



# Interaction between chikungunya and dengue viruses during co-infection in *Aedes* mosquito cells and in *Aedes aegypti* mosquito

Margot Enguehard

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# **Interaction between chikungunya and dengue viruses during co-infection in *Aedes* mosquito cells and in *Aedes aegypti* mosquito**

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## Abbreviations list

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HIV	Human Immunodeficiency Virus
HTLV	Human T – lymphotropic Virus
DENV	Dengue virus
WNV	West Nile Virus
USA	United States of America
CI	Cytoplasmic Incompatibility
RNA	RiboNucleic Acid
DCV	Drosophila C Virus
CrPV	Cricket Paralysis Virus
FHV	Feline herpes Virus
wAlbA	<i>Wolbachia</i> strain Clade A infecting <i>Aedes albopictus</i>
wAlbB	<i>Wolbachia</i> strain Clade B infecting <i>Aedes albopictus</i>
wMel-Pop	<i>Wolbachia</i> stain Clade A Infecting <i>Drosophila Melanogaster</i>
CHIKV	Chikungunya Virus
SINV	Sindbis Virus
SFV	Semliki Forest Virus
RRV	Ross River Virus
YFV	Yellow Fever Virus
WEEV	Western Equine Encephalitis Virus
VSV	Vesicular Stomatitis Virus
NSP	Non Structural Proteins
IL-10	InterLeukine 10
JAK-STAT	Janus Kinase STAT
Imd	Immune Deficiency
RNAi	RNA interference
miRNA	micro RNA
piRNA	Piwi RNA
siRNA	small interfering RNA
dsRNA	double strain RNA
TLR	Toll Like Receptor
PRR	Pattern Recognition Receptor
AMP	Antimicrobial Peptide
PCD	Program Cell Death
CASPS7	Caspase 7
CASPS8	Caspase 8
PI3P	Phosphatidylinositol – 3 - Phosphate
NIAID	National Institute of Allergy and Infectious Diseases
ONNV	O’Nyong Nyong Virus
VEEV	Venezuelan Equine Encephalitis Virus
DC-SIGN	Dendritic cell Specific ICAM grabbing non integrin
L-SIGN	Lectin Specific ICAM grabbing non integrin

TSPAN9	TetraSpanin 9
RT-PCR	Retro Transcription Polymerase Chain Reaction
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IFA	ImmunoFluorescence Assay
ELISA	Enzyme linked immunosorbent assay
HI	Hemagglutination Inhibition
DHF	Dengue Hemorrhagic Fever
JEV	Japanese Encephalitis Virus
SLEV	Saint Louis Encephalitis Virus
ZIKV	Zika Virus
TBEV	Tick-Born Encephalitis Virus
ssRNA	single strand RNA
ER	Endoplasmic Reticulum
WHO	World Health Organization
DF	Dengue Fever
DSS	Dengue Shock Syndrome
ADE	Antibody Dependent Enhancement
cDNA	complementary Deoxyribonucleic Acid

## **General Introduction and Objectives**

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Pathology associated to infection is often considered as resulting from the reciprocal interaction of a given pathogen with a given host. This paradigm looks however oversimplistic since the pathology, as well as the epidemiology of a pathogen, relies also on the interactions between several infectious agents present within an organism. It is therefore crucial to consider to which extent a host infected by a first microorganism is modified and whether its reaction to the infection by a second microorganism is consequently altered. Most of the emerging infectious diseases are generated by arboviruses (Arthropod-borne viruses). Among these viruses, the genus *alphavirus* (i.e. Chikungunya CHIKV virus), and the genus *flavivirus* (i.e. Dengue DENV and Zika ZIKV viruses) belong to the Togaviridae and Flaviviridae families, respectively.

CHIKV has recently caused important outbreaks with more severe symptoms than previous contagions, with reports of complications and death. During the outbreak in 2005, 1 million human cases in the Indian Ocean islands have been reported. The outbreak extended in the Indian subcontinent and southeast Asia (Santhosh et al., 2008), and in Africa (Leroy et al., 2009). Often considered as an exotic disease, the outbreak reached firstly the Europe continent in 2007 (Rezza, 2008). Currently, a large-scale outbreak occurs in the Caribbean islands and in American continent following introduction of a CHIKV Asian genotype, related to strains identified in Asia. Because of the lack of licensed vaccine or specific treatment, million people in endemic areas are at risk.

