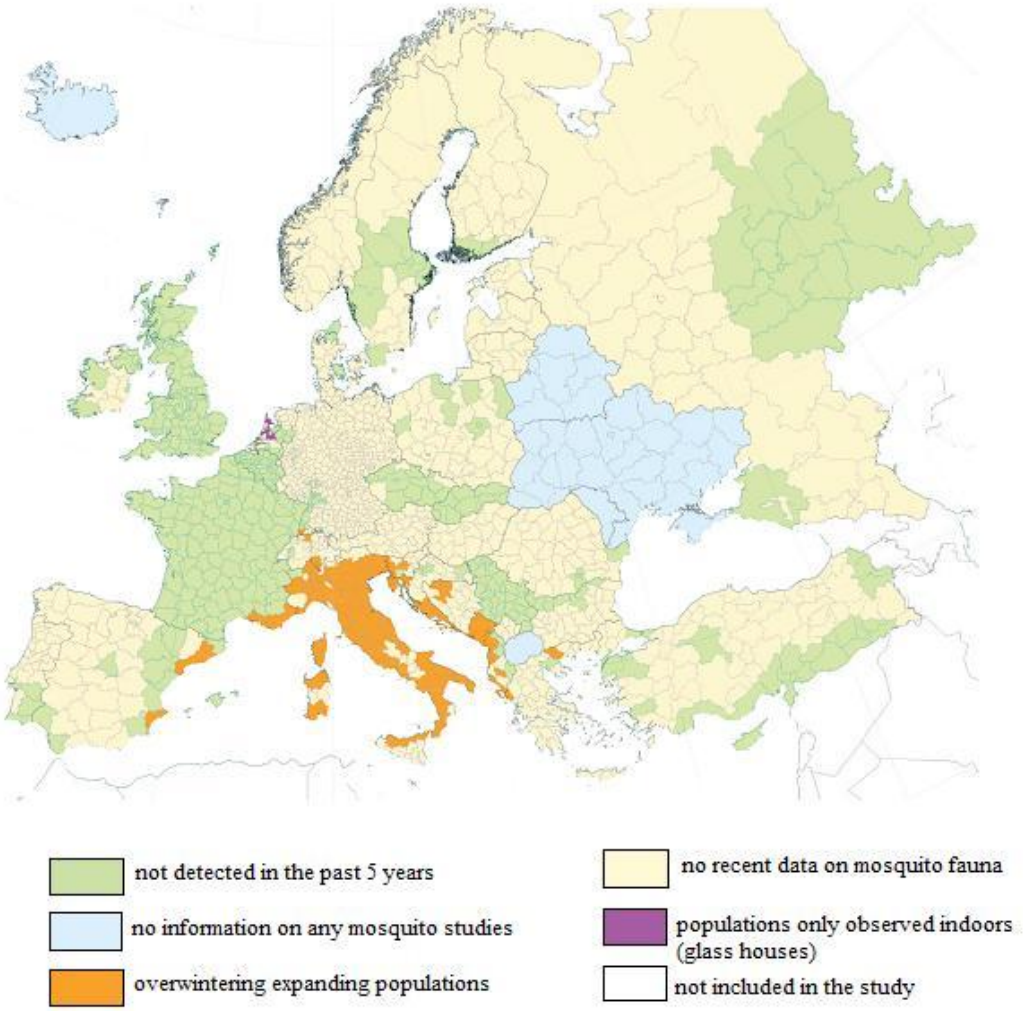


**Figure 23:** Distribution of *Aedes albopictus* in Europe, as of 2009 **(adapted from Reiter, 2010).**

As shown above, *Ae. albopictus* has been found all over Europe, with particular emphasis in Italy where it has been established for over a decade, and has also been recorded since 2005 in France, all down the length of the Italian border (Lapied *et al*., 2008). For that reason, it is not surprising that major outbreaks have occurred with this mosquito as the potential vector incriminated for the 2007 Chikungunya outbreak, which resulted in the infection of 205 individuals (Rezza *et al*., 2007; Lapied *et al*., 2008). Additionally, in August 2004, following complaints of the presence of "black flies" in a residential area in the capital city of Catalonia, the search for which started by sampling breeding sites that lead to the finding of an adult male and many immature forms to be collected (Aranda *et al*., 2006). From the immature forms, another male emerged and after identification, both were confirmed as being *Ae. albopictus* (Aranda *et al*., 2006). Consequently, many other searches for these mosquitoes resulted in the discovery of other adults, males and females, an also immature forms; thus suggesting that this species was already established in the country (Aranda *et al*., 2006). Following these events, further investigations took place leading to the conclusion that these mosquitoes had been settled for at least two years (Aranda *et al*., 2006).

West Nile and Usutu virus activity has been reported in Europe, and recently a highly lethal (for humans) WN strain was circulating in Northern Greece in 2010 and more cases have been reported during this year in other parts of the country (Chaskopoulou *et al*., 2011; Vázquez *et al*., 2011a). WNV was also detected in Russia and Spain affecting humans and horses (Zeller *et al*., 2010; Vázquez *et al*., 2011a). Usutu virus was detected in Italy in the summer of 2009 and caused neurological involvement in humans, among other symptoms; and although it has been actively circulating in Spain, no cases have been detected so far (Vázquez *et al*., 2011a). It has been postulated that WNV and Usutu virus are circulating endemically around the Mediterranean, and in some areas co-circulation of these two flaviviruses has been reported (Vázquez *et al*., 2011a).

### 1.7. Portugal: the imminent risk

A potentially dangerous tropical disease vector is now present and well established in the Iberian Peninsula, in Spain (Aranda *et al*., 2006). Spain and Portugal present favourable ecological and climatic conditions for the development of certain arthropods that may act as disease vectors, especially those of tropical origins brought by travelling or transportation of goods (Aranda *et al*., 2006). The main means of dispersal of *Aedes albopictus* are known to be the transportation of immature forms in tyres and, more recently, it was found that the importation of the Lucky Bamboo plant (*Dracaena spp*.) was also to blame (Aranda *et al*., 2006). However, it has been suggested that the transportation of adult mosquitoes in cars or lorries may have played an essential part in their geographical spread (Aranda *et al*., 2006). Furthermore, the Iberian Peninsula is directly under the flight path of migratory birds coming from Africa (figure 24), this alone presents a relatively high risk of the introduction of arboviruses, since there is a vast fauna of ornitophilic mosquitoes established in Portugal and Spain, such as *Culex* species mosquitoes. Usually, warmer temperatures and rainy seasons determine mosquito density peaks; however, climate changes due to global warming could bring storms, flash floods, heat waves and droughts, as has been predicted to occur more frequently and intensely in Portugal and other countries with temperate climates in Europe (Epstein, 2000; Santos *et al*., 2002; Santos and Miranda, 2006). These extreme climatic events would greatly benefit high population densities due to the reduction of mosquito predators in nature, the hatching of pre-existing desiccated eggs (floods), creation of new breeding sites and larval habitats (Epstein, 2000; Santos *et al*., 2002). Conditions in Portugal favour the breeding of large populations of anthropophilic mosquitoes as well as, for example, *Culex* species mosquitoes that may act as bridge vectors of West Nile. The *Culex pipiens* complex is composed of two forms, the "pipiens" and "molestus" forms; hybrids of these two species have been described as opportunistic feeders that, for that reason, are considered epidemiologically important as they may act as bridge vectors of viruses such as WNV (Gomes *et al*., 2009). Moreover, since the only member of the complex present in our collections was the ornitophilic "pipiens" form that reaches the highest densities in estuarine regions, some of which harbour migratory birds from Africa, their presence in conjunction with

infected birds represents a major risk of introduction of pathogenic flaviviruses (Gomes *et al*., 2009).

Taking into account that Portugal has also seen temperature rises as a consequence of global warming, and that outbreaks of arboviral disease have occurred in neighbouring countries it is only a matter of time until arboviral activity is detected (Abrantes and Silveira, 2009).

Taken together, these facts lead to the conclusion that Portugal faces a serious risk of introduction of exotic mosquito species, such as *Ae. albopictus*, thus increasing the possibility of arboviral transmission within the country (Almeida *et al*., 2008).

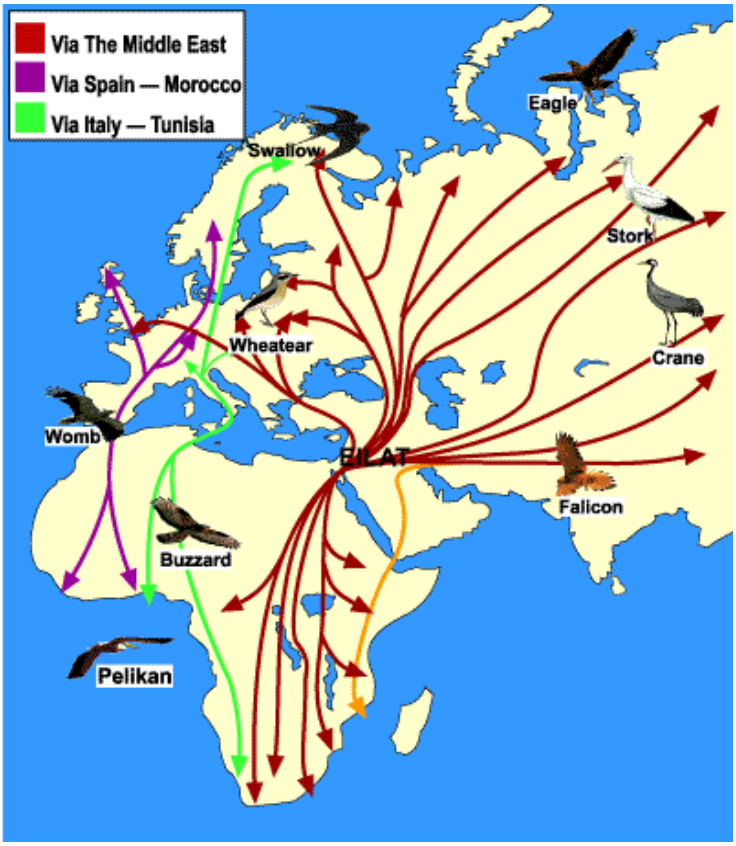


**Figure 24:** Bird migratory routes **(http://www.eilat-guide.com/birds.html - accessed on the 18th August 2011)**.

Arboviruses have previously been active and detected in the Iberian Peninsula, as is the case of West Nile virus isolated from mosquitoes collected in southern Portugal in 2004 (Esteves *et al*., 2005) and suspected human and equine cases in 2010.

In addition, WNV and Usutu virus were also detected in Spain and Italy, and outbreaks have been reported in other countries including: the WNV outbreaks in Hungary (2004-5/2010), Austria (2008), Russia (2010-11) and Northern Greece (2010-11) (figures 25 and 26) (Esteves *et al*., 2005; Parreira *et al*., 2007; Vázquez *et al*., 2011a).

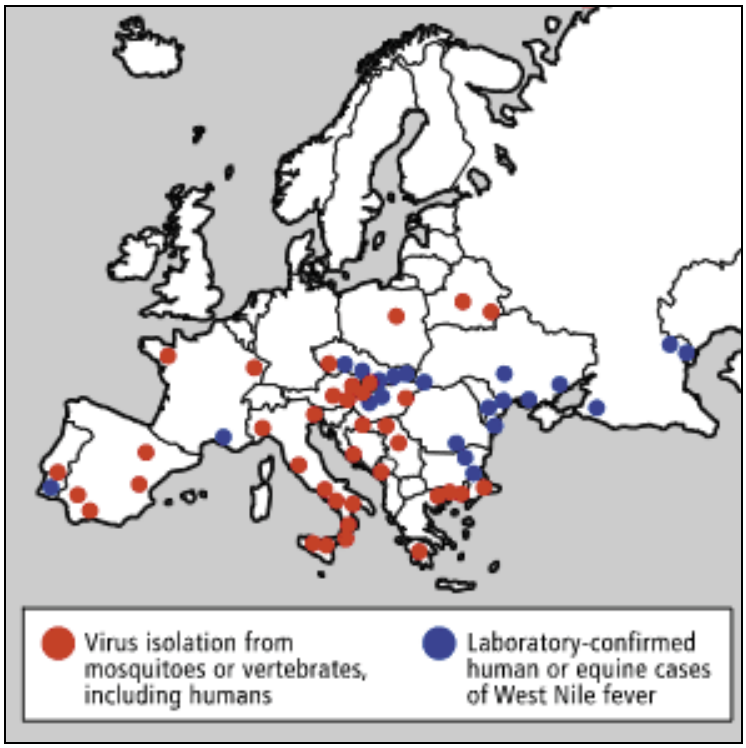


**Figure 25:** European distribution of West Nile virus based on virus isolation from vertebrates or mosquitoes **(http://www.interceptbloodsystem.com/blood-safety-and-pathogen-inactivation/new-a-emerging-pathogens.html – accessed on 18th August 2011)**

West Nile virus is now spread through many European countries via autochthonous transmission (figure 25). To further enhance public health importance regarding arboviral transmission, *Ae. aegypti* is also present in countries linked to Portugal. Cape Verde, a Portuguese colony until 1975, has recently seen a dengue epidemic that in just over a month registered six fatalities, 13,000 suspected cases with more than 3,000 confirmed, in four different islands (DREF, 2009; WHO, 2009). Furthermore, this mosquito species is also present in Madeira (Portugal), having been detected for the first time in 2004. However, and despite their presence, there is no record of endemic or autochthonous transmission of flaviviruses, up to this day (Almeida *et al*., 2007).

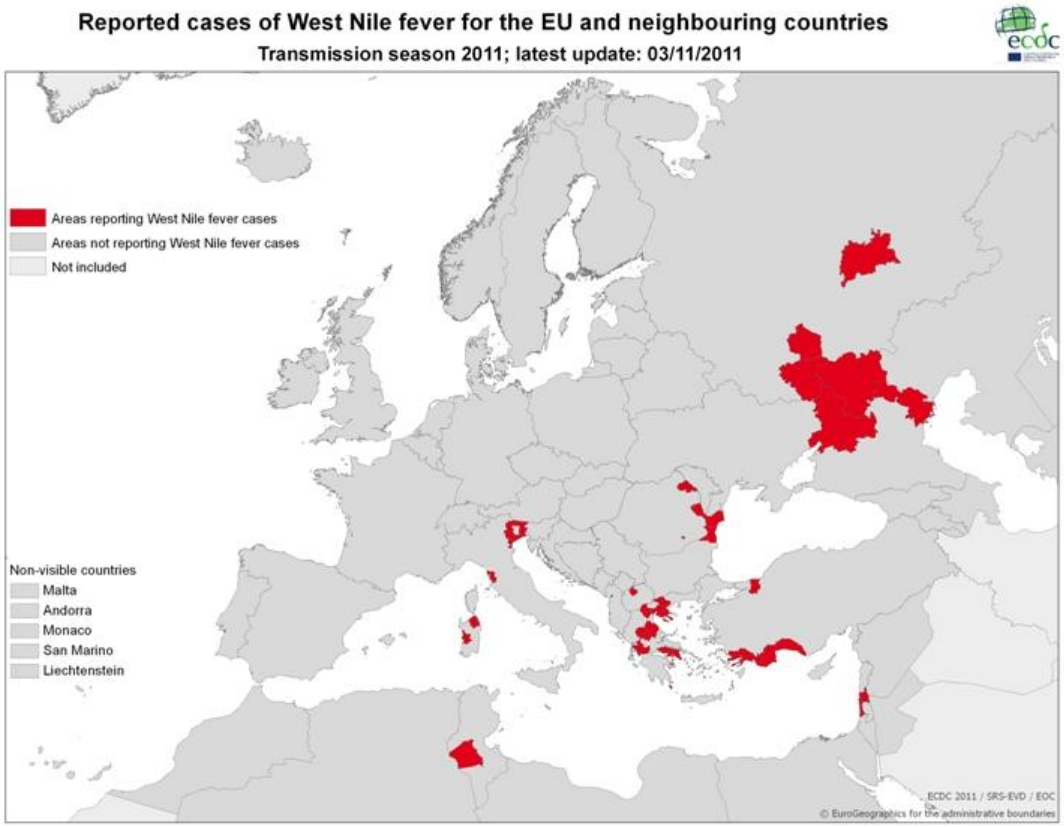


**Figure 26:** West Nile virus transmission in European countries.
**(http://ecdc.europa.eu/en/activities/diseaseprogrammes/emerging_and_vector_borne_diseases/Pages/West_Niles_fever_Risk_Maps.aspx – accessed on 4th of November 2011).**

Besides, WNV was first detected in Portugal in the 1970's but was first isolated in 1971 from *An. maculipennis* s.l. and, more recently, in 2004 from *Cx. univittatus* and *Cx. pipens* s.l., which are usually part of the normal mosquito fauna within the country, as shown on figures 27 and 28 (Almeida *et al*., 2010). West Nile virus detected in 2004 affected two Irish tourists who developed influenza-like symptoms after returning home from a holiday in the Algarve region; with one of the tourists ending up developing central nervous system infection (encephalitis) (Connell *et al*., 2004). The permanent presence of *Ae. albopictus* in Europe and *Ae. aegypti* in Madeira and Cape Verde and other potential vectors such as *Culex spp*., the continuing but seasonal outbreaks of arboviral disease within neighbouring countries, together with the autochthonous and non-autochthonous transmission of arboviruses pose a major public health risk for Portugal that calls for regular entomological and epidemiological surveillance in order to predict and, if possible, avoid major epidemics of deadly arboviral diseases.