Dengue fever, caused by the infection of DENV is considered as the major global public health by arboviruses. Infection with any of four serotypes of DENV (DENV-1, -2, -3 and -4) result in disease ranging from mild, flu-like disease, dengue fever (DF), to dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). According to recent study, DENV is endemic in a large part of countries in Africa, Asia, Central and South of America, and 400 million people are infected annually (Bhatt et al. 2013). Only one licensed DENV vaccine is available for specific conditions and no effective antiviral treatment is currently available.

Transmitted by *Aedes aegypti* mosquito, both viruses are also associated with the invasive vector, *Aedes albopictus*, which the expansion was rapidly increased since several decades. So capable of co circulate during outbreak, CHIKV and DENV may share

same vectors and same hosts. Some cases of humans co-infection with both viruses are frequently observed, in habitants or in travelers, confirming former data (Chahar et al., 2009; Chang et al., 2010; Myers and Carey, 1967; Parreira et al., 2014). Moreover, experiments performed in the field showed the simultaneous presence of CHIKV and DENV in *Ae. albopictus* mosquito (Caron et al., 2012), suggesting the potential co-transmission of viruses to a novel host. Performed on laboratory, studies confirm this data by the demonstration of presence of viruses in saliva after orally simultaneous infection (Vazeille et al., 2010), and more recently, by the demonstration of infection with three distinct arboviruses (CHIKV, DENV and ZIKV) (Ruckert et al., 2017). These co-infected mosquitoes present both viruses in their saliva and can potentially transmit both viruses together. When we started this work, at the exception of the successful co-infection of *Aedes albopictus* by Failloux's group, no studies focused on the consequences of *alphavirus* and *flavivirus* co-infection.

At the cellular scale, CHIKV and DENV viruses share a common cellular tropism in mosquito and humans (Gasque et al., 2015; Gregson and Edelman, 2003). Arboviruses infect the mosquito midgut, replicate and disseminate in the whole organism, until the salivary glands, for the transmission by the saliva. The midgut and salivary glands act as barriers to virus infection and escape. However, no tissue tropism difference in insects has been reported, except for germinal cells that are not always infected. However, nothing is known about the interaction between CHIKV and DENV during co infection of the same insect cell. The interaction of both viruses with common pathways can potentially mediate an interference, anergy or synergy between CHIKV and DENV.

In this work, the main objective was to explore the interaction between *alphaviruses* (CHIKV) and *flaviviruses* (DENV) during a sequential infection. This topic was developed taking in account some observations from previous studies in laboratories and in the field, where the presence of both viruses in the same time can lead to co-infections.

In a first part, we define an artificial sequential infection model *in cellulo*. Cells lines from *Aedes albopictus* and *Aedes aegypti* mosquitoes were infected sequentially with CHIKV and DENV-2. A generalization of the phenotype was established by using another *alphavirus* (SFV) and *flaviviruses* (YFV and ZIKV).

To go further, these *in vitro* results were confirmed by *in vivo* experiments. For this, we developed an *in vivo* model of sequential infection with CHIKV and DENV-2 in *Aedes aegypti* mosquitoes in collaboration with Valérie Choumet in Pasteur Institute of Paris. Previous work showing the potential simultaneous transmission were performed during a simultaneous infection with CHIKV and DENV-2 (Göertz et al., 2017; Ruckert et al., 2017; Vazeille et al., 2010). However, the model of simultaneous infection is not the only one happening during simultaneous outbreaks. The originality of this study consists to sequentially infect *Aedes aegypti* mosquitoes. In this second part, we determined the kinetic of DENV-2 infection in orally infected *Aedes aegypti* mosquito in mono and co-infection conditions. Even if simultaneous and sequential infection models result in the co-infection, the timing of infection could be an important factor. A pre-infection by a first virus may influence the vector competence, the viral replication, or the dissemination for the second virus. Such modification may impact the transmission, and the pattern of the current outbreak, with frequent severe cases observations.

Finally, in a third part, experiments aiming to identify the molecular mechanisms involved in the co-infection phenotype are presented.

In insect and mammalian cells, these viruses are interacting with different cellular factors, especially with proteins of the innate immune system (Fusco et al. 2013; Yasunaga et al. 2014). The majority of studies performed in mammal cells indicates that CHIKV and DENV viruses bypassed antiviral strategies into cells to successfully and efficiently replicate their genome. In comparison, the infection in the vector became persistent and nonpathogenic infection, which results in a continuous transmission of these viruses in nature. Since several decades, it is known that arboviruses persist in insect cells through the suppression and/or by evading immune defense (RNA interference, Toll, Jak/STAT and Imd/Jnk) (Lin et al. 2004). Since several decades, investigations suggest that the inhibition of the mosquito innate immune pathways seems to be implying in this persistence of arboviruses (Fragkoudis et al., 2007) (Jupatanakul et al., 2017; Xi et al., 2008). All these antiviral strategies are activated following infection, and probably by the interaction between viral proteins and cell factors. Interactions could probably play a crucial role in observed effects during co-infection.

Altogether, we suggest that the modulation of viral infection during co-infection is the resulting of equilibrium between viral replication and cellular pathways or antiviral response. This study can be crucial for a better understanding of disease and epidemiology during simultaneous outbreaks.

## **Bibliographic synthesis**

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## Chapter 1. Pathogens interactions in mosquitoes

### I. Interactions – definitions

Animals and plants host many microorganisms within establish symbiotic relationships. Described by Anton de Bary in 1879, symbiosis is the fact that dissimilar organisms are living together. This definition takes care of the size of partners. The smaller is named symbiont and the bigger is the host. All micro-organisms (bacteria, fungi, virus, parasites...) associated to a host constitute the microbiote. Since several decades, the microbiote is described as an important factor playing a role in the biology of the host. It is becoming apparent that organisms are not isolated. A complex of pathogens resides with one host, which influences the individual's phenotype. This is particularly true and relevant for insects as around 70 % of insects have an endosymbiont (Bourtzis and Miller, 2003). The presence of this endosymbiont is able to influence vital functions as the development and nutrition (Baumann, 2005; Zientz et al., 2004) the reproduction (Werren et al., 2008), the speciation (Hurst and Warren, et al., 2001), and may influence the immune response against a pathogen infection (Dong et al., 2009). Up to now, it's crucial to adopt a global view of a host, taking in account of the association of the microbiote and the interactions between them. These interactions are frequently complex and variations may occur.

Interaction	Host	Symbiont
Parasitism	-	+
Predation	+	-
Amensalism	-	0
Competition	-	-
Neutralism	0	0
Commensalism	0	+
Mutualism	+	+

**Table 1. Interactions between host and symbiont.**

Beneficial effects are represented with +; harmful with -, and 0 represents neutral effects.

The concept of the holobiome has been proposed for interactions between symbionts, and concerns the whole association between bacteria, fungi, virus or parasites in a host (Rohwer et al. 2002). Principally analyzed on *Drosophila* models, numerous studies allow to increase our knowledge on genetic and the insect immunity developed against pathogen infection. In this part, we review the current knowledge on interactions in the holobiome, and the consequences of them on the biology of host and the maintenance of pathogens.

In natural, an infection event is not isolated and a host is rarely infected by one pathogen. Novel Sequencing technics called NGS, and numerous studies showed the evidence of co-infections in animals and humans. The probability that two or more pathogens may infect simultaneous or sequentially a host is highly possible. During an infection, pathogens induce changes in their hosts. The pathogen may use common cell pathways for their multiplication, and it can speculate that pathogens interactions may be critical to the viral pathogenesis and viral evolution. Host conditions and fitness may be affected and interactions may have important implications for pathogen transmission dynamics. The modification can be observed at the body level or at the cell level.

According to immune-compromised state of Human Immunodeficient Virus (HIV, Retroviridae family, *lentivirus* genus) infected individuals, it's not surprising that their susceptibility to other diseases is enhanced. For example, a lot of reports established interactions between HIV and various pathogens, including parasites, *Leishmania*, *Trypanosoma*, or *Plasmodium* (Andreani et al., 2012; Okwor and Uzonna, 2013). The immune system is modulated, reciprocal effect can be observed on each multiplication. Interactions with other viruses is not rare. A lot of publications refer the interaction between HIV and other opportunist viruses such as Human T-Lymphotropic Virus (HTLV, Retroviridae family, *deltaretroviridae* genus) and herpes virus 8 (HHV8 or Kaposi's sarcoma-associated herpesvirus (KSHV)) which causes Kaposi's sarcoma, a cancer commonly occurring in AIDS patients. The opportunistic viruses take advantage of the environmental changes and the diminution of immune system induced by the first infection. Other famous example is the *Staphylococcus aureus* necrotizing pneumonia disease that is frequently preceded by influenza infection and is a life-threatening disease.