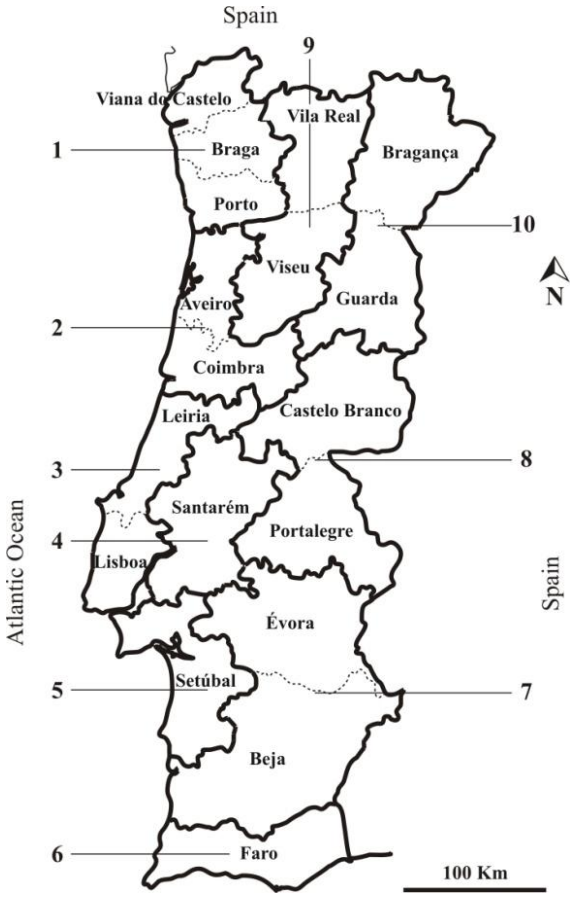


**Figure 27:** Areas where mosquitoes were collected in Portugal, by district **(Almeida et al., 2008)**.

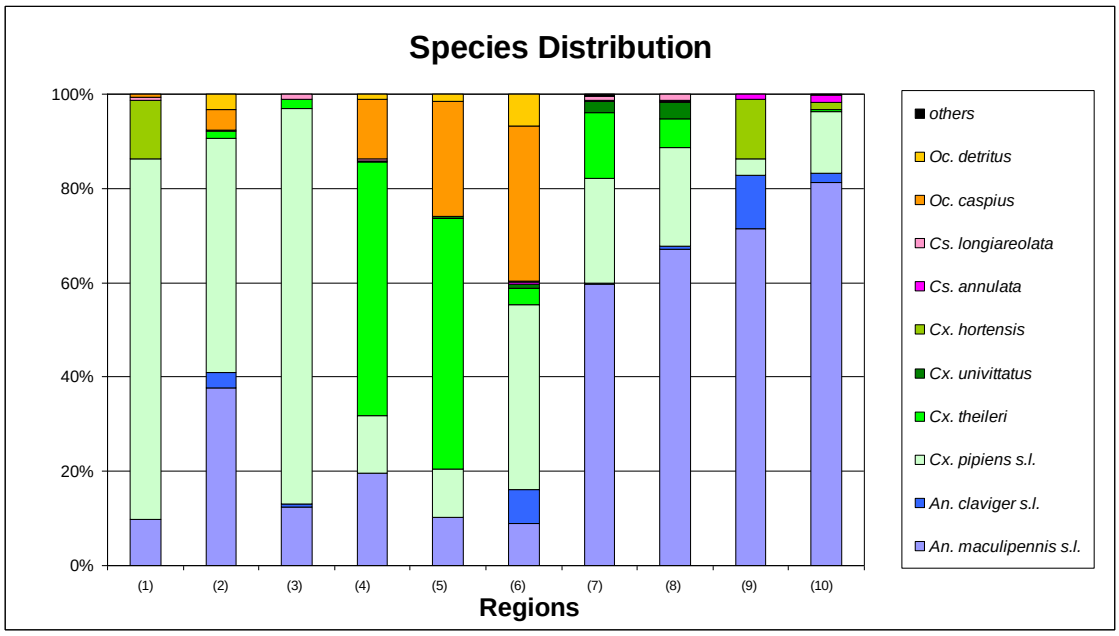


**Figure 28:** Species distribution of mosquitoes collected in Portugal **(Almeida *et al*., 2008)**.

# 2.

# OBJECTIVES

# 2. Objectives

The main purpose of this work was to search for flaviviruses in mosquitoes of two distinct areas of continental Portugal where West Nile virus and other flaviviruses have previously been found.

## 2.1 Specific objectives

- To study mosquito densities and seasonal dynamics of mosquitoes collected in wetlands of the Algarve, during two consecutive years.
- To screen collected mosquitoes, both from Setúbal and The Algarve, for flavivirus sequences.
- To study the phylogeny of potentially detected flaviviral sequences.

# 3.

# MATERIALS AND METHODS

# 3. Materials and methods

## 3.1. Mosquito surveys

Mosquito collections in the Algarve were carried out monthly, from April to October, during 2009 and 2010, in 20 locations forming three main groups: the Western, Central and Eastern Algarve, each one comprising 6 or 7 collection sites (figure 29). All areas where collections were made are located along the southernmost coast of Portugal, in lowlands and wetlands vicinity.

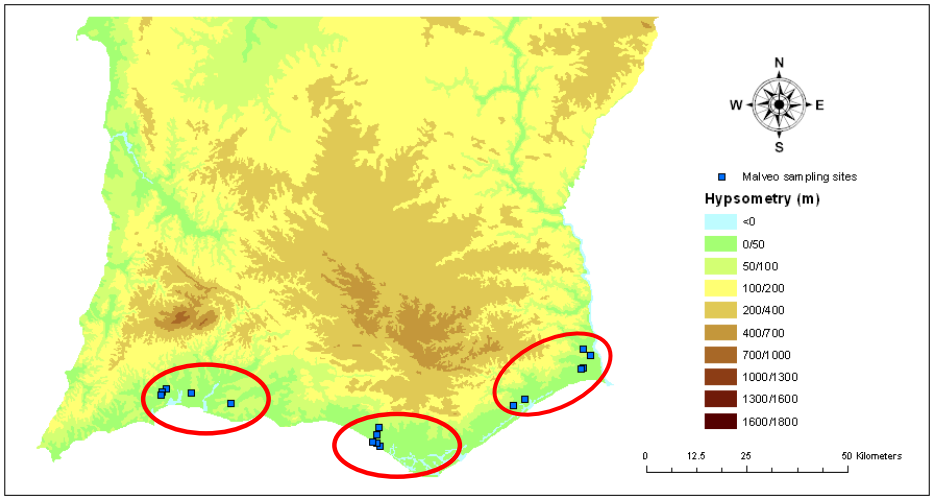


**Figure 29:** Areas where mosquitoes were collected in The Algarve region during 2009-2010.

For this thesis, all collections made in fixed sampling sites within that time period were analysed. However, for logistical reasons a few sampling sites were discontinued and therefore will not be taken into account regarding comparison of mosquito population densities. Collections made in the Setúbal district are not connected to the Algarve surveillance project; and were carried out sporadically, due to complaints of mosquito nuisance at a university in November 2009, and following the detection of suspected human and equine cases of West Nile virus in a locality, Gâmbia, within the district, in July 2010.

### 3.1.1. Characterisation of the study areas

An initial prospective study was carried out in order to select the most appropriate sampling sites, in Faro district (Algarve). Collections were also made in the district of Setúbal (City centre, Comporta, the Estate of Gâmbia); however, no prospective study was carried out. These two regions, where mosquitoes were collected, present different ecological and geographical characteristics. Nevertheless, both districts include wetlands characterised by fresh and salt-water marshes, saltpans, dunes, extensive beaches and small islands (Osório *et al*., 2010). In these areas rice cultivation and reed plantations are abundant, but large areas of cork oak (*Quercus suber L.)* and pine forest (*Pinus pinaster A.*, *Pinus pinea L.*), as well as bird sanctuaries that harbour some species of migratory birds, amongst others are also present (Almeida *et al*., 2010). These areas display the typical Mediterranean climate (according to Koppen Classification System, group C- temperate/mesothermal climates) with a humid sub-tropical climate with a dry warm summer and a mild winter. Overall, these areas comprise beneficial conditions for the establishment and maintenance of mosquito breeding sites.

### 3.1.2. Mosquito sampling

Mosquitoes were collected using CDC light traps baited with dry ice (as a source of $CO_2$ used as an olfactory stimulus) for a period of at least 12 hours (in order to include sunset and sunrise) thus covering mosquito activity peaks. CDC light traps were placed mainly outside animal shelters or in their close proximity. In addition, indoor resting (IR) mosquitoes were sampled, in every location, with the use of battery operated handheld mechanical aspirators, inside those same locations.

Field-collected mosquitoes were kept in refrigerated boxes for transportation to the Medical Entomology Laboratory at the Institute of Hygiene and Tropical Medicine, in Lisbon, where they were frozen at -80ºC. Some characteristics such as the site and time of collections, number of collectors, the presence or absence of potential hosts, both at and in the vicinity of the collection sites, as well as the duration of each IR collection, were registered for every location.

### 3.1.3. Specimen identification

Collected specimens were morphologically identified to the species level by trained staff, according to Ribeiro & Ramos (1999) identification keys. Mosquitoes were then pooled into groups of approximately 50 specimens based on their species, sex, gonotrophic stage (for females) and collection site, were assigned numbers, placed into 1.5ml flat cap tubes, and stored at -80ºC until further processing.

### 3.1.4. Mosquito density and seasonal dynamics study

Mosquito densities were determined by group, month and year according to collection method, IR (indoor resting) or CDC light traps baited with $CO_2$.

Mosquito monthly densities per group within the Algarve region were calculated as the arithmetic mean of collection yield obtained for each individual collection. Densities are presented as the number of mosquitoes captured per trap-night for CDC collections, or as the number of mosquitoes collected per collector/hour for IR collections. For all collections made in each geographical group, the mean density and the standard error were estimated.

### 3.1.5. Statistical analysis

Statistical analysis was carried out using the statistical package SPSS version 19.0 for Windows (SPSS Inc., Chicago, USA).

Kolmogorov-Smirnov and Shapiro-Wilk tests were used to analyse data for normality, while Levene's test was used to test for homogeneity of variance. Due to the lack of normality of the data, the huge standard deviations and the lack of homogeneity of variance, non-parametric tests were used to analyse mosquito densities (Siegel and Castellan, 1988). Mean densities in the different regions where compared for each year with the Kruskal-Wallis test for independent samples, and mean monthly densities were tested with Wilcoxon Signed Ranks test for related samples, thus comparing mean densities for all surveyed regions corresponding to the same month in subsequent years.

## 3.2. Viral screening

### 3.2.1. Mechanical maceration of mosquitoes

Mosquito pool maceration was performed following the protocol previously described by Huang *et al* (2001), with modifications. Briefly, for each mosquito pool to be processed a 15ml Falcon tube was prepared with 5 glass beads (previously washed with hydrochloric acid and sterilised by autoclaving), 0.25g of alumina and 1.5ml of phosphate–buffered saline solution supplemented with 4% Bovine Serum Albumin (BSA). These pools were homogenised by vortexing for about one minute each, and were then centrifuged at low speed (≅1,800 g), for 10 minutes at 4ºC. Following centrifugation, the supernatant was transferred into a 1.5ml flat-cap tube, which was centrifuged for a second time, at higher speed (≅10,400 g) for clarification. Clarified supernatant (150µl) were transferred into a 2 ml flat-cap tube and kept at -80ºC until RNA extraction. The remaining volume was distributed into two aliquots of approximately equal volume for long-term storage at -80ºC.

Alumina has two distinct functions in maceration, one is due to its sand-like texture which aids the mechanical maceration of mosquitoes; secondly it works as a matrix for the adsorption of positively charged molecules, therefore facilitating their elimination. BSA is a serum albumin protein that is used as a protein stabiliser as well as to prevent proteins from adhering to tube walls. It was used at 4% since this concentration is known to increase Polymerase Chain Reaction (PCR) sensitivity (Huang *et al*., 2001).

### 3.2.2. RNA extraction

Total RNA was extracted from mosquito homogenates using the *Instant Virus RNA Extraction Kit* (*Analytikjena*, Germany) following the manufacturer's instructions, starting with the 150µl of clarified supernatant previously stored at -80ºC.

In summary, mosquito homogenates were defrosted at room temperature and added to 450µl of lysis solution (*RL*) in an extraction tube. This procedure took place under a laminar flow cabinet of Bio Safety Level 3. This mixture was homogenised by vortexing and then submitted to 15-minute incubation, at room temperature, after which

a binding solution (*RBS*) was added. This was homogenised, and 650µl was applied onto a spin filter, where the RNA was retained, and quickly centrifuged at approximately 16,000 g, for one minute. This step was repeated for the residual volume of the RNA-containing mixture. After two washes with the washing solutions provided (*HS and LS*), the extracted RNA was eluted in 60µl of RNase-free water, added 30µl at a time, followed by incubation at room temperature (2 minute) and centrifugation (1 minute). The eluate containing the extracted RNA was split into three different aliquots with volumes of 5µl, 25µl and 30µl, respectively. The 5µl aliquot was kept for reverse transcription (RT) while the others were stored at -80ºC.

### 3.2.3. cDNA synthesis

Extracted RNA molecules were used to synthesise cDNA using the *RevertAid™ H Minus First Strand cDNA Synthesis Kit* (*Fermentas*, Lithuania), according to the manufacturer's instructions. In sum, 5µl of extracted RNA was added to a reaction mixture consisting of 1µl of random hexamer primers (mixture of oligonucleotides representing all possible hexamer sequences) and 6µl of diethylpyrocarbonate (DEPC)-treated water. This mixture was incubated in a water bath at 70ºC for 5 minutes (for RNA denaturation) and then placed in ice for an additional 5 minutes. A second reaction mix containing 4µl of reaction buffer, 1µl of *Ribolock™ RNase Inhibitor*, 2µl of dNTP Mix (10mM) and 1µl of reverse transcriptase enzyme (*RevertAid™ H Minus M-MuLV Reverse Transcriptase*, in a concentration of 200U/µl) was prepared and later added to the first one in a laminar flow cabinet. It is important to emphasize that all the reagents and samples were kept on ice at all times. Negative controls were included in each set of RT reactions in order to assess for genomic DNA contaminations.

The RT reactions took place in a Thermal Cycler (*iCycler*, BioRad, USA), in accordance with the programme shown in table 2.

**Table 2:** Conditions employed for the reverse transcription reaction.

| | Temperature | Duration | Number of cycles |
|---|---|---|---|
| **Hybridization** | 25ºC | 5 minutes | 1 |
| **Extension** | 42ºC | 60 minutes | 1 |
| **Enzyme inactivation** | 72ºC | 15 minutes | 1 |

The resultant cDNA was kept at – 20ºC, for PCR reactions and long–term storage.

### 3.2.4. Preliminary tests

Two separate preliminary tests were performed to evaluate whether the modifications made to the original protocol would still allow for the amplification of genomic viral RNA. In addition, the primers were tested to verify if they could be amplified for viral DNA amplification. In the first test, positive and negative controls were prepared. In sum, we macerated a pool of mosquitoes from a colony and spiked it with *O'Nyong nyong* virus (*Togaviridae, Alphavirus*), to serve as a positive control. Total RNA was extracted using the kit referred to in section 2.2.2. The extracted product was used for the RT and the cDNA used as matrix for amplification by PCR. For first round amplification we used the primer pair Seq3 and nsP2_RO for amplification of a fragment of 723bp, expected size, whilst for the second round, the expected fragment size was 606bp and the primers used were Seq3 and nsP2_RI. For primer sequences please refer to table 3.

After amplification and separation by electrophoresis, the gel was observed under UV light and the band pattern analysed.

**Table 3:** Oligonucleotide primers used for viral sequence amplification.

| Primer Name | Oligonucleotide Primer Sequence 5'- 3' | Primer Direction |
|---|---|---|
| **Seq3** | GTGAGAGGGGAAAGAATGGAATGGCTG | Forward |
| **nsP2_RO** | TGACCGCACATGATTGTCTTTGCAGTCC | Reverse |
| **nsP2_RI** | CGGCGTTTACCACGCATTCCTCAGTGTTC | Reverse |

For the second test, West Nile and Dengue viruses obtained from our Virology Unit were used. The RNA extraction, and RT-PCR reactions were carried out as described previously. For the PCR reactions, the primers used were Flavi1RY and Flavi2RY, for the first round, and primer pair Flavi2RY and Flavi3RY for the second round amplification reaction (please refer to the following section). Once again, the amplified product was separated by electrophoresis, the gel was observed under UV light and the band pattern analysed.

### 3.2.5. Amplification of viral sequences by PCR

After synthesis of cDNA from RNA extracted from mosquito macerates, amplification of viral sequences by PCR was carried out using the primers designed to target the highest conservation region of the flavivirus genome, the NS5 gene. However, due to high genetic diversity within the *Flavivirus*, the primers were degenerated in order to allow the detection/amplification of a larger number of flavivirus sequences. These primers were designed using, as a starting point, the primer sequences as published by Moureau *et al* (2007) and Sánchez-Seco *et al* (2005), having been modified based on the analysis of a multiple alignment sequences including pathogenic arboviruses and insect-specific flaviviruses (R. Parreira, personal communication). The degenerate primers used for amplification of flaviviruses sequences were as shown on table 4.

**Table 4:** Primers used for amplification of flavivirus sequences.

| Primer name | Primer sequences 5'-3' | Primer direction | Temperature |
|---|---|---|---|
| **FLAVI 1** | TGY RTN TAY AAC AYV ATG GG | Forward | 58.6 ºC |
| **FLAVI 2** | GTG TCC CAN CCD GCD RYR TC | Reverse | 69.6 ºC |
| **FLAVI 3** | ATH TGG TWY ATG TGG YTN GG | Forward | 59.6 ºC |

M= A+C; W= A+T; R= A+G; K= G+T; D= G+A+T; N= A+C+G+T.

In general, nested–PCR reactions use two sets of primers. The so-called inner primers target DNA sequence located within the region limited by another set of primers – the outer primers. In the first PCR reaction, the cDNA yielded by RT was used as the

matrix for amplification of viral sequences, using the outer set of primers. The following PCR reaction was carried out with the product obtained from the first reaction, using the inner set of primers, thus increasing the assay's sensitivity and specificity (since this primer set only amplifies the DNA yielded in the first reaction; figure 30). In each PCR reaction, each primer in solution is at a concentration of 0.6 pmol/µl.