In this chapter, we will describe only interactions between vector borne pathogens, in particular the ones transmitted by mosquitoes. Multiple vector borne pathogens can circulate in the same mosquito, and potential interactions may occur resulting in modification of the infection, the dissemination, the transmission or biology of host.

## II. Interaction Virus-parasites

Five species of *Plasmodium* can infect and be spread by humans. Most deaths are caused by *P. falciparum* because *P. vivax*, *P. ovale*, and *P. malariae* generally cause a milder form of malaria. The species *P. knowlesi* rarely causes disease in humans. *Anopheles* mosquitoes transmit this parasite. In Nigeria, malaria and dengue outbreaks are sometimes co-circulating. It's common to detect both pathogen in patients, as described a recent study, showing a 2,84% of co-infected patients (Kolawole et al., 2017). Symptoms and degree of severity can be impacted by this interaction. In 2017, a report established a correlation between co-infection with dengue virus (DENV, Flaviviridae family, *flavivirus* genus) and an acute febrile illness (Chong et al., 2017). However, the common vector for DENV is *Aedes* species, and we can underestimate the real importance of interactions.

Among arthropod-borne alphaviruses transmitted by mosquitoes, only the O'Nyong-Nyong virus (ONNV, Togaviridae family, *alphavirus* genus) is transmitted primarily by *Anopheles* mosquitoes. Both O'nyong-nyong virus and *Plasmodium* can be found in the same mosquito (Brault et al., 2004), but interactions between pathogens are still unclear.

In *Culex* species, avian malaria and West Nile Virus (WNV, Flaviviridae family, *flavivirus* genus) have been isolated from mosquitoes and birds. Birds are considered as major reservoir for this virus (Medeiros et al., 2016). Previous work has shown a negative association between these pathogens in adult birds, unlike juvenile birds (Medeiros et al., 2014). Among 13 species collected in Chicago (USA), 1714 individual birds were analyzed for the presence of WNV and *Plasmodium* parasite. A higher prevalence of *Plasmodium* was observed in the absence of the WNV infection. This is suggesting a competition between pathogen but the molecular mechanisms have to be clarified. Since

several years, the potential of co-infection between *Plasmodium* and arboviruses has been highlighted inside mosquitoes (Hughes et al., 2010). Interactions could have important consequences on fitness and physiology of host, but also spreading of the pathogen in term of epidemiology.

### III. Interaction Virus-bacteria

Interactions between virus and bacteria in host are certainly the most common interaction. In this part, we focused to discuss of the most important bacteria in mosquito known to date.

*Wolbachia* is an obligate intracellular alpha-proteobacteria predicted to infect a wide range of insect species (Hilgenboecker et al., 2008; Jeyaparakash and Hoy, 2000).

*Wolbachia* lives in the cytoplasm of host cells and is dependent of host cell resource for its survey. The mode of transmission of *Wolbachia* is principally the vertical transmission from an infected female to its progeny through the cytoplasm of the maternal line. The horizontal transmission is also possible and contributes to the *Wolbachia* prevalence.

The characteristic of this bacterium is its ability to alter the reproduction of its host in various ways, such as the cytoplasmic incompatibility (CI). The molecular description of CI is still not completely clear. CI occurs when the bacteria strain is absented in egg during the embryogenesis and the embryonic development is disrupted. This system gives advantage to infect females and permits a rapid enhancement of *Wolbachia* expansion.

It was initially discovered in *Drosophila melanogaster* (Hedges et al., 2008; Teixeira et al., 2008) that *Wolbachia* plays a role of antiviral agent. In presence of the bacteria, flies survived to a pathogenic infection with RNA viruses: Drosophila C virus (DCV, *Dicistroviridae* genus), Cricket paralysis virus (CrPV, *Dicistroviridae* genus) and Flock House virus (FHV, *Nodaviridae* genus). It had shown that accumulation of viral particles was decreased, and the host mortality following the infection was also reduced.

In mosquito, *Wolbachia* was first detected in *Culex pipiens* mosquito (Hertig and Wolbach, 1924). Currently, the bacterium has been shown to be present in populations of various wide mosquito species. *Aedes albopictus* mosquito is naturally infected with

two *Wolbachia* strains, wAlbA and wAlbB, but the bacteria has not been detected in *Aedes aegypti* (Sinkins et al., 1995a; Sinkins et al., 1995b).

The presence of *Wolbachia* in common arbovirus vectors raises the question of the impact of *Wolbachia* infection on arboviruses transmission in natural vectors population. This is a complex tripartite system with contributions from the host, *Wolbachia* and virus on the outcome of virus infection.

To study the impact of *Wolbachia* on arboviruses transmission, experiments on *Aedes albopictus* were conducted with the dengue virus (DENV, Flaviviridae family, *flavivirus* genus) in the presence or absence of *Wolbachia* (Mousson et al., 2012). The clearance of *Wolbachia* in mosquitoes was obtained by a tetracycline treatment of larvae and rifampicin of adult stage. After an infected blood meal, the midgut, wings and salivary glands were collected until 14 days. The results indicated that there was no significant impact of *Wolbachia* in midgut and wings. However, the viral load in salivary glands was lower in presence of *Wolbachia*. It suggested that the bacteria do not affect the replication of dengue virus in *Ae. albopictus*, but the viral transmission is limited by the reduction of viral infection in salivary glands (Mousson et al., 2012). In studies using another vector of dengue virus, *Ae. aegypti*, the bacteria was introduced by trans-infection. The invasion and the fixation within the next generations were evaluated until 18 days after the infection. Contrary to the results obtained with *Ae. albopictus*, the wAlbB *Wolbachia* strain seems to inhibit the DENV dissemination (Bian et al., 2010), in *Ae. aegypti*. The difference could be due to the low density of *Wolbachia* in midgut and salivary gland to induce reduction and resistance. This hypothesis is supported by another studies showing the density dependence of *Wolbachia* in the reduction of DENV in *Aedes albopictus* mosquito and in *Aedes aegypti* Aag2 cells (Lu et al., 2012).

Finally, in 2011, the group of Hoffmann, Walker et al., showed that two different *Wolbachia* strains from *Drosophila Melanogaster*, wMel and wMel-Pop (a pathogenic strain wMelPop that over replicates in insect hosts and causes severe life shortening) induces the diminution of DENV-2 infection in whole mosquitoes and it reduces the prevalence of DENV-2 in saliva (Walker et al., 2011). Without *Wolbachia*, the prevalence reaches 80%, whereas the diminution is drastic by addition of wMelPop strain (0% of prevalence).

Altogether, these studies show the key role played by *Wolbachia* in viral inhibition. This interference is still unclear, some hypothetic mechanisms have been proposed such as

the stimulation of host immune response, the competition of resources in mosquitoes (Bian et al., 2010; Frentiu et al., 2010; Moreira et al., 2009).

However, the restriction is not conserved for every arbovirus. For example, the modulation of another arbovirus transmitted by *Aedes albopictus*, the chikungunya virus (CHIKV, *Togaviridae* family, *alphavirus* genus) by the bacteria is debated. Although a study showed the modulation of CHIKV replication by *Wolbachia* in 2010 and in 2015 (Mousson et al., 2010; Raquin et al., 2015), a recent study seems to prove the contrary (Ahmad et al., 2017). Mosquitoes were treated by tetracycline to allow the clearance of wAlbA and wAlbB strains. Midgut and salivary glands were analyzed by PCR method until 10 days post CHIKV infection. Authors didn't show any differences of infection in presence or in absence of the bacteria. However, in 2017, Tan *et al.*, suggests that the wMel strain of *Wolbachia* is capable to limit the transmission of CHIKV and ZIKV (Zika Virus, *flavivirus*) in *Aedes aegypti* mosquito. In presence of the bacteria, viruses are not detected in salivary glands after 14 days post infection.

Transmitted by *Culex* species, West Nile virus (WNV *Flaviviridae* family, *flavivirus* genus), infection is enhanced when wAlbB is introduced by trans-infection in *Culex tarsalis* mosquitoes (Dodson et al., 2014). However, another study conducted in *Aedes aegypti* mosquito showed a reduction of infection rate, viral load and transmission after the trans-infection of wMelPop strain or wMel to a lesser extent (Hussain et al., 2013).

Altogether, the impact of *Wolbachia* on arboviruses seems to be dependent of various criteria: the *Wolbachia* strain, the recent or long-term infection by bacteria, the mosquito, and the virus. It suggests a real specific interaction between partners.

#### IV. Interaction Virus-Virus

A phenomenon called super-infection exclusion has been discovered in arboviruses. Super-infection exclusion or homologous interference is a phenomenon where prior infection with a virus reduces or prevents a subsequent infection with a closely related virus. This phenomenon was first described with two closely related genotypes of tobacco mosaic virus, where plants previously infected with common-mosaic virus could not be subsequently infected with the yellow-mosaic tobacco virus (Swanson et al., 1998).