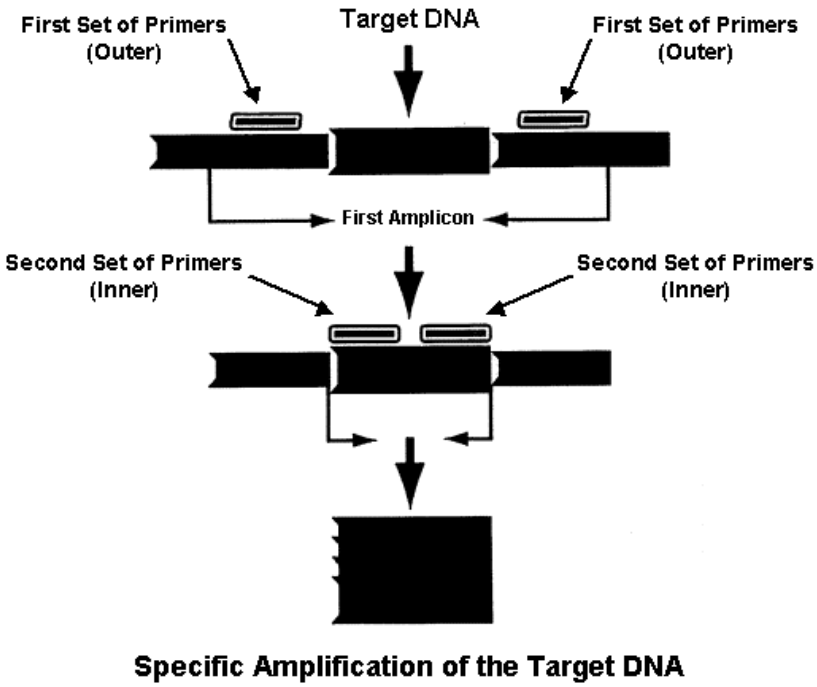


**Figure 30:** Specific amplification of viral DNA with inner and outer set of primers **(http://www.ivpresearch.org/nested_pcr.htm – accessed on 22nd March 2011)**

For PCR reactions, the commercial system *pureTaq Ready-To-Go PCR Beads* (*illustra*™, GE Healthcare, United Kingdom) was used. This included 0.2ml flat capped, thin-walled tubes containing a white bead of lyophilised reagents such as dNTPs, reaction buffer and *pureTaq* DNA polymerase. Briefly, a solution of primers was prepared for hydration of the components referred to above, making up a total volume of 25µl which consisted of 3µl of each primer (forward and reverse) and 14µl deionised water, to which 5µl of sample cDNA was added.

For flavivirus-positive samples, for DNA purification purposes, viral sequence amplification was carried out using the same kit, but making up a total volume of 50µl. In this case, each primer in solution was at a concentration of 0.4 pmol/µl.

The PCR reaction mixtures were prepared in a laminar flow cabinet, and all the reagents were kept on ice, at all times. After cDNA addition, the PCR tubes were placed on a thermal cycler (*iCycler*, Bio-Rad, USA) for amplification under the conditions presented on table 5.

**Table 5:** PCR conditions for viral sequence amplification.

| | Temperature | Duration | Number of cycles |
|---|---|---|---|
| **Initial denaturation** | 95ºC | 2 minutes | 1 |
| **Denaturation** | 94ºC | 45 seconds | 35 |
| **Annealing** | 50ºC | 45 seconds | |
| **Extension** | 72ºC | 30 seconds | |
| **Final extension** | 72ºC | 5 minutes | 1 |

Amplified PCR products were separated by electrophoresis in agarose gel at 1.5% stained with 1µg/ml of ethidium bromide, with 0.5x TAE buffer solution. A 100bp molecular weight marker (*100bp GeneRuler™ Express DNA Ladder*) was used as a reference. Finally, the gel was observed under UV light using the *Gel Doc XR System* (Bio-Rad, USA).

### 3.2.6. Purification and sequencing of PCR products

The amplicons obtained from the amplified DNA fragments were used as basis for analysis of the nucleotide sequences for the NS5 region of the flavivirus genome. The amplified products were purified using a commercial kit (*Zymoclean™ Gel DNA Recovery Kit*, Zymo Research, USA) according to the manufacturer's instructions.

Briefly, after separation in agarose gel (1.5%), the DNA fragment was excised and dissolved in a buffered solution (*Agarose Dissolving Buffer*) at approximately 55ºC, for 5 to 10 minutes. The dissolved mixture was transferred onto a column (*Zymo-Spin*™ *Column*) where the DNA was fixed, followed by two washes with *Wash buffer* that allows for the removal of non-specifically bound molecules. The DNA was eluted in 10µl of 10mM Tris-HCl, pH 8.0. In order to assess the yield of the extraction and purification processes, the purified product (1µl) was analysed by electrophoresis in agarose gel (1.5%).

STAB VIDA biotechnology company (Portugal) sequenced the purified products using one of the primers utilised for viral DNA amplification. The primers used were either the reverse primer (FLAVI 2) for products obtained from the first and second rounds reactions simultaneously, since this primer is common to both reactions; or the forward primer used for products yielded in second round amplification reactions only.

### 3.2.7. Phylogenetic analysis of viral sequences

In order to analyse the sequences obtained from the amplified products, these were aligned with reference flavivirus sequences available in free access public databases, such as *GenBank*, where our reference sequences were obtained.

BLAST (**B**asic **L**ocal **A**lignment **S**earch **T**ool) was used for basic alignment and comparison of our sequences with those of flavivirus reference. The editing and multiple alignments of sequences were performed with the aid of bioinformatics software *BioEdit Sequence Alignment Editor version 7.0.5.3* and *MAFFT version 6*, respectively. Once aligned, these sequences were used to generate phylogenetic trees obtained by the neighbour-joining method from a matrix of genetic evolutionary distances calculated using Kimura's two parameter estimate (Tamura *et al*., 2007), with the use of *Mega version 5*. Bootstrap analysis, based on 1000 replicates of the original sequence data, was carried out in order to evaluate the consistency of tree topology. The groups that displayed bootstrap values equal or greater than 70% were considered consistent form a phylogenetic perspective.

### 3.2.8 Extraction controls

In order to evaluate for contaminations, randomly selected samples were used as extraction controls; firstly, to each extracted RNA sample (5 µl), 1µl of RNase (*Ribonuclease A*, USB Corporation, USA) was added at a concentration of 0.1µg/µl. The mixture was left to incubate at room temperature for two hours. The treated samples underwent a similar RT-PCR protocol as that described in 3.2.3 and 3.2.5. PCR products of RNase-treated samples were charged into a 1.5% agarose gel containing ethidium bromide alongside the untreated samples for separation by electrophoresis.

# 4.

# RESULTS

# 4. Results

## 4.1. Mosquito collections

During this study, 36,273 mosquitoes were collected from April through October, in the wetlands of the Algarve (20 locations) and the district of Setúbal (3 locations) (figure 31).

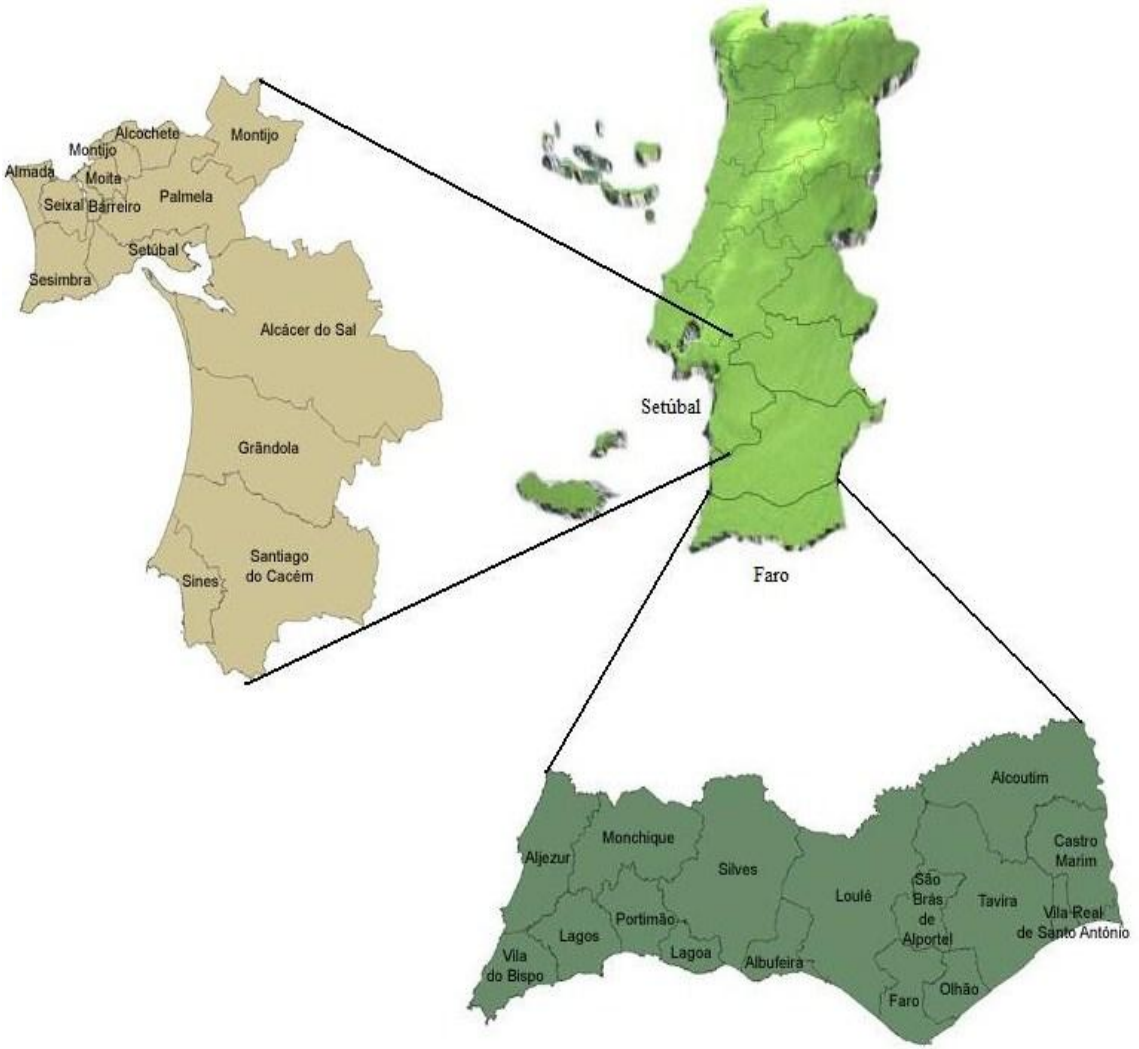


**Figure 31:** Schematic representation of the district of Setúbal and the district of Faro (Algarve) (adapted from **http://codigopostal.ciberforma.pt/** - accessed on the 20th of August 2011). Sampled areas include Lagos, Portimão, Lagoa, Albufeira, Loulé, Olhão, Tavira and Castro Marim (District of Faro) and Setúbal (District of Setúbal).

Mosquito specimens collected belonged to six genera and fifteen different species, namely *Anopheles algeriensis, An.atroparvus, Aedes berlandi, Ae. caspius, Ae.detritus, Ae. mariae, Coquillettidia richiardii, Culex laticinctus, Cx.pipiens, Cx.theileri, Cx.univittatus, Culiseta annulata, Cs.longiareolata, Cs.subochrea,* and *Uranotaenia unguiculata*.

In Setúbal 2009, a total of 135 mosquito specimens were captured; however, only two species were collected, both belonging to the same genus. The most abundant species was *Ae. caspius* with 132 specimens collected against 3 specimens of *Ae. detritus* (table 6).

**Table 6:** Summary of collections made in the district of Setúbal.

| Collected mosquito species | Total mosquitoes captured | | Total | Percentage |
|---|---|---|---|---|
| | 2009 | 2010 | | |
| ***Aedes caspius*** | 132 | 565 | 697 | 36.45% |
| ***Aedes detritus*** | 3 | 1 | 4 | 0.21% |
| ***Anopheles atroparvus*** | 0 | 114 | 114 | 5.96% |
| ***Culex pipiens*** | 0 | 106 | 106 | 5.54% |
| ***Culex theileri*** | 0 | 876 | 876 | 45.82% |
| ***Culex univittatus*** | 0 | 115 | 115 | 6.01% |
| **Sub-total** | 135 | 1,777 | 1,912 | 100.00% |

Mosquito collections in the Algarve region yielded the 35,967 mosquitoes of 15 different species (table 7). The three predominant species captured were *Ae. caspius*, *Cx. pipiens* and *Cx. theileri*, in that order. *Anopheles atroparvus* was also present in significant numbers corresponding to approximately 6% of all collections.

**Table 7:** Summary of collections made in the Algarve, 2009-2010, per geographic group.

| Collected mosquito species | Total mosquitoes collected | | | Total | Percentage |
|---|---|---|---|---|---|
| | West | Centre | East | | |
| ***Anopheles algeriensis*** | 116 | 0 | 76 | 192 | 0.53% |
| ***An.atroparvus*** | 627 | 1,175 | 306 | 2,108 | 5.86% |
| ***Aedes berlandi*** | 0 | 1 | 0 | 1 | 0.00% |
| ***Aedes caspius*** | 11,297 | 690 | 12,915 | 24,902 | 69.24% |
| ***Ae.detritus*** | 298 | 241 | 208 | 747 | 2.08% |
| ***Ae. mariae*** | 9 | 0 | 0 | 9 | 0.03% |
| ***Coquillettidia richiardii*** | 0 | 0 | 1 | 1 | 0.00% |
| ***Culex laticinctus*** | 0 | 186 | 0 | 186 | 0.52% |
| ***Cx.pipiens*** | 817 | 1,551 | 1,730 | 4,098 | 11.39% |
| ***Cx.theileri*** | 661 | 1,358 | 1,215 | 3,234 | 8.99% |
| ***Cx.univittatus*** | 11 | 123 | 15 | 149 | 0.41% |
| ***Culiseta annulata*** | 47 | 40 | 7 | 94 | 0.26% |
| ***Cs.longiareolata*** | 65 | 97 | 63 | 225 | 0.63% |
| ***Cs.subochrea*** | 11 | 2 | 5 | 18 | 0.05% |
| ***Uranotaenia unguiculata*** | 0 | 0 | 3 | 3 | 0.01% |
| **Sub-total** | 13,959 | 5,464 | 16,544 | 35,967 | 100.00% |

### 4.2. Mosquito seasonal dynamics in the Algarve, 2009-2010

For all surveyed regions within the Algarve, IR and CDC collection yield's densities were compared according to geographical group and year of collection. It is important to note that collection data for all methods displayed a wide variation, ranging from zero to extremely high numbers. Due to the lack of normality and homogeneity of the data, exemplified by large standard deviations, statistical analysis was carried out using non-parametric tests (as described in section 3.1.5.). The mosquito seasonal dynamics is analysed and presented per collection method, month/year of collection and geographical group (figures 32 to 35).

Mean mosquito densities by CDC trap collections were analysed for all three geographical groups – East, Centre and West (21 separate collection sites). These revealed that in 2009 the distribution of mosquitoes per trap-night was not significantly different between those three groups (Kruskal-Wallis test = 2.427, DF= 2, *P*= 0.297); however, in 2010 the distribution of mosquitoes per CDC was shown to be significantly different between the Western, Central and Eastern groups (Kruskal-Wallis test = 11.688, DF= 2, *P*= 0.003). Pairwise comparison of regions revealed that the Western and Eastern areas have similar mosquito distribution registering higher values of mosquitoes/trap-night, whereas their distribution in the Central group was significantly different, registering lower mosquito distribution than both other areas.