In the case of *alphavirus* genus, some old studies on the *alphavirus*, Sindbis virus (SINV, Togaviridae family), have demonstrated the incapacity of another Sindbis virus to infect cells already infected. This exclusion has been also reported for other *alphaviruses*. A first infection with SINV excluded a second infection with CHIKV, Una and SFV (Eaton, 1979). Another study using three different cell types derived from *Ae. albopictus* (C6/36, C7/10 and U4.4) precise the restriction. In presence of SINV in cells, they observed a reduction of different SINV strain, but also other *alphaviruses* (AURA, SFV and RRV). This reduction was specific to *alphavirus* as the viral production of YFV virus was not impacted by the presence of SINV in cells (Karpf et al., 1997).

The super infection exclusion is not well understood, but numerous factors may contribute to the restriction, such as the competition for host cell receptors, the production of immune response factors or the production of a trans-acting protease by the first virus.

It has shown that only the non-structural proteins is required to the establishment of the exclusion (Adams and Brown, 1985). Moreover, the RNA of the second virus seems to fail to replicate (Stollar and Shenk, 1973). The direct transfection of RNA is not efficient, and it suggests that the blockage is intracellular, and not dependent of the entry or the attachment of the virion to the cell (Igarashi et al., 1977).

However, recent data are suggesting that infection evoked several independent mechanisms for blocking the entry and uncoating of superinfecting viruses (Singh et al., 1997). Finally, recently, Eilat virus (EILV), was described that readily infects mosquito but not vertebrate cell lines (Nasar et al., 2012). In C7/10 cells (*Aedes albopictus*), this mosquito specific *alphavirus* induced homologous and heterologous interferences, reducing the virus titers of heterologous superinfecting viruses (SINV, VEEV, EEEV, WEEV, and CHIKV) by ~10–10,000 fold and delaying replication kinetics by 12–48 h. Similar to *in vitro* infection, prior *in vivo* EILV infection of *Aedes aegypti* mosquitoes delayed dissemination of chikungunya virus for 3 days (Nasar et al., 2015).

Recently, investigations have been performed on dengue virus serotypes (*flavivirus* genus). Indeed, during outbreaks, various serotypes of dengue virus may cause disease. Co-infections performed in *Ae. aegypti* showed a competition between serotype 4 and 1, in favor of the serotype 4. The authors compared the infection, dissemination and transmission between mono and co-infection, in different organs crucial for infection. In midgut level, there is a higher dissemination of the DENV-4. Moreover, the transmission

is also drastically increased in co-infection model, due to the unique presence of DENV-4 in saliva (Vazeille et al., 2016).

Other studies have been conducted on West Nile virus in order to clarify the mechanism of super-infection inclusion Exclusion of West Nile virus super-infection through RNA replication (Zou et al., 2009). The authors introduced a replicon composed of all non-structural viral proteins into cell and after selection of cells by selective medium, cells expressed stably the non-structural viral proteins with a replicative system. The presence of the replicon into cells suppressed the WNV infection, but also other *flaviviruses*, such as yellow fever virus (YFV), dengue virus (DENV-2) (Zou et al., 2009). However, this process was specific to *flavivirus* as the replicon replication had no impact on Western Equine Encephalitis Virus (WEEV, *Togaviridae* family, *alphavirus* genus) or Vesicular Stomatitis Virus (VSV, *Rhabdoviridae* family, *vesiculovirus* genus). The viral attachment and the entry process were not affected, suggesting that exclusion occurs at the RNA synthesis step. More investigations have shown that viral proteins NS4a and 2k confer the super-infection restriction capacity of the replicon (Zou et al., 2009). A positively selected mutation in the WNV 2K peptide confers resistance to super-infection exclusion *in vivo* (Campbell et al., 2014). This work suggests the existence of competition for intracellular host factors required for viral RNA synthesis during viral replication. This phenomenon of super-infection exclusion, like for *alphaviruses*, has been also described for mosquito specific *flavivirus* (Goenaga et al., 2015). These super-infection exclusion mechanisms are interesting antiviral approach against human threats.

This phenomenon of super-infection exclusion has been also well documented for VSV (Simon et al., 1990) that infect insect cells. The *alphavirus* or *flavivirus* has been used as control of specificity and different interactions described above have been observed.