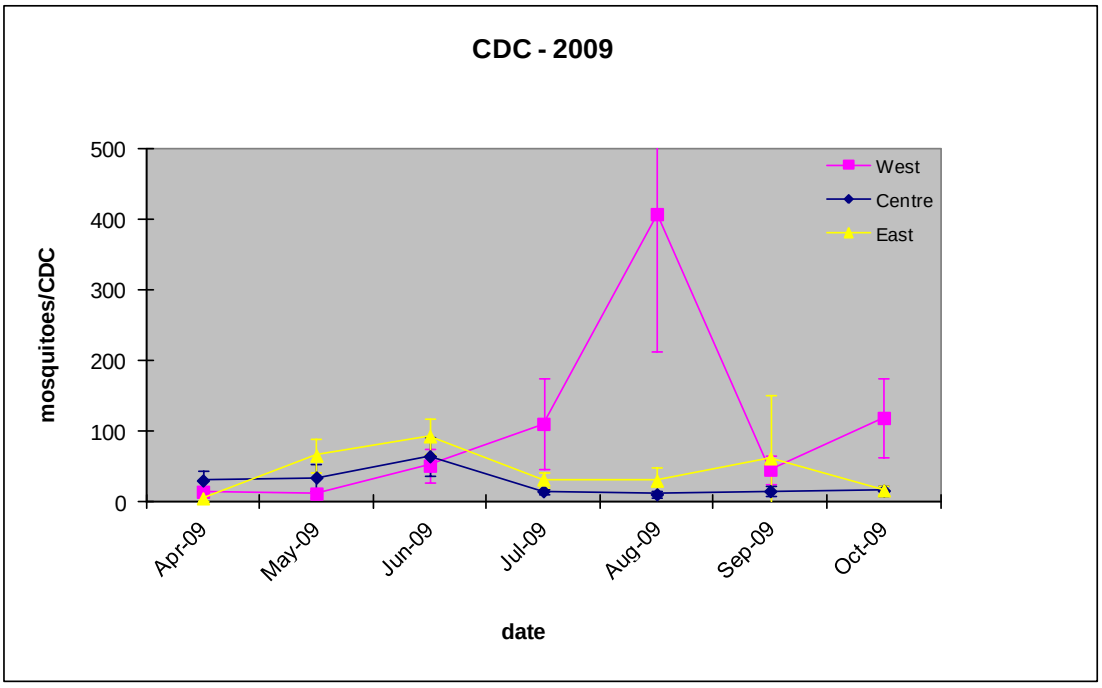


**Figure 32:** Mosquito density by CDC, in the Eastern, Western and Central groups of the Algarve region during 2009.

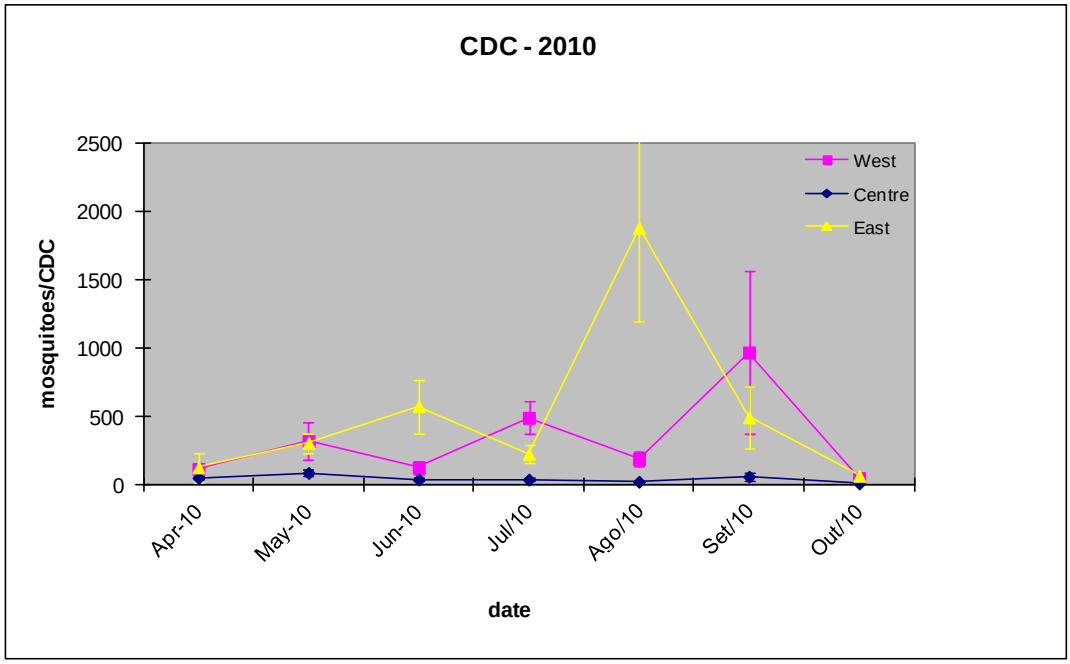


**Figure 33:** Mosquito density by CDC, in the Eastern, Western and Central groups of the Algarve region during 2010. As opposed to figure 31, the y-axis goes up to 2500 mosquitoes/CDC.

Mean mosquito densities by IR collections varied according to month and area of collection yielding peak densities between June and September, whereas the lowest densities were recorded in April and October.

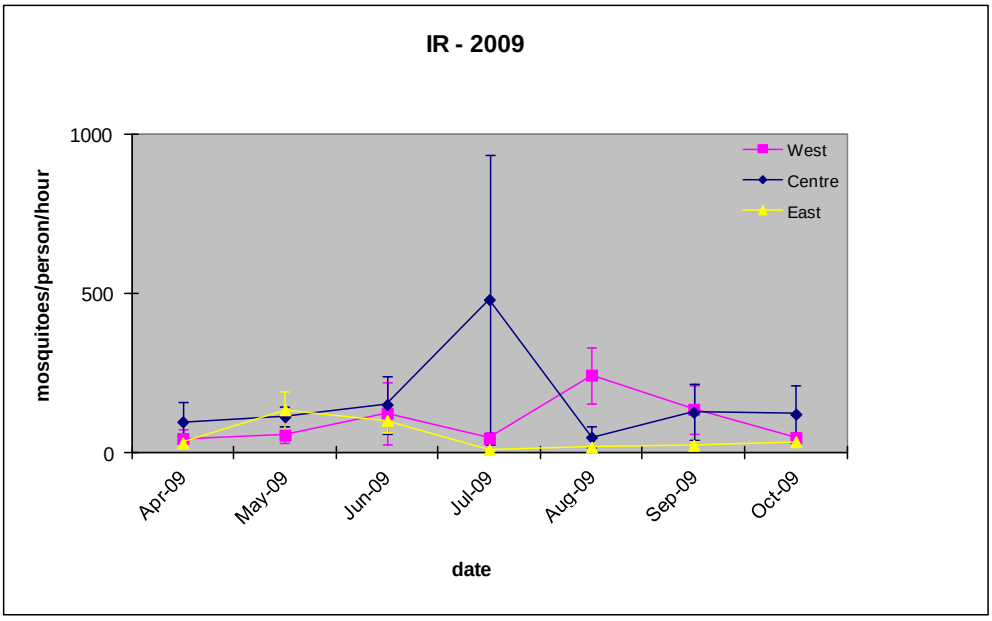


**Figure 34:** Mosquito density by IR, in the Eastern, Western and Central groups of the Algarve region during 2009.

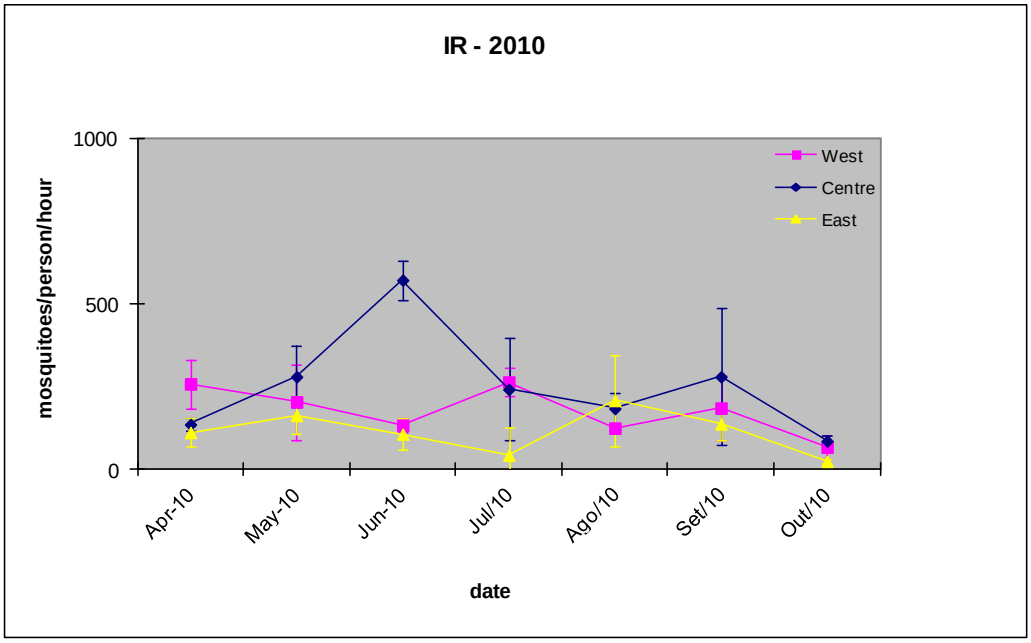


**Figure 35:** Mosquito density by IR, in the Eastern, Western and Central groups of the Algarve region during 2010.

In 2009, the yield of IR collections presented as mosquitoes per collector/hour was found to be significantly different between geographical groups (Kruskal-Wallis test = 6.731, DF= 2, *P*= 0.035). Pairwise comparisons revealed that the Western and Eastern groups have a similar distribution, registering lower values of mosquitoes/collector/hour, whereas their distribution in the Central group was significantly different, registering higher values of mosquitoes/collector/hour. However, in 2010 the yield of IR collections was similar across all geographical groups (Kruskal-Wallis test = 4.876, DF= 2, *P*= 0.087).

In this study, IR collections yielded higher mean mosquito densities in Central Algarve in 2010 in relation to 2009 (149 and 253 mosquitoes/collector /hour, respectively), followed by the West with mean densities ranging between 97 and 161 mosquitoes/collector /hour. The lowest densities yielded by the same method, were registered in the East with values comprised between 47 and 118 mosquitoes/collector/hour. Mean densities yielded by CDC traps were highest in the Eastern group, registering a maximum value of 539 mosquitoes/trap-night in 2010, as opposed to 43mosquitoes/trap-night in 2009. Western Algarve presented maximum mean densities of 328 mosquitoes/trap-night as opposed to 121 mosquitoes/trap-night in

2009, whereas the lowest values registered in the Centre, and varied between 25 and 41 mosquitoes/trap-night, in 2009 and 2010 respectively.

Mosquito mean densities per IR and CDC were compared per month and year of collection. Mean monthly values in CDC traps, were found to be significantly higher in 2010 in relation to 2009 (Wilcoxon Signed Ranks test: Z= -3.007, *P*= 0.003). Mean densities in IR were also significantly higher in 2010 in relation to 2009 (Wilcoxon Signed Ranks test: Z= -2.555, *P*= 0.011). Consequently, collections made in 2010 represented a remarkable increase in mosquito densities, when compared to the previous year, ranging from 64% (41/25) to 1153% (539/43), depending on collection method and geographical group (figure 36).

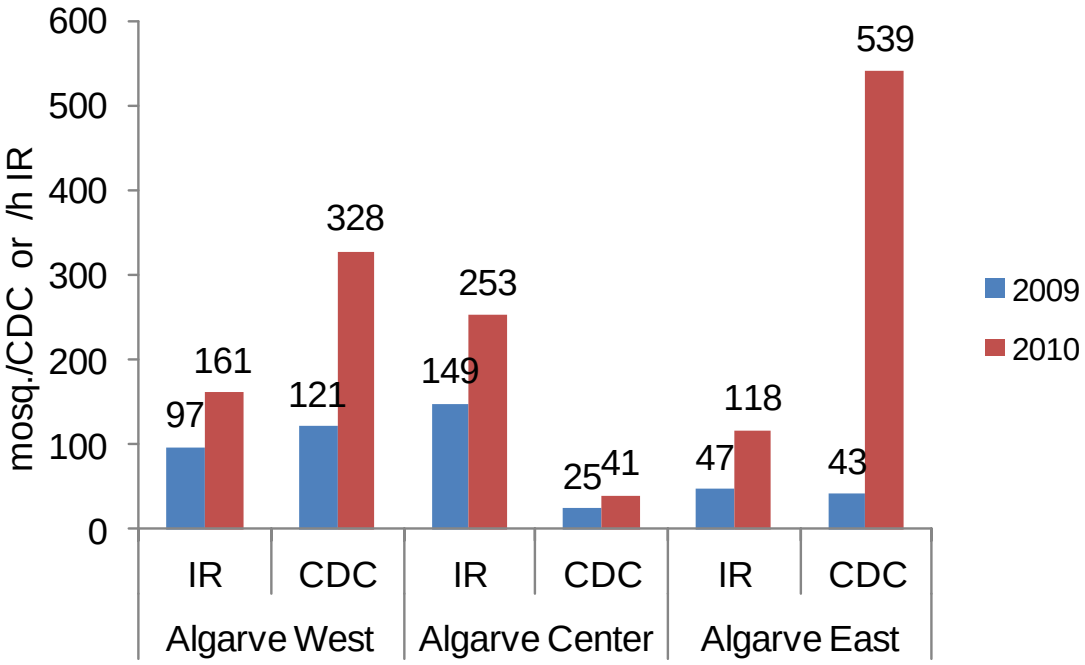


**Figure 36:** Mosquito densities by method yield, per geographical group in 2009-2010.

### 4.3. Mosquito relative species distribution in the Algarve, 2009-2010

During 2009, in the Algarve, a total of 6,677 mosquitoes belonging to six genera and eleven different species were captured. The most abundant species were *Ae. caspius*, *Cx. pipiens* and *Cx. theileri*, respectively (figure 37). In 2010, collections yielded markedly higher numbers of mosquitoes, over 29,000 specimens. The most abundant species were the same as in the previous year; however, *Culex theileri* was more abundant than *Culex pipiens* (figure 38).The relative proportion of all mosquito species collected in the Algarve in 2009 and 2010 are represented below (figure 37 and 38). However, mosquito species distribution varied according to collection methods and, within these, according to geographic group.

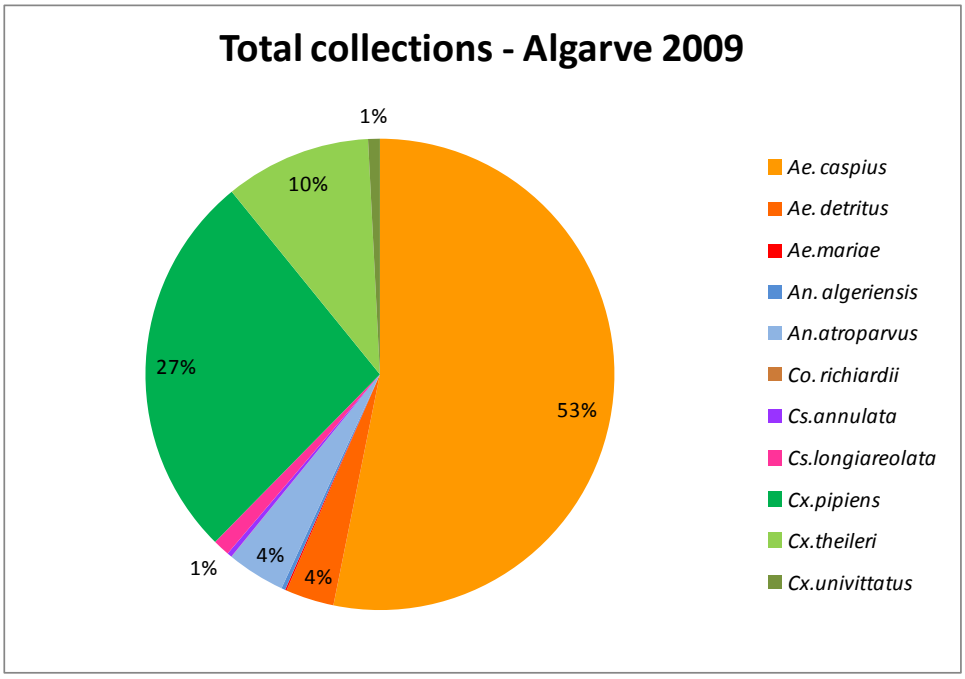


**Figure 37:** Mosquitoes species collected in the Algarve, during 2009.

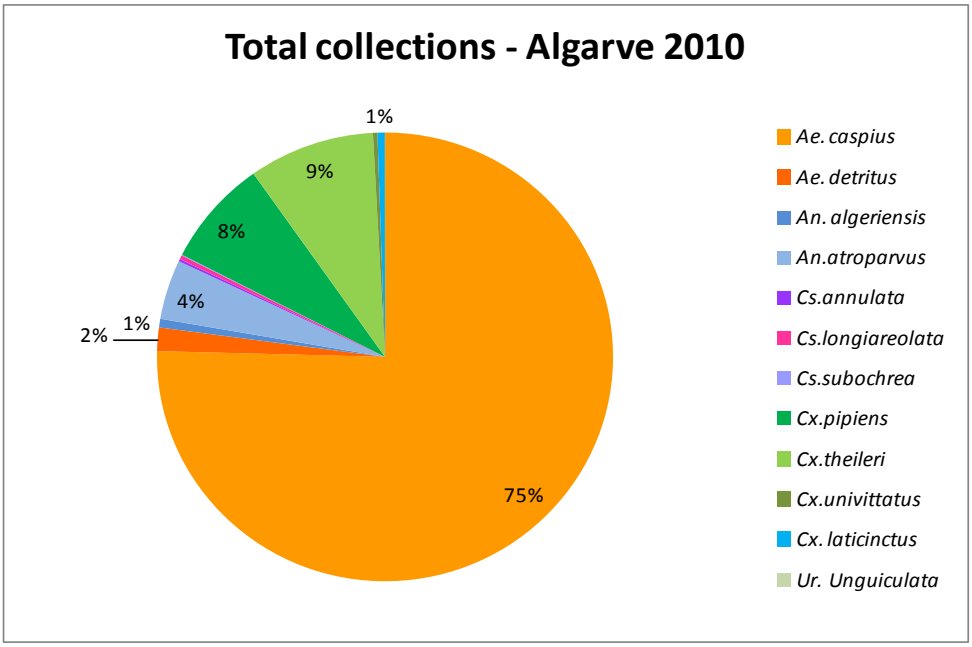


**Figure 38:** Mosquitoes species collected in the Algarve region during 2010

The relative proportions of mosquito species collected by CDC traps, in 2009 and 2010, are represented below by year and geographical group (figure 39 and 40). The predominant species was *Cx. pipiens*, in 2009, in the East and Central groups. Whereas, in the following year, *Aedes caspius* was the prevalent species in the Western and Eastern groups. Although *Culex theileri* was the prevalent species in the Centre in 2010, a significant increase in *Aedes caspius* proportions was observed.

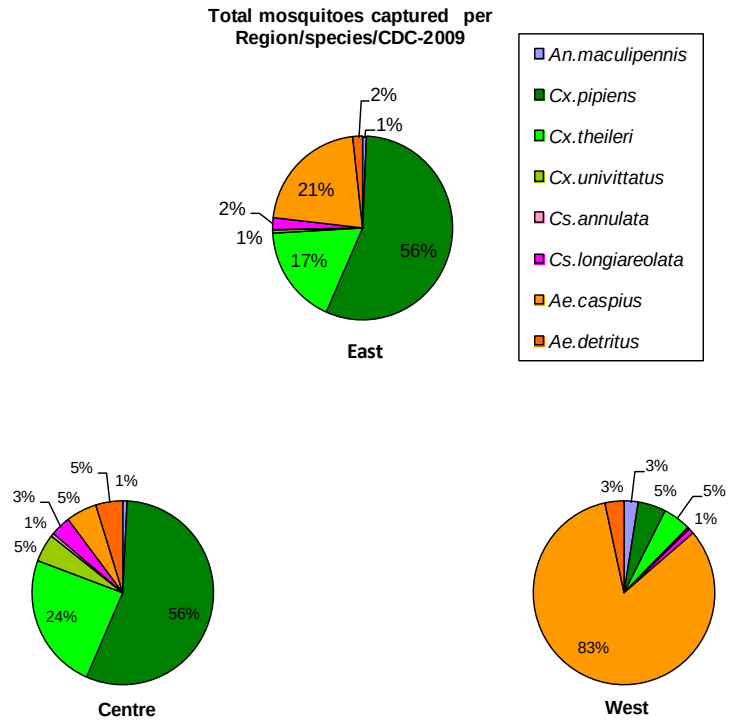


**Figure 39:** Relative proportion of mosquito species captured by CDC traps in 2009.

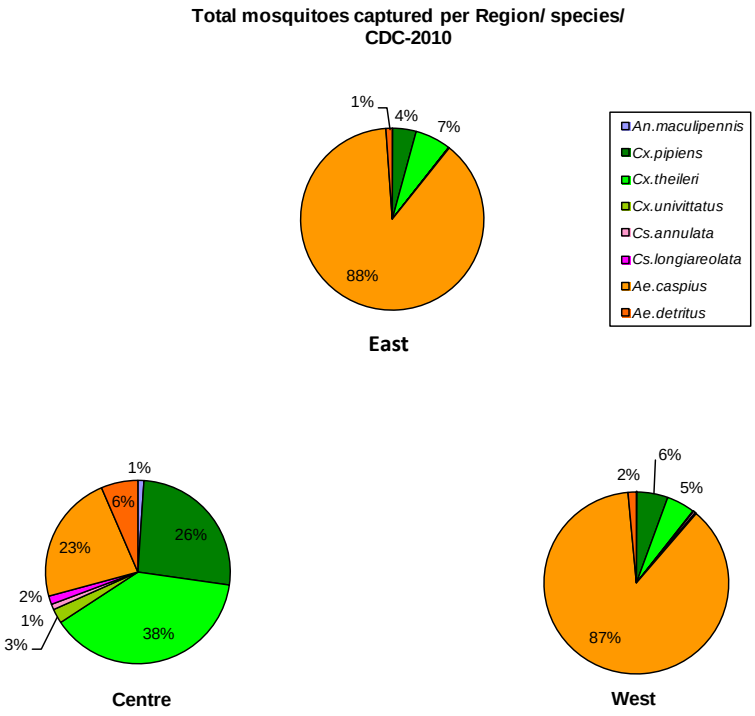


**Figure 40:** Relative proportion of mosquito species collected by CDC traps in 2010.

The relative proportions of mosquito species collected by IR are represented below (figure 41 and 42). Overall, the predominant species collected by this method in both years and in all the geographical groups, except the Eastern group in 2010, was *An. atroparvus*. However, an increase in *Aedes caspius* was seen in the Eastern and Western groups.

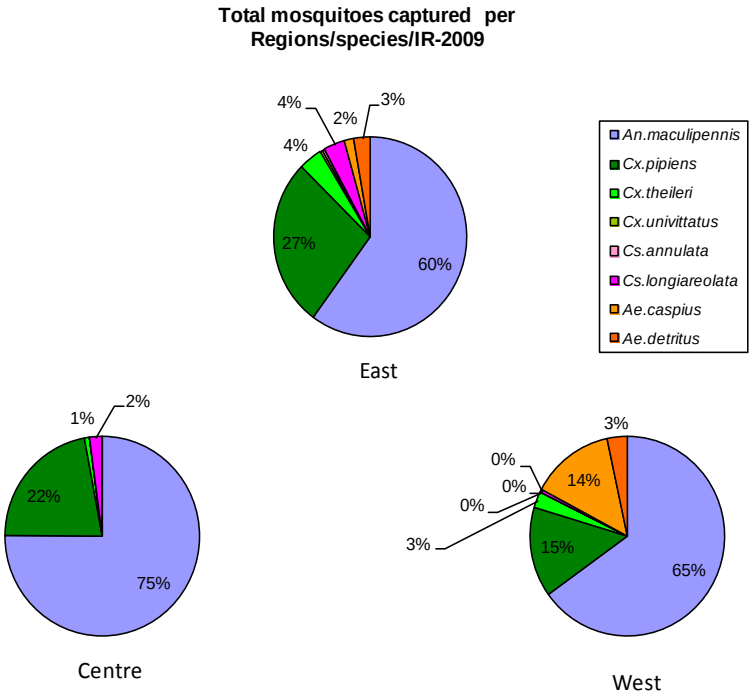


**Figure 41:** Relative proportion of mosquito species captured by IR in 2009.

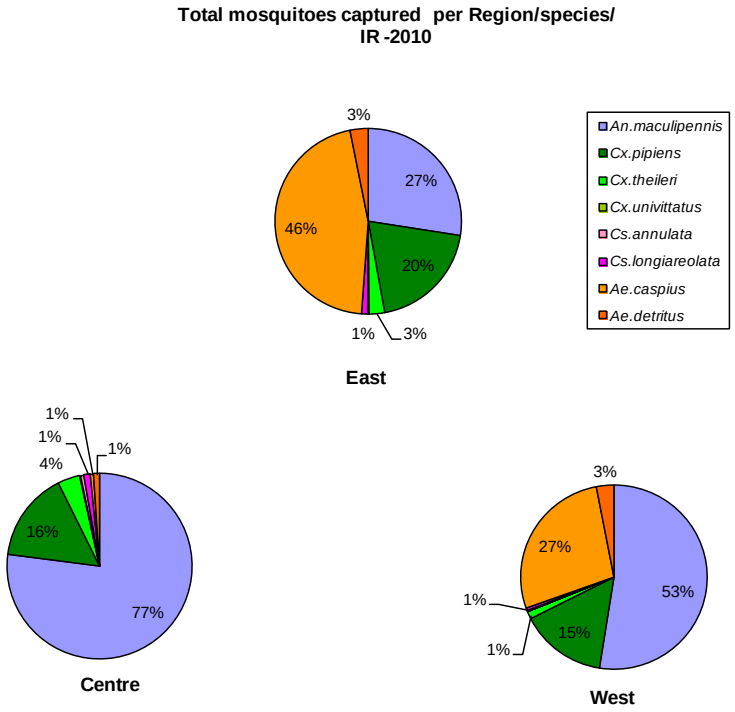


**Figure 42:** Relative proportion of mosquito species captured by IR in 2010.

### 4.4. Mosquito screening for flaviviral sequences

From 36,273 mosquitoes collected, 35,420 were screened for flaviviruses. These included all species named on the table 8 below, including male and female, fed and unfed. Mosquitoes collected were grouped into pools according to species, sex and gonotrophic stage.

**Table 8:** Summary of mosquitoes processed for flaviviruses.

| Collected mosquito species | Mosquitoes analysed | | Total | Percentage |
|---|---|---|---|---|
| | Setúbal | Algarve | | |
| ***Anopheles algeriensis*** | 0 | 188 | 188 | 0.53% |
| ***Anopheles atroparvus*** | 114 | 1,407 | 1,521 | 4.29% |
| ***Aedes caspius*** | 697 | 23,798 | 24,495 | 69.16% |
| ***Aedes detritus*** | 3 | 687 | 690 | 1.95% |
| ***Aedes mariae*** | 0 | 7 | 7 | 0.02% |
| ***Coquillettidia richiardii*** | 0 | 1 | 1 | 0.00% |
| ***Culex laticinctus*** | 0 | 90 | 90 | 0.25% |
| ***Culex pipiens*** | 102 | 3,860 | 3,962 | 11.19% |
| ***Culex theileri*** | 863 | 3,141 | 4,004 | 11.30% |
| ***Culex univittatus*** | 115 | 129 | 244 | 0.69% |
| ***Culiseta annulata*** | 0 | 67 | 67 | 0.19% |
| ***Culiseta longiareolata*** | 0 | 134 | 134 | 0.38% |
| ***Culiseta subochrea*** | 0 | 15 | 15 | 0.04% |
| ***Uranotaenia unguiculata*** | 0 | 2 | 2 | 0.01% |
| **Total** | 1,894 | 33,526 | 35,420 | 100.00% |

The vast majority of mosquito pools analysed included *Ae. caspius* species that comprises more than 69% of all processed mosquitoes, followed by *Cx. theileri* and *Cx. pipiens* with around 11%. A significant proportion of *An. atroparvus*, corresponding to over 4% of all collections was also analysed. Apart from *Ae. detritus* (1.95%), all other species analysed represented less than 1% of the total number of mosquito species processed for flaviviruses. These mosquitoes were macerated in order to obtain a clear homogenate from which RNA could be extracted.

### 4.4.1. RNA extracted from mosquito homogenates

The clarified mosquito homogenates obtained from the maceration process were used for the extraction of total RNA. During this process, unspecific binding of, for example, soluble macromolecular cellular components, salts and metabolites to the extraction column where RNA is fixed usually occurs and that can potentially cause inhibition of subsequent RT-PCR reactions. Even though this procedure is carried out to eliminate unspecific bound molecules, some potential contaminants may not be removed. Therefore, in order to ensure that no contaminants are present in the samples and the extracted RNA was viral and, consequently, the positive pools obtained were a result of viral DNA amplification extraction controls were carried out. Due to cost restrictions, extraction controls were performed only for a few, randomly selected samples. These RNA extracts were first treated with Ribonuclease A before undergoing a retro-transcription reaction in order to assess whether all RNA had been digested, and consequently if any cDNA would be synthesized. The RT reaction product was used as matrix for amplification and the PCR products were subject to electrophoresis. After separation in agarose gel with ethidium bromide, it was possible to observe that none of the RNAse-treated samples produced any bands, thus confirming that the there were no genomic DNA contaminants in our samples suggesting that there was viral RNA only.

### 4.4.2. Amplification reactions preliminary tests

The first preliminary test was carried out in order to verify whether there was any step in the protocol that would, somehow, interfere with our ability to detect viral RNA in the analysed samples. For this, two types of experiments were performed.

In the first, a mosquito macerate including mosquitoes maintained in a colony at IHMT was spiked with *O'Nyong nyong* virus GULU strain (*Togaviridae, Alphavirus*) genomic RNA. Total RNA was then extracted from the clarified macerate, and submitted to RT-PCR, using specific primers. After amplification and separation by electrophoresis, the gel was observed under UV light. Analysis of the band pattern obtained revealed the expected size bands indicating that the detection of *O'Nyong nyong* virus RNA had been successful.

A second preliminary test was carried out in order to verify if the Flavi1RY, Flavi2RY and Flavi3RY oligonucleotide primers would amplify viral RNA. For this test two positive controls namely Dengue virus type 2 (from our Virology Laboratory) and West Nile virus isolated in our laboratory in 2004 (Esteves *et al*., 2005) were used. The main aim was to verify whether the positive controls would yield an amplified DNA fragment that, after separation in agarose gel (1.5%) and under UV light, would produce a 200bp band, as expected for known flaviviruses with the primers used. The RNA extraction, retro-transcription and PCR reactions were carried out as described previously. For the PCR reactions, the primers used were Flavi1RY and Flavi2RY, for the first round, and primer pair Flavi2RY and Flavi3RY for the second round amplification reaction (please refer to section 3.2.5, table 5). As observed in figure 43, we were able to amplify viral DNA sequences corresponding to the WNV and DENV-2 used as positive controls (1:1 and 1:100), which yielded expected size bands of 270bp and 200bp for first and second round amplification reactions, respectively (after electrophoresis). These results suggest that the primers used were suitable for amplification of flavivirus sequences and could be applied in general.

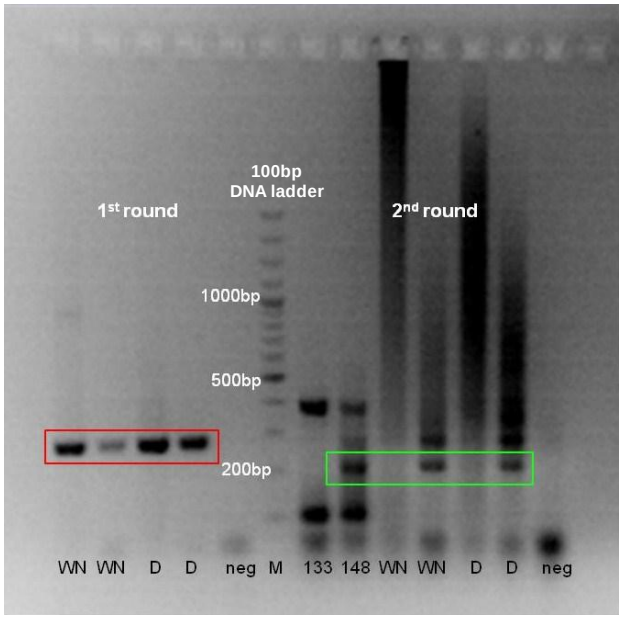


**Figure 43:** Viral DNA amplification.

**Red box:** 270bp size band expected for first round amplifications; **Green box:** 200bp size band expected for second round amplifications. **WN:**West Nile virus; **D:** Dengue virus; **Neg:** negative control; **M:** molecular weight marker.

Since the negative controls produced no bands, we can also conclude that there was no contamination during the course of reactions performed. Once the primers were confirmed suitable for amplification of viral DNA, we proceeded to the analysis of our mosquito pools.

A total of 745 pools were subsequently processed for the presence of flaviviruses, particularly the medically important members of the *Flavivirus* genus. Those 745 pools comprised mosquitoes collected in 2009, which were grouped into 148 pools and mosquitoes collected in 2010, which allowed us to form four times more pools than the previous year - 597 pools (table 9).

**Table 9:** Mosquito pools screened for flaviviruses, per year of collection and positivity rates.

| Year of collection | Mosquitoes screened | Total of pools | Positive pools | % positive pools |
|---|---|---|---|---|
| **2009** | 6,812 | 148 | 47 | 32 |
| **2010** | 28,608 | 597 | 187 | 31 |
| **Total** | 35,420 | 745 | 234 | 31 |

From the 745 pools screened for flaviviruses, 234 produced positive flavivirus RT-PCR amplification results, corresponding to an overall positivity rate of around 30% for all processed mosquito pools.

Minimum infection rate (MIR) was calculated for all positive pools. According to CDC, MIR is an estimate of infection rates, usually presented as the number of infected mosquitoes per 1000 tested, calculated as follows:

(number of positive pools/total specimens tested) x 1000

assuming that a positive pool includes a minimum of one infected mosquito (CDC, 2011).

MIR was calculated for positive pools of mosquitoes captured in both the Setúbal and Faro districts, during 2009-2010, and the results are shown on the graphs 44 and 45 below.

In the Algarve region, in 2009, the lowest MIR values were recorded for the following species: *Cx. pipiens* (MIR= 2.24/1000), *Ae. caspius* (MIR= 3.66/1000) and *Ae. detritus* (MIR= 8.97/1000). The highest values were registered for *An. algeriensis* with a MIR of more than 50 infected mosquitoes per 1000 tested, followed by *Cx. univittatus* and *An. atroparvus*, both showing MIR values of around 20 infected mosquitoes in a thousand.

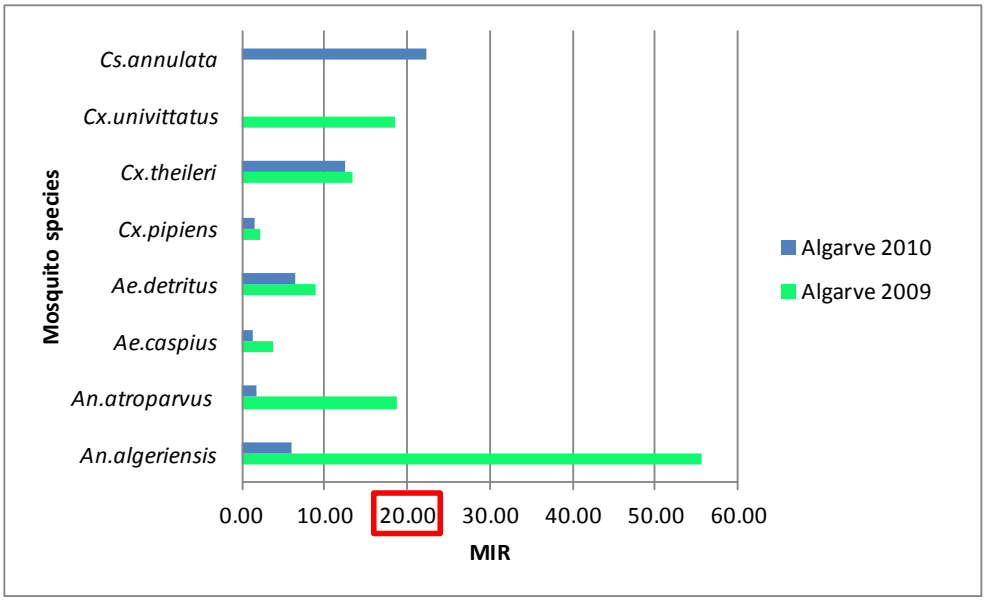


**Figure 44:** Minimum infection rates per species, in the Algarve 2009-2010.

In 2010, the highest MIR values were obtained for *Culiseta annulata* (22.22 mosquitoes infected/1000 tested) and *Cx. theileri* with approximately 12.6 infected mosquitoes in a thousand. The lowest registered MIR values belonged to *Ae. caspius* (MIR= 1.23/1000), *Cx. pipiens* (MIR= 1.44/1000) and finally *An. atroparvus* (MIR= 1.68/1000). *Anopheles algeriensis* and *Ae. detritus* showed similar MIR values of around 6 infected mosquitoes per a thousand tested.