Interactions between micro-organisms (bacteria, parasites, virus, fungi) in the same organism are not a rare phenomenon. In the future, more investigations probably will demonstrate the importance of these interactions in the evolution of microorganisms, the severity of pathogenesis.

## Chapter 2. *Aedes* mosquitoes

### I. Introduction

*Aedes* mosquitoes are arthropod belonging to Insecta Class and Culicidae family, including *Anopheles* and *Culex* genus (Table 2). Created by Meigen in 1818, almost 800 species are identified, with 43 sub-genera (Mosquitoes classification 2010, [www.mosquitocatalog.org](http://www.mosquitocatalog.org)). Some of them are able to transmit a range of pathogens that cause human morbidity, mortality and suffering. Some of them are anthropophilic and have been identified as vector of viruses, named arboviruses, for arthropod-borne viruses. *Aedes aegypti* is one of the most efficient mosquito vectors for arboviruses, because it is highly anthropophilic (preferring human being over other animals) and thrives in close proximity to humans. With the other specie, *Ae. albopictus*, they are visually distinctive from other species by their black and white markings on their body and legs. These mosquitoes are known to be implied in the spread of severe viral diseases such as Yellow Fever, Chikungunya, Ross River and Zika Viruses. West Nile Virus can be transmitted by several *Aedes* species, such as *Ae. atropalpus*, *Ae. Brelandi* or *Ae. duprei* but *Culex* is the main vector. In this chapter, we will focus on the two species *Aedes aegypti* and *Aedes albopictus* mosquitoes.

Taxonomie	
<b>Kingdom</b>	Animalia
<b>Subkingdom</b>	Metazoa
<b>Phylum</b>	Arthropoda
<b>Sub phylum</b>	Hexapoda
<b>Super class</b>	Endopterygota
<b>Class</b>	Insecta
<b>Sub class</b>	Pterygota
<b>Infra class</b>	Neoptera
<b>Order</b>	Diptera
<b>Sub order</b>	Nematocera
<b>Family</b>	Culicidae
<b>Subfamily</b>	Culicinae
<b>Tribe</b>	Aedini
<b>Genus</b>	<i>Aedes</i>
<b>Sub genus</b>	<i>Stegomyia</i>
<b>Species</b>	<i>Aegypti, albopictus</i>

Table 2. classification of *Aedes* mosquitoes

## II. Biology, anatomy and ecology

### 1. Development cycle

#### 1. 1. Stages

Mosquitoes are capable to metamorphosis. The different developmental stages are present on Figure 1, including egg phase, larval phase, pupae phase, and finally adult phase. The duration of the biological cycle of mosquitoes varies considerably according to the sorts and ecological conditions such as the temperature, the pluviometer, predators, the larval competition or still the availability in resources (Armistead et al., 2008; Hawley, 1988; Mohammed and Chadee, 2011; Murrell and Juliano, 2008; Shirai et al., 2002). The eggs-laying is done on various wet support, not far from surface of water. Eggs resist to the low temperature, and to the desiccation (Farnesi et al., 2009; Hawley,

1988). They are said quiescent, that mean they are able to survive few months, by stopping their development, due to unfavorable environmental conditions (Dégallier et al., 1988). These characteristics are crucial for the survey and for the geographical expansion of species.

In optimal conditions of development, the duration of development of larval and nymph (or pupa) is from 6 to 8 days for *Ae. Aegypti*, until 10 days for *Ae. albopictus* (Dégallier et al., 1988; Hawley, 1988). Larval and nymph grow up in aquatic medium, the nymph has many transformations leading to the emergence of adult in 10 days. Generally, males emerge before females, and have a shorter life (Dégallier et al., 1988). The coupling follows by few the emergence. Females have only one coupling during their life, sperm cells are then store, until the fertilization during the successive eggs-laying (Biology of Mosquitoes : Development Nutrition and | A. N. Clements | Springer). In laboratory, the male lives one week in overall whereas the female lifetime varies from a few days to several weeks whereas in nature, predators can eat female, blown around by storms or slapped during the blood meal.