Regarding infection rates for mosquitoes collected in Setúbal during 2009, the only species for which the MIR was calculable was *Ae. caspius* registering approximately 7.6 infected mosquitoes in a thousand.

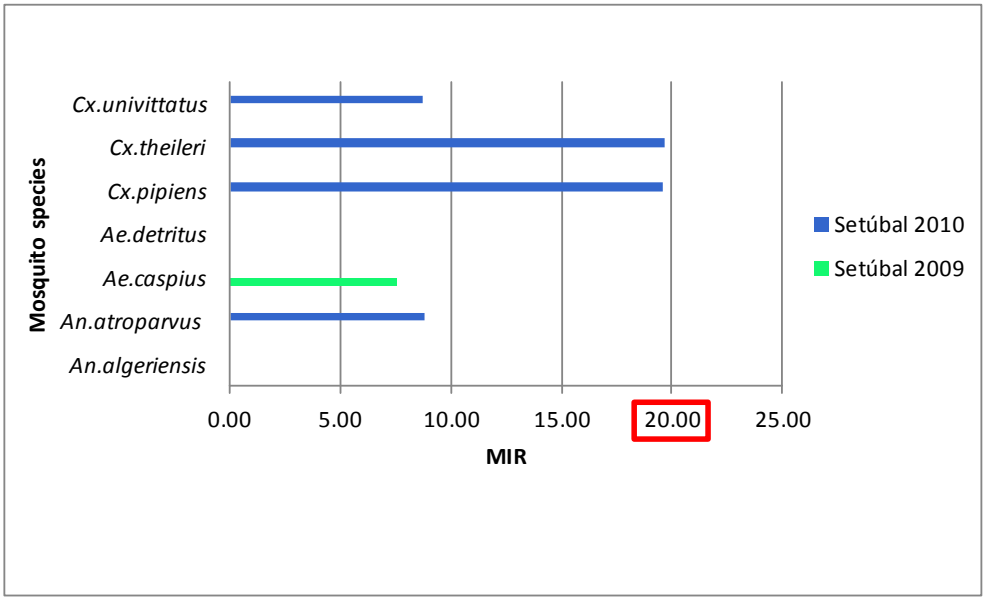


**Figure 45:** Minimum infection rates per species, in Setúbal 2009-2010.

The highest minimum infection rate was registered for *Cx. theileri* and *Cx. pipiens*, both containing around 20 infected mosquitoes per a thousand tested; whereas the lowest MIR values belonged to *Cx. univittatus* and *An. atroparvus* achieving an estimate of 9/1000.

To conclude, overall MIR of screened pools were similar for both years and locations with maximum values of approximately 20 infected mosquitoes per a thousand, except for *An. algeriensis* for which the calculated MIR was the highest of all, corresponding to approximately 56 infected mosquitoes in a thousand specimens tested. This was due to the fact that low number of mosquitoes were captured. Only two pools were prepared, of which one was positive, corresponding to 50% of positivity for this species.

Selected positive pools were re-amplified in order to obtain a higher concentration of DNA for purification. Purified DNA was obtained by excision and purification of either the 270bp or the 200bp amplicon (whichever was strongest) yielded on the first or second round amplification reactions (figure 46).

However, after purification, not all positive samples contained enough pure DNA to produce a band when 1µl of the purified product was charged into an agarose gel (1.5%) to evaluate yield of the purification reaction. Consequently, those samples were not used for further study, while some of the samples that yielded positive results consistently were sent for sequencing.

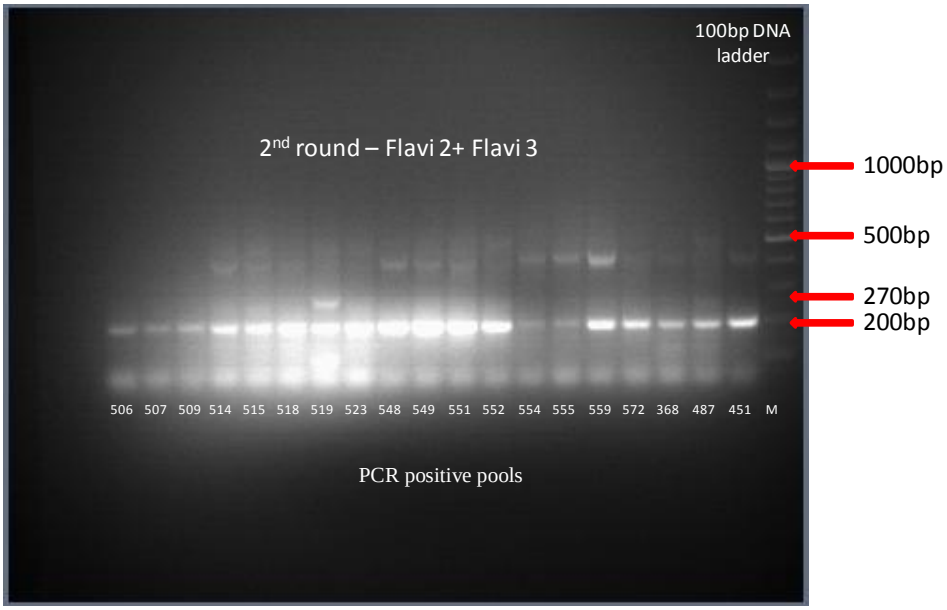


**Figure 46:** PCR amplification of positive pools for DNA purification. **M:** Molecular weight marker; the numbers represent the flavivirus-positive pools.

Out of the 234 pools that tested positive for flaviviruses, only 32 NS5 amplicons were sequenced. Among those, there were DNA fragments obtained from pools of *Ae. caspius*, *Ae. detritus*, *An. atroparvus*, *Cx. pipiens*, *Cx. theileri* and *Cx.univittatus*. However, after sequence analysis, the majority of sequences were excluded, as they did not present enough quality to be included in phylogenetic inference studies. In total, 11 out of 32 sequences were used for further analysis; these belonged to 3 different species, *Ae. caspius* (n=5), *Cx. pipiens* (n=1) and *Cx. theileri* (n=5).

Sequence analysis was based on the comparison of a short fragment (270bp or 200bp) of the viral sequence amplified from the highly conserved region of the flavivirus genome, the NS5 gene, with reference sequences deposited in public databases.

BLAST was used to perform similarity searches against the flavivirus reference sequences. Sequence analysis via BLAST (high similarity search) revealed that all our sequences showed significant similarity with insect-specific flavivirus genome sequences deposited in databases. The results of this search are shown on the table 10 below.

**Table 10:** Flavivirus sequences identity results.

| Pool number | Mosquito species | Related sequences (NS5 gene) | Query coverage | Maximum identity |
|---|---|---|---|---|
| **188** | *Aedes caspius* | Mosquito flavivirus isolate OccaFV5 | 78-90% | 93-98% |
| | | Mosquito flavivirus isolate FE-Ac3 | 78-90% | 93-98% |
| **207** | | Mosquito flavivirus isolate FE-Ac2 | 78-90% | 93-98% |
| | | Mosquito flavivirus isolate FE-Ac1 | 78-90% | 93-98% |
| **219** | | Mosquito flavivirus isolate FE-Cp | 78-90% | 93-98% |
| | | Culicinae flavivirus PoMoFlav_A95 | 78-90% | 93-98% |
| **350** | | Mosquito flavivirus isolate OccaFV1 | 78-90% | 93-98% |
| | | Mosquito flavivirus isolate OccaFV4 | 78-90% | 93-98% |
| **357** | | Culicinae flavivirus PoMoFlav_A163 | 78-90% | 93-98% |
| | | Culicinae flavivirus PoMoFlav_A155 | 78-90% | 93-98% |
| | | Culicinae flavivirus PoMoFlav_A153 | 78-90% | 93-98% |
| | | Culicinae flavivirus PoMoFlav_A136 | 78-90% | 93-98% |
| | | Culicinae flavivirus PoMoFlav_A128 | 78-90% | 93-98% |
| | | Culicinae flavivirus PoMoFlav_A126 | 78-90% | 93-98% |
| | | Flavivirus Phlebotomine/76/Arrabida/07 | 78-90% | 93-98% |
| | | Culicinae flavivirus PoMoFlav_A103 | 78-90% | 93-98% |
| | | Culicinae flavivirus PoMoFlav_A91 | 78-90% | 93-98% |
| | | Mosquito flavivirus isolate OccaFV3 | | |
| **387** | *Culex pipiens* | *Cx. theileri* flavivirus PoMoFlav | 83% | 96% |
| | | Wang Thong virus | 81% | 90% |
| **871** | *Culex theileri* | *Cx. theileri* flavivirus PoMoFlav | 80% | 95% |
| | | Wang Thong virus | 78% | 89% |
| **869** | *Culex theileri* | *Cx. theileri* flavivirus | 80-81% | 96-97% |
| **200** | | PoMoFlav_A131 | | |
| **154** | | | | |
| **383** | | | | |

### 4.5. Phylogenetic analysis of flaviviral sequences obtained

Classification of flaviviruses is an issue yet to be resolved, which has been complicated by the inclusion of "non-classical" flaviviruses such as the heterogeneous group that make up ISFs as members of the genus.

Following the production of multiple alignments of flavivirus sequences, they were used to generate phylogenetic trees in order to ascertain the relatedness of the viral sequences obtained in the course of this work, and those of numerous other flaviviruses.

Moreover, it is noteworthy that these sequences were compared and analysed based on pairwise nucleotide sequence identity of a short and highly conserved fragment of the flavivirus genome, which does not allow many conclusions to be drawn from tree topology alone. In addition, in this tree, some members of the *Flavivirus* genus such as the YFV, Apoi virus, Sepik virus and Kamiti River virus, among others, were excluded since they added entropy to the tree thus diminishing tree robustness.

All sequences obtained in this study from pools of *Ae.caspius*, *Cx. theileri* and *Cx. pipiens* were included into the ISF group (figure 47). These sequences formed a sister group separate from the medically important flaviviruses such as TBEV, WNV and DENV.

The sequences detected in *Ae. caspius* grouped together and, not only did they show close relation to "Culicinae flaviviruses" sequences detected in Portugal (direct submission, accession numbers displayed on the tree) but they were also closely related to "Mosquito flaviviruses" (Calzolari *et al*., 2010a; Calzolari *et al*., 2010b) detected in Italy. In both occasions, the sequences were obtained from a group of various species including *Ae. caspius*, *Cx. pipiens*, *Cx. theileri* and *Cx. univittatus*.

Our sequences also showed similarities to sequences from "Mosquito flavivirus" derived from either *Culex* or *Aedes* species mosquitoes, whereas a more distant relation to the group composed of *Culex* flavivirus (CxFv), *Aedes* flavivirus (AeFv) and CFAV was revealed.

The sequences detected from *Culex* species mosquitoes were incorporated in a large group that includes *Culex theileri* flavivirus sequences, CFAV and AeFv (figure 47).

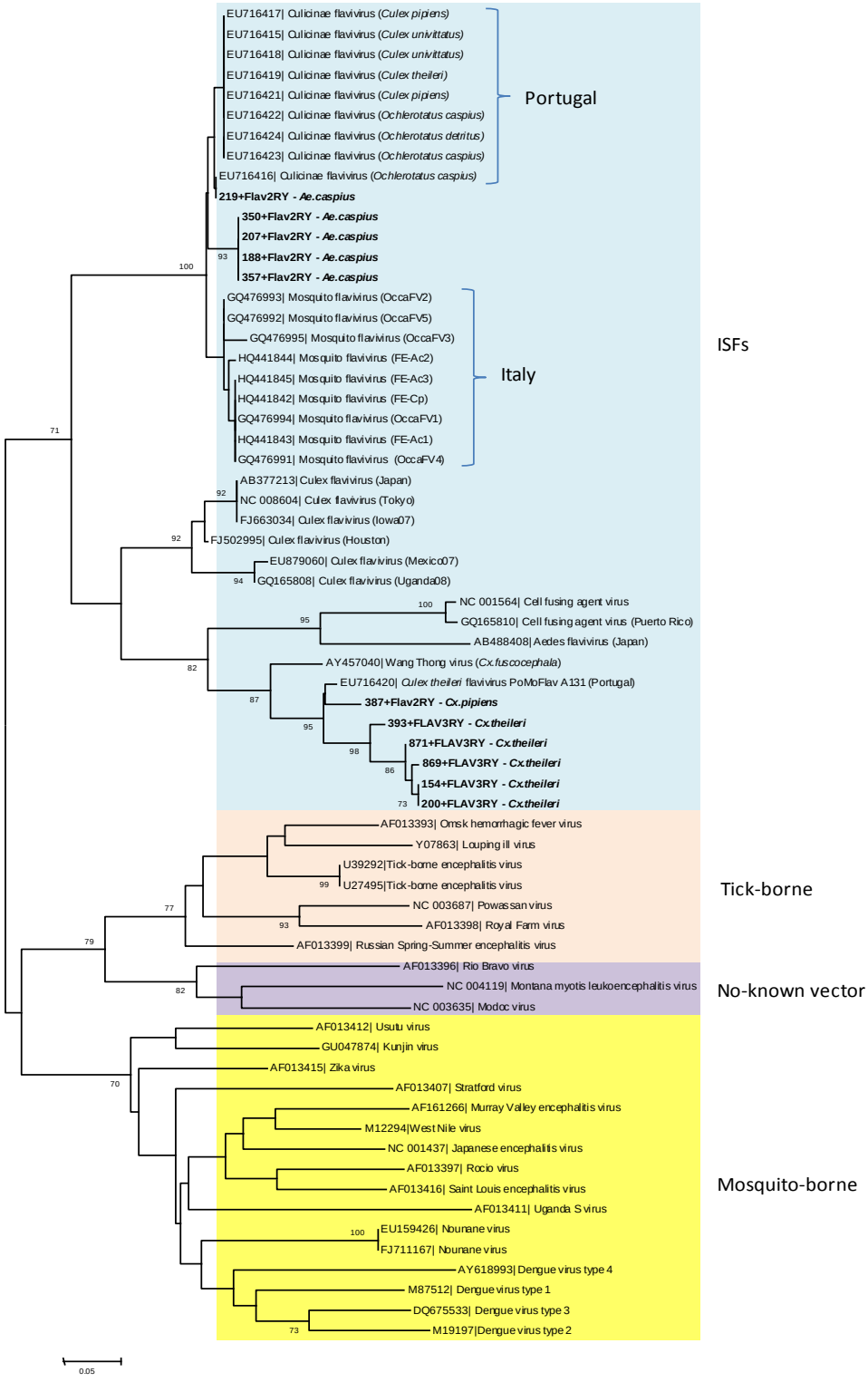


**Figure 47:** Neighbour-joining tree based on the analysis of a short fragment of the NS5 gene. Bootstrap analysis was based on a thousand replicates of original sequence data. Groups presenting bootstrap values below 70 are not shown. Sequences in bold correspond to those detected in this study. Accession numbers of sequences used are displayed on the tree.

The sequences detected from *Cx. pipiens* and *Cx. theileri* were incorporated close to one another within the ISF group, as expected. They show higher sequence identity to a *Culex theileri* flavivirus sequence (95-97%) from Portugal and to Wang Thong virus (89-90%) than to the CFA/AeFv group, which was likely since those sequences where all obtained from *Culex* species mosquitoes, as opposed to CFA/AeFv, which derive from *Aedes* species.

Surprisingly, overall the sequences derived from *Culex* species mosquitoes show a weaker relation to CxFv strains found in Japan, North America, Mexico and Uganda than to the CFA/AeFv group, even though they derive from different mosquito species.

Taking the BLAST results into account, the relative position of our sequences in the phylogenetic tree does, in the vast majority, coincide with the homology of sequences shown on table 10 presented above.

Similarity search results for *Ae. caspius* sequences are in accordance with the relative position they occupy in the phylogenetic tree. With regards to *Culex pipiens* and *Culex theileri* sequences, similarity searches indicate homology *Culex theileri* flavivirus sequences and with Wang Thong virus (*Cx.fuscocephala*), to which the close relation is clearly shown by the position they possess within the tree.

In regards to tree topology, the consistency of relative position and composition of the three main groups is well supported by bootstrap values (based on 1000 replicates of the original sequence data) of 70% for the mosquito-borne group, 77% for the tick-borne and 82% for the no-known vector group.

The insect-specific flavivirus group is mainly separated into two groups, one of which presents a bootstrap value of 100%; this group includes the five sequences obtained from *Aedes caspius*, the "Culicinae flavivirus" and "Mosquito flavivirus" reference sequences obtained from Genbank. However, the group including "*Culex* flavivirus", CFAV, AeFv and the obtained sequences detected in *Culex* species mosquitoes is not a phylogenetically consistent group, since it presents a bootstrap value of 57%.

# 5.

# DISCUSSION AND CONCLUSIONS

# 5. Discussion and conclusions

### 5.1. Mosquito abundance and seasonal dynamics

Collections made for this study, both in the Algarve and Setúbal regions of Southern Portugal, yielded specimens belonging to fifteen species from the forty (38%) taxa previously described to exist in Continental Portugal (Ribeiro *et al*., 1988). This is in agreement with previous studies regarding species distribution in Portugal, with 38% and 40% of the forty known species found by Almeida *et al* (2008) and Osório *et al* (2010), respectively.

In the Algarve, both in 2009 and 2010, the most abundant species were *Aedes caspius*, *Culex pipiens* and *Cx. theileri*, which have been considered to be amongst the commonest, most abundant and widespread mosquitoes caught in Portugal (Almeida *et al*., 2008; Osório *et al*., 2010). Although collections made in Setúbal were sporadic, a total of 1,777 mosquitoes were sampled, five different species belonging to three genera were identified, and *Cx. theileri* was the prevalent one representing approximately 45% (n=876) of all specimens collected, followed by *Ae. caspius* with around 36% (n=697). All other species, namely *An. atroparvus*, *Cx. pipiens* and *Cx. univittatus*, display an almost equal proportion within the total mosquito collections. The presence and high prevalence of both *Aedes caspius* and *Culex theileri* over the other species is an expected finding in southern, coastal and estuarine regions such as the districts of Setúbal and Faro (Almeida *et al*., 2008; Osório *et al*., 2010). From this point onwards, the mosquitoes collected in Setúbal will not be discussed because the collections, as referred to above, were made sporadically, and thus, no conclusions can be drawn regarding mosquito seasonal dynamics.

In the Algarve, regardless of collection method, the abundance of all species captured varied according to the geographical group. More specimens were collected in the Western and Eastern groups than in the Centre. The highest number of mosquitoes was collected in Eastern Algarve with 46% (n=16,544), followed by the Western group with 36% (n=13,959) while the Central group yielded only 15% (n=5,464). In both years, *Aedes caspius* was the most abundant species in the Eastern and Western geographical groups, while in the Centre the predominant species was *Culex pipiens* closely followed

by *Culex theileri*. In addition, it was in the Central group that higher numbers of *Anopheles atroparvus* were collected.

Although the most abundant mosquito species in the Algarve were *Ae.caspius* (69%), *Cx. pipiens* (11%) and *Cx. theileri* (9%), an enormous gap of more than 50% separating the relative abundance of *Ae. caspius* from that of the other two species was observed, clearly disclosing an unmistakable predominance over all other mosquitoes.

Mosquito collections made in 2010 corresponded roughly to a four-fold increase in mosquito densities when compared to 2009. Differences in mean mosquito densities were registered by both methods.

Mosquito abundance and seasonal dynamics are partially determined by climatic and ecological conditions, with warmer temperatures and rainy seasons usually defining the peak mosquito densities. In this study, overall mosquito densities were higher in warmer months whereas the lowest were registered in April and October, as these presented cooler temperatures from 16 to 21ºC in 2009 and approximately 18ºC in 2010 (figure 48). Accordingly, the highest densities in collections using CDC traps were observed in August (usually the warmest summer month) 2009 and 2010, with the highest mean temperatures registered (25.6ºC and 26.8ºC, respectively), and no precipitation. In IR collections the peak densities were observed between June and September where temperatures varied from 23ºC to 25.6ºC, with low precipitation (1.7 to 2.5mm).

Regarding mosquito seasonal dynamics by CDC light trap collections, the highest densities were observed in August, reaching 400 mosquitoes/trap-night in the Western group in 2009. Despite registering the highest densities, there was also high variability. The Central and Eastern groups reached higher densities in June, with values not exceeding 100 mosquitoes/trap-night. In 2010, the peak month was maintained from the previous year. In the Eastern group, mosquito densities were highest in August, with over 1800 mosquitoes captured per trap-night although, once again, a high variability was observed as we can see from figure 33 (results section), there are wide error bars and y-axis values reach 2500, as compared to 500 in the 2009 graph (figure 32). In addition, for that same group, densities between 490 and 580 mosquitoes/trap-night were registered in June and September 2010. The Central group registered a reduction

in mosquito densities comparing to the previous year, especially between April and July, since the rest of the year seems to maintain the same low densities. The Western group had a peak mosquito density of nearly a 1000 mosquitoes/trap-night in September.

The IR collection data from 2009 revealed a peak density registered in August with values not surpassing 200 mosquitoes/collector/hour, for the Western group. The highest mosquito density was recorded in the Centre of the Algarve where approximately 500 mosquitoes/collector/hour were captured (as represented in figure 34, results section). In contrast, the Eastern group showed consistently low-density values, having been slightly higher in May 2009 but not surpassing 135 mosquitoes/collector/hour. In 2010, the peak mosquito density was registered in June where around 570 mosquitoes per collector/hour were collected, in the Centre of the Algarve. However, IR collections made in July and September in the Central group yielded considerable densities between 240 and 270 mosquitoes/collector/hour. In the West, the highest densities were detected in April and July with values of no more than 250 mosquitoes/collector/hour; whereas the lowest densities were observed in October. In the East, August was the month when highest mosquito densities were recorded; however, significant densities were obtained in May 2010. The lowest density in this area was also registered in October that had a mean temperature of 18.8ºC, a drop from the 23.4 ºC recorded for September, which may explain the lower density yields.

Collectively, mosquito densities were usually lower in April and October when lower temperatures were recorded, although precipitation values were higher than on all other months before and when collections were made. That trend was once again clear in the CDC collections in both years, except for the Western group. It was also clear that, for IR collections peak densities were usually observed between June and September, whereas for CDC collections the peak densities were recorded mainly in August.

In conclusion, mosquito density values recorded for CDC and IR collections contrasted consistently between collection years. Mean mosquito densities by CDC traps were similar for all three geographical groups in 2009, whereas in 2010 the Western and Eastern groups were similar (higher) but different from the Central group (lower). Whereas mean mosquito densities by IR were different amongst geographical groups in

2009, having been lower in the Western and Eastern groups and higher in the Central group, in contrast to 2010 when the yield of IR collections was similar in for all groups.

As mosquitoes are highly influenced by the atmospheric conditions for their breeding, such as temperature, precipitation and humidity, this patent increase of mosquito densities from 2009 to 2010 was investigated, regarding the climatological variables registered for this district (figure 48).

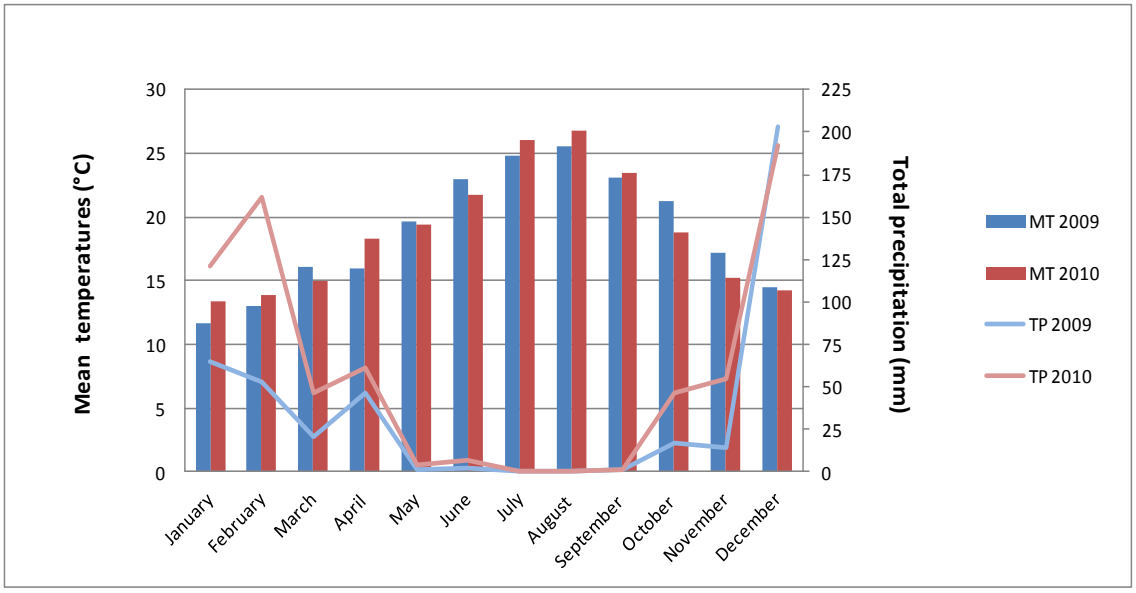


**Figure 48:** Mean temperatures and total precipitation registered for the Algarve region, during 2009 and 2010. Climate data obtained from **http://clima.tiempo.com/clima-en-faro+aeropuerto-085540.html** (accessed on the 8th of November 2011).

In this graph it can be observed that the transition from 2009 to 2010 was marked by reasonably high temperatures for the season, between October and December 2009. This was followed by the maintenance of high temperatures in the beginning of 2010, between January and April, with the exception of March, with average mean temperatures higher than those registered for the previous year. These high temperatures may have permitted the survival of mosquito populations through the winter, and their maintenance in nature. This was further enhanced by a peak in precipitation from January to April, considerably higher than those registered in 2009 for the same period. Taken together, this strongly suggests that the significant increase in mosquito

populations from 2009 to 2010 could be due to the combined effects of higher temperatures at the end of 2009 and beginning of 2010, and the increase in total precipitation before the beginning of spring, thus providing optimal conditions for the survival and reproduction of mosquitoes in 2010. In fact, Calheiros and Casimiro (2006) have already stated that climate change in the Algarve may result in an increase of mosquito population density, particularly of *Anopheles atroparvus* and *Culex pipiens* during cold months, thus causing an increase in the vectorial capacity and, consequently, the risk of transmission of vector-borne diseases especially West Nile fever.

As well as mean densities, the relative proportions of all mosquito species collected also varied according to collection method, geographical group and month/year of collection. This is a direct consequence of the fact that different collection methods target different types of mosquito behaviour. For example, while CDC traps aim at active host-seeking mosquitoes, whether indoors or outdoors, the main targets of IR collections are mosquitoes resting indoors after feeding, that is, endophilic mosquitoes.

In CDC trap collections, in 2009, *Cx. pipiens* was the predominant species representing 56% of all mosquitoes collected, followed by *Cx. theileri* 17-24% and *Ae. caspius* 21-25%, in Eastern and Central groups, respectively, as represented in the figures 39 in the results section. However, the Western group was dissimilar to the other two, with *Ae. caspius* as the most abundant species 83% and all other species taking a share of 5% or less each.

In 2009, *Ae. caspius* represented more than 80% of all CDC collections in the West, and that was maintained in the following year. A remarkable increase in *Ae. caspius* relative proportion was registered in the Eastern group where a rise of 67% was recorded, from 21% in 2009 to 88% in 2010 (as shown on figure 40, in the results section). This trend was also observed in CDC collections in the Central group, where an increase of *Ae.caspius* relative proportion of nearly 20% from 2009 to 2010 was registered. In addition, in this same area, the most abundant species was *Cx. theileri* with 38%, which also registered a significant increase of 14% from the previous year, closely followed by *Cx. pipiens* (26%) and *Ae. caspius* (23%).

In IR collections, the predominant species in both years was *An. atroparvus*, the only member of the *An. maculipennis* complex captured, in all three groups, ranging between 27 and 77% (as represented in the figures 41 and 42 in the results section). The second most abundant species, collected by this method, was *Cx. pipiens* with over 20% of all mosquitoes collected, except in the Western group where it represents only 15%, in the following year. In 2010, the most abundant species was *An. atroparvus* in the Central and Western groups, while in the East, *Ae. caspius* took over as the predominant species, with a 46%, followed by *An. atroparvus* and *Cx. pipiens* with 27% and 20%, respectively. The prevalence of *Anopheles atroparvus* in IR collections, in most geographical groups in both years, is a reflection of their animal feeding behaviour and endophily (Cambournac and Hill, 1938; Sousa, 2001). The relative proportion for this species by IR collections suffered a significant reduction from 60% (2009) to 27% (2010) in the Eastern group, which may reflect fewer IR collections made (47 in 2009 to 36 in 2010), and possibly even a lesser availability of vertebrate hosts as was confirmed in at least two collection sites.

Moreover, the relative proportions of *Ae. caspius* collected by IR in the Western group nearly doubled from 2009 to 2010 (from 14% to 27% ). The values for the Central area suffered only minor alterations; this species appeared in 2010 and was not recorded in the previous year, however such slight changes would be predictable in nature, and could be simply due to seasonality.

In sum, in 2009, the predominant species in IR collections, in all geographical groups, were *An. atroparvus* and *Cx. pipiens*, respectively. This was maintained in 2010 only in the Centre, as in the Western and Eastern groups the predominant species were *An. atroparvus* and *Ae. caspius.* Regarding CDC collections, an absolute predominance of *Ae. caspius* was registered for the West in both years, and in Eastern Algarve in 2010. In the East and Centre 2009, the most abundant species was *Cx. pipiens*. The Central Algarve (2010) was the only area where a predominance of *Cx. theileri* was registered.

The considerable higher numbers of *Ae. caspius* captured by CDC traps, in contrast to IR collections, clearly reflects their predominant exophilic behaviour characteristic of this species, as was also described by Almeida *et al* (2008, 2010).

*Aedes caspius* is mainly found in coastal and wetland areas, reaching the highest densities in estuarine regions in the districts of Faro and Setúbal (Shaffner *et al*., 2001), which can be explained by their halophilic[6] nature. However, it also breeds in rice fields that are vastly spread over both districts, especially in, and around, the Comporta area in the district of Setúbal (Almeida *et al*., 2010). This mosquito species is described as opportunistic and highly anthropophilic, often found in high numbers in outdoors and human baited collections (Almeida *et al*., 2008; Osório *et al*., 2008). *Aedes caspius* is considered an aggressive mosquito species commonly incriminated of nuisance biting (Shaffner *et al*., 2001; Beker and Zgomba, 2007) in many densely populated areas, which is the case of the districts of Setúbal and Faro. For these reasons, its public health importance cannot be disregarded since its human biting rate can reach up to 300 bites/person/hour in the Comporta region (Sousa, 2008). Furthermore, it is also a competent vector for WNV (Hubalek and Halouzka, 1999), Chikungunya virus (Vazeille *et al*., 2008), Tahyna virus (Lundstrom, 1999), and Rift Valley fever virus (Turell *et al*., 1996).

*Culex theileri* has been found to breed, among others, in rice farming areas (Novo, 2008), reaching high densities when climatic conditions are propitious (Almeida *et al*., 2010). It is considered one of the most predominant, and widespread, mosquitoes present in Portugal, especially in the southern regions (Almeida *et al*., 2008). This mosquito feeds mainly in mammals, humans included, and is capable of transmitting West Nile virus, Sindbis viruses and Rift Valley fever (Jupp *et al*., 1972; McIntosh *et al*., 1980; Jupp, 1985). Its public health impact is reflected by the aggressive behaviour, reflected by the human biting rate of approximately 400 bites per person per hour (in the Comporta area, Setúbal), making it an incommodity agent responsible for nuisance biting complaints (Sousa, 2008).

*Culex pipiens* was one of the most abundant and widespread species captured in estuarine areas in both regions. This mosquito species is known as the main vector of WNV in Europe and exists in Portugal in both the "pipiens" and "molestus" forms, with high interbreeding which heightens their importance as bridge vectors between birds,

[6] Requires a salty environment for breeding and development.

humans and other hosts due to their ornitophilic and anthropophilic behaviour (Gomes *et al.*, 2009),.

*Anopheles atroparvus* was found in both the Setúbal and Faro districts. This mosquito is mainly found in animal shelters due to its zoophilic behaviour (Cambournac and Hill, 1938; Sousa, 2001). This species has been previously found infected with WNV in Portugal (Filipe, 1972), hence its public health relevance.

*Anopheles algeriensis* was also collected in the Algarve, in agreement with other studies (Ramos *et al*., 1982; Osório *et al*., 2010); however, none was found in Setúbal, perhaps due to the limited number of collections as this species has previously been found in that region (Osório *et al*., 2010).

*Culex univittatus* was, in agreement with Almeida and others (2008) present in the Southern Portugal in low densities. This species has previously been found infected with WNV (Esteves *et al*., 2005), and although it was collected in smaller numbers, its ecology, geographical distribution and ability of vertically transmit WNV (Miller *et al*., 2000) may constitute a potential cause for concern from a public health viewpoint.

*Aedes detritus* were collected in estuarine regions, though in low numbers when compared to other species. However, in accordance with past studies, in itself this values would not be an unexpected finding since *Ae. detritus* is also usually present in wetlands and coastal areas (Ribeiro *et al*., 1988; Almeida *et al*., 2008). Although its medical importance has not yet been clearly confirmed, it has, at least, been found susceptible to infection by the Chikungunya virus (Vazeille *et al*., 2008).

*Culiseta annulata* and *Cs.longiareolata* were only collected in the Algarve region, though in similarly low numbers.

*Culiseta annulata* was previously found mainly in inland regions (Almeida *et al*., 2008) and, perhaps, the low numbers collected in this study could be because coastal and estuarine areas, where collections were made, may not present the ideal conditions for their breeding and development.

*Cs. longiareolata* has been considered one of the most widespread mosquitoes in Portugal (Almeida *et al*., 2008).

Other species of mosquitoes such as *Ae. berlandi, Ae. mariae, Coquillettidia richiardii, Cx. laticinctus, Cs.subochrea,* and *Uranotaenia unguiculata* were found in much lower numbers. Most of these have no known medical importance, except for *Co. richiardii* that, although very poorly represented in Portugal, it is a vector of WNV and bunyaviruses, such as Calovo and Batai viruses (Hubalek and Halouzka, 1999; Lundstrom, 1999; Higgs *et al*., 2004). This mosquito species has been found to share habitats with migratory birds from Africa, which may be infected with arboviruses, they feed on humans as well as birds, and thus may act as bridge vectors enhancing the possibility of transmission of any of the above mentioned viruses to humans (Service, 1971).

Importantly, the spread of flaviviruses via arthropod vectors and/or their natural reservoirs, is influenced by changing climatic patterns due to global warming, thus expanding their distribution northwards as temperatures become higher. A study by Epstein and Defilippo (2001) provided evidence that a correlation exists between WNV outbreaks and drought. According to these investigators, the large outbreaks that occurred in Romania (1996), USA (1999) and Russia (1999) followed short or long periods of drought. Therefore, since an increase in the frequency and intensity of periods of drought has been predicted to occur in Portugal because of climate change (Calheiros and Casimiro, 2006), the probability of WNV outbreaks eventually occurring in the country would also increase, if these changes were verified.

### 5.2. Mosquito screening for flaviviral sequences and respective phylogeny

The screening for flaviviral sequences was performed using, as starting point, the clarified homogenates obtained from mosquito pool maceration, from which total RNA was extracted. It is important to take into account that the products of total RNA extraction are most certainly not pure. Indeed, apart from viral RNA (if present), other RNA molecules from mosquitoes (at least) and other organisms (fungi, bacteria and ectoparasites infecting the mosquitoes themselves) are most probably present in these extracts. Furthermore, the primers used for viral sequence amplification were degenerated in order to allow the amplification of a larger number of flaviviral NS5 sequences, and although this strategy may work in our favour, it may also be considered

risky. These risks translate in the form of amplification products that result from retro-transcription of non-targeted RNA molecules from total RNA extracts. As a result, we could be amplifying a variety of RNA molecules from different organisms and not only those specifically targeted by the primers used. As a result, the possibility remains that the obtained amplification products were produced from RNA contaminants and not from viral RNA. Therefore, to rule that out, extraction controls were performed (as described in section 3.2.8). Since these extraction controls produced negative results (as expected), we can conclude that, under the conditions used, these RNA contaminants present in the analysed samples were not having a significant impact in the obtained amplification results.

In the past few years, studies conducted by Crochu *et al* (2004), Roiz *et al* (2009) and Vázquez *et al* (2011b) have reported the integration of viral RNA sequences like that of insect-specific viruses, genetically similar to that of CSAV, and other particles into the mosquitoes' genomes. As speculated for *Ae. albopictus*, sequence integration might possibly occur following infection by a flavivirus, resulting in a novel mechanism by which genetic diversity in eukaryotic cells may be produced (Crochu *et al*., 2004; Roiz *et al*., 2009). However, though it is known that integration events occur, the respective mechanism has not yet been determined. In addition, it is not known whether the genetic diversity potentially created in eukaryotic cells would be beneficial or detrimental and what effects it could generate on viral transmission and in vector susceptibility and competence. Taken together, these findings also pose two other potential problems to our analysis. If the prepared RNA extracts would be contaminated with either mosquito genomic DNA (integrated viral sequences), or RNA molecules expressed from these putative DNA forms, then amplification results might translate the amplification of these "secondary contaminants", and not from viral RNA contained within virions, thus producing false positive results. This is a major pitfall of this type of viral detection method since there are no guarantees that the positive results are not due to integration events. Therefore, the only way to clearly prove that positive amplification pools resulted exclusively from the presence of virion RNA is to perform viral isolation in cell cultures followed by search of flavivirus virions by electron microscopy and their complete sequence analysis. In their absence, one cannot conclude

that positive pools resulted solely from viral RNA since these procedures were not systematically carried out for this study.

Whatever the nature of the obtained amplification results, the sequences obtained resulted from the specific amplification of the target region of the NS5 gene of the flavivirus genome, as previously suggested by preliminary control experiments (section 4.4.2.). However, their small size, ranging between 165bp and 170bp, limits the accuracy of subsequent phylogenetic analysis considerably (bootstrap certainty is compromised). Since our PCR products resulted in the amplification of several molecules apart from the targeted one (which can be observed by the appearance of multiple bands for the same sample, after gel electrophoresis), DNA had to be purified from the agarose gel, after separation of the amplified molecules. This method is usually associated with the loss of a fraction of the purified product, in contrast to DNA purification directly from the PCR product, which would only be applicable if the purification products were resultant from a single targeted molecule.

The use of oligonucleotide primers that amplify, in average, a fragment of approximately 200bp of the flavivirus NS5 gene is quite common among research groups. For example, Scaramozzino *et al* (2001) and Pabbaraju *et al* (2009) analysed 250bp sized amplimers, Calzolari *et al* (2010) reported NS5 sequences ranging between 200bp and 262bp, whereas Sánchez-Seco *et al* (2005) obtained even shorter sequences of 143bp in length. However, as such small fragments do not allow for accurate phylogenetic analyses, some research groups have designed and adapted primers in order to amplify larger fragments for better and more precise phylogenetic characterisation of sequence sets. Some of these groups aimed to obtain partial sequences of large sizes of, for example, 1000bp (Kuno *et al.*, 1998), 1075bp (Ayers *et al*., 2006), 800bp (Maher-Sturgess *et al*., 2008), 720bp (Morales-Betoulle et al., 2008) and 1019bp (Vázquez *et al*., 2011b). Though these groups used different primer sets, the methodology used was usually similar with only a few variations.

The flaviviruses are known to be globally widespread viruses, with the exception of Antarctica (Weaver, 2006). Since the beginning of the 21$^{st}$ century, the circulation of a variety of pathogenic and insect-specific flavivirus sequences from different mosquito species (as described in sections 1.5 and 1.6) has been discovered all over the world,

including Europe. Insect-specific flaviviruses recently detected in Portugal (directly deposited in the sequence database), Italy and Spain are included in the phylogenetic tree presented below (figure 49). The majority of the sequences obtained were detected in *Aedes* mosquitoes (mainly *Ae. caspius*) and in *Culex* species mosquitoes.

Similarly to what occurs in phylogenetic analysis of the genus *Flavivirus* in agreement with Kuno *et al* (1998) and Gaunt *et al* (2001) (as described in section 1.3.3, figure 7), all these sequences grouped together mainly according to vector species. Consistent with the separation of viruses by vector species, most sequences derived from *Culex* mosquitoes grouped together at the top half of the tree, where the sequences we detected in *Culex* species mosquitoes clustered together with the Spanish "Mediterranean *Culex* Flavivirus" sequences.

The "Mediterranean *Culex* Flavivirus" group includes sequences detected in Spain mainly in *Cx. theileri* and *Cx. pipiens* (Vázquez *et al*, 2011b); this is in agreement with our findings, where a higher prevalence of positive pools composed of *Cx. theileri* over those of *Cx. pipiens* was verified. Consequently, more sequences were detected in the former (n=5) than in the latter species (n=1). Although a "*Culex theileri* flavivirus" sequence from Portugal (EU716420) is included in the analysis, all our sequences derived from *Culex spp*. show closer relation to their Spanish counterparts. The entire group of sequences derived from *Culex* mosquitoes is well supported by a 96% bootstrap value; this is in accordance with the positioning of the *Culex* group of sequences in the tree shown in figure 47 (section 4.5).

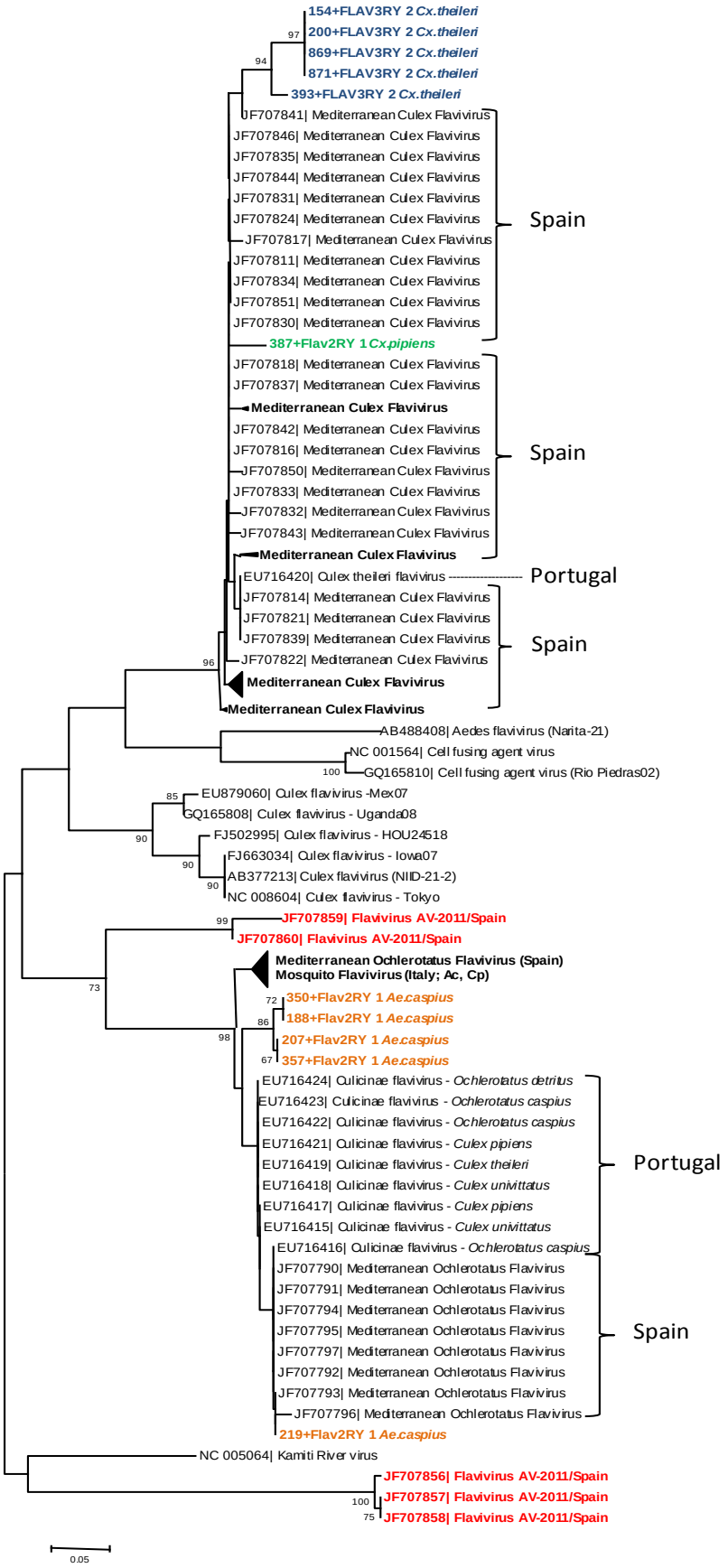


**Figure 49:** Neighbour-joining tree of some insect-specific flaviviruses based on the analysis of a short fragment of the NS5 gene. Bootstrap analysis was based on a thousand replicates of original sequence data. Groups presenting bootstrap values below 70% are not shown. Sequences in blue, green and orange correspond to those detected in this study. Those in red represent "DNA forms". Accession numbers of sequences from Spain not displayed on the tree can be obtained in Vázquez *et al* (2011b). For accession numbers of "mosquito flavivirus" sequences from Italy, please see figure 47.

In contrast, the sequences detected in *Ae. caspius* including the Spanish, the Italian and those obtained in the course of this work, formed a large group at the bottom half of the tree, which is well supported by solid statistical certainty. However, within the "Mosquito Flavivirus" group (Italy) there is a single sequence derived from *Cx. pipiens*. Apart from this isolated case within the group, all other RNA viruses in this cluster were detected in *Aedes caspius*. The viral sequences detected in this study (from *Ae. caspius*) show a stronger relation to the group including "Culicinae flavivirus" from Portugal and "Mediterranean Ochlerotatus Flavivirus" from Spain than to that composed of Spanish and Italian sequences from the same mosquito species. This could be due to the relative geographical proximity of collection sites, since sampled sites are located in southern Spain (Huelva) and southern Portugal (Faro district, Algarve), and mosquito dispersal between these two regions is feasible.

Notably, the group of *Culicinae* flaviviruses from Portugal also includes sequences derived from *Culex* species mosquitoes (5 out of 9); however, these also clustered with the other "Culicinae flavivirus" sequences detected in *Ae.caspius*.

It is noteworthy that some groups of viral sequences have derived from different vector species, as is the case of the "Mosquito flavivirus" group from Italy and "Culicinae flavivirus" group from Portugal. The fact that a certain type/species of virus infects more than one vector/host species and that some mosquito-borne viruses have been isolated from ticks in the wild and some tick-borne viruses have been detected in mosquitoes in the wild, invalidates the theory that viruses evolve progressively with their hosts. In addition, since some viruses share the same host/vector species the simultaneous circulation of different viruses may occur (Sánchez-Seco *et al*., 2005) in the same geographical areas, thus making the viruses difficult to identify.

The classification of flaviviruses presents many difficulties that partly stem from the widespread distribution of these viruses as well as with the variety of vectors species and vertebrate hosts associated with their biological transmission (Kuno *et al*., 1998). Thus, the distribution of flaviviruses usually overlaps that of their arthropod vectors and is accompanied by rapid genetic alterations, which facilitate host range expansion. Importantly, under optimal climate conditions RNA viruses are capable of undergoing rapid genetic changes through mutations, which contributes to the genetic variability

and facilitates the adaptation of those viruses to a high diversity of hosts, thus promoting their host range expansion in distinct geographical areas. In addition, according to an *in vitro* study conducted by Kuno (2007), the diversity of host range strongly correlates with the standard classification of flaviviruses (Karabatsos, 1985) as well as with the segregation of viral sequences in flavivirus phylogeny (Kuno *et al*., 1998), in agreement with the positioning of flaviviruses sequences obtained in this study. Another factor that influences host-specificity is the composition of the viral genome. Greenbaum *et al* (2008) discuss that a trend exists in the expression of flaviviruses sequences composition (CpG expression) and the host species, that is, that flaviviruses suppress CpG because their hosts also suppress it. However, Cook and Holmes (2006) and Schubert and Putonti (2010) disagree and affirm that according to sequence composition, a correlation does not exist between codon usage and vector species, thus there is no co-variation of codon usage within a vector group.

Other factors may play important roles in the emergence or re-emergence of viruses worldwide. For example, DENV has been remerging in new tropical and subtropical areas globally, most probably due to human activity that contributes to the expansion of their vectors, *Ae. aegypti* and *Ae. albopictus*, into new areas; whereas WNV has been circulating and become established in new geographical areas due to natural factors such as bird migration, amongst others (Mackenzie *et al*., 2004). From its African origin where it was first reported in 1937 WNV has since been extending its distribution, the virus caused a large-scale epidemic in the USA, where it entered in 1999 and quickly spread throughout the country, though the means of entry are still unclear (Epstein and Defilippo, 2001). Following repeated isolations of WNV throughout Europe, it has been suggested that the virus has become endemic in many European countries, and both lineage 1(widespread) and 2 (previously found only in Africa) strains have been detected (ECDC, 2011). The most likely means of dispersal of WNV is by migratory birds as Europe sits right below the main migration routes (as shown on figure 24, section 1.7). This is also the principal means of dispersal of WNV through Asia via the Africa - Middle East route. Therefore, potentially infected migratory birds can spread flaviviruses when resting and settling in new geographical areas in search of warmer temperatures, thus infecting local populations of ornitophilic mosquitoes capable of acting as bridge vectors and potentially causing "spill-over" to humans and animals.

Since co-circulation (Vázquez *et al*., 2011a) and co-infection (Kihara *et al*., 2007; Kanthong *et al*., 2010) of flaviviruses have previously been reported, it is important to investigate the influence of co-infection of flaviviruses on the capacity and competence of vectorial transmission, whether the co-infection consists of antigenically distinct flaviviruses, such as MBV/MBV or MBV/ISF. Especially since infection of mosquitoes by insect-specific flavivirus does not seem to have a negative effect on their fitness, and infection with one virus reduces the probability of infection and transmission with a second antigenically related virus, as recently evidenced by Pesko and Moores (2009). Therefore, it would be interesting to see if the infection by an insect-specific flavivirus has any effects (positive or negative) on subsequent infection by arboviruses and on their virulence.

### 5.3. Final conclusions

In this study, we were able to detect viral sequences in a significant proportion of the screened mosquito pools (≅30%). Our results strongly suggest that these sequences relate to insect-specific flaviviruses. As evidenced by the high minimum infection rates (MIR) registered (≤ 22/1000), there was a large proportion of mosquitoes exhibiting these sequences, thus potentially infected with these viruses.

A remarkable increase in mosquito densities was registered in the Algarve, from 2009 to 2010, and although no sequences of human pathogenic flaviviruses were detected, several insect-specific flavivirus sequences were found in considerably high percentages. The future importance of these is still to be known as these viruses are widely dispersed and may integrate mosquito genomes. Furthermore, *Aedes albopictus* and *Aedes aegypti* were not detected, despite the fact that the former has been established in Europe for a few years, and the latter is settled in the Madeira Archipelago (Portugal). However, there are other mosquito species that due to their presence, high abundance and ability to transmit flaviviruses such as West Nile virus, may be a cause for concern to Public Health. This importance is enhanced by the vector's choice of vertebrate hosts since Portugal harbours many migratory bird species from Africa, and a diversity of ornitophilic mosquitoes that may act as bridge vectors,

some of which have been reported to share a common habitat, thus increasing the probability of exotic arboviral transmission to animals and humans.

Portugal is a country that gathers many favourable climatic and ecological conditions for the establishment of invasive species such as *Aedes albopictus*, and consequently the introduction and transmission of arboviruses. Mosquito surveys are essential for monitoring of mosquito species distribution, abundance and seasonal dynamics that gives glues as to what potential vectors are present and what viruses are circulating in association with those vectors, thus allowing for the risk assessment of arboviral transmission. Epidemiological surveys have, thus, become indispensable tools for the prediction, risk assessment and management of arboviral outbreaks, and all the above-mentioned factors emphasize the need for continuous surveillance programmes in order to ensure Public Health, especially in Southern Mediterranean countries where pathogenic flaviviruses circulation has increased in recent years causing many fatalities.

In conclusion, the screening for viral sequences revealed the presence and high prevalence of these, in mosquito pools. Further analysis of a limited number of positive pools demonstrated that the detected sequences did not correspond to arboviruses (NS5 amplifiable by the employed method), but to a group of flaviviruses generally designated by insect-specific flaviviruses (ISF). While their biology remains unclear, they are dispersed over a wide geographical area, and their prevalence appears to be high in natural mosquito populations of different species. Moreover, as a direct result of this work, R. Parreira has isolated an insect-specific flavivirus, from our mosquito homogenates, in cell culture (R. Parreira, personal communication).

# 6.

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# 7. List of figures

# 8. List of tables