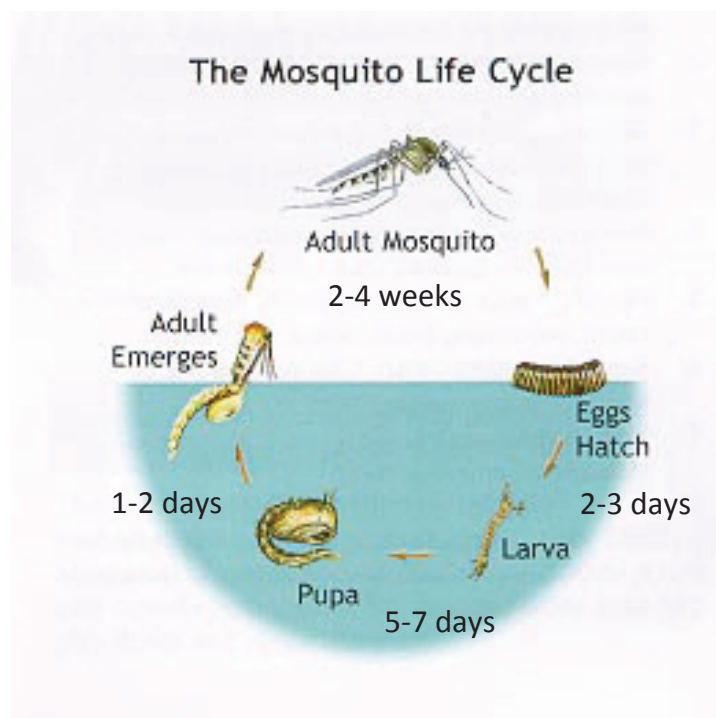


Figure 1. Life cycle of *Aedes* mosquitoes

### 1. 1. Blood meal

Only the female is bloodsucker. For mosquitoes, the blood meal is crucial to the egg maturation, due to the proteins and iron contribution. The majority of mosquitoes are nocturnal, with a peak of activity at nightfall. However, *Ae. aegypti* and *Ae. albopictus* species show a down and crepuscular diurnal rhythm (Hawley, 1988). They are capable of biting all day long in the wet and shaded zones.

As all hematophagous arthropods, mosquitoes, must overcome physical barriers and the physiological responses to successfully obtain a blood meal from a vertebrate host. Indeed, host develops a complex hemostatic and inflammatory system in order to prevent blood loss in case of lesion, or to combat infection. Therefore, mosquitoes develop mechanisms to neutralize hemostatic responses based on the release of saliva during the blood feeding.

The saliva contains a complex pharmacological mixture of proteins and peptides, can affect a multitude of factors, and can have a multitude of properties (Ribeiro, 1987). All hematophagous arthropods have at least one substance for each characteristic. Common features include vascular constriction, blood coagulation, platelet aggregation, inflammation, immunity and angiogenesis.

In contact with the host skin, the mosquito must locate very quickly the blood vessels. The exploratory phase consist to the introduction of the proboscis into the skin for searching the blood, by repeated movements, which lacerate blood vessel (Ribeiro, 1987). This phase can take few seconds. The saliva is ejected during this intradermal probing phase. The laceration and the saliva induce the formation of skin hematomas during the probing phase, and by this fact, reduce the time required to find blood. Besides the antihemostatic properties, the saliva has an antiplatelet activity. Indeed, rapidly after the laceration of blood vessels, platelets come into contact with collagen to reduce the hemorrhage. In blood-feeding arthropods, the salivary apyrases are nucleotide-hydrolysing enzymes that are implicated in the inhibition of host platelet aggregation through the hydrolysis of extracellular adenosine di-phosphate. The quantity of blood ingested can reach few microliters in few minutes.

After the blood meal, females belly becomes red. The blood meal is followed by a period of digestion leading to the complete development of eggs. The number of eggs depends on species and blood quantity absorbed during the blood meal.

If the blood meal is interrupted, mosquito is able to bite successively several hosts. The epidemiology study of the chikungunya fever outbreak in Montpellier in 2014 revealed that four patients from the same family, living in the same house, developed symptoms and present multiple mosquito bites (Delisle et al., 2015). The diagnosis of chikungunya had been confirmed for all of members. These temporally and geographically linked cases reveal the potential multiple mosquito bites in short term.

## ***2. Geographical distribution: an invasive specie: Aedes albopictus***

*Aedes* mosquitoes are considerate as invasive mosquitoes, by their ability to colonize new territories and to cause harm to the economy, environment or human health. Since the late 1990's, the environmental and climatic changes, the increase of the commercial exchange and travel around the world, have significantly increased the expansion of invasive mosquitoes.

### **2. 1. First invasions and expansion**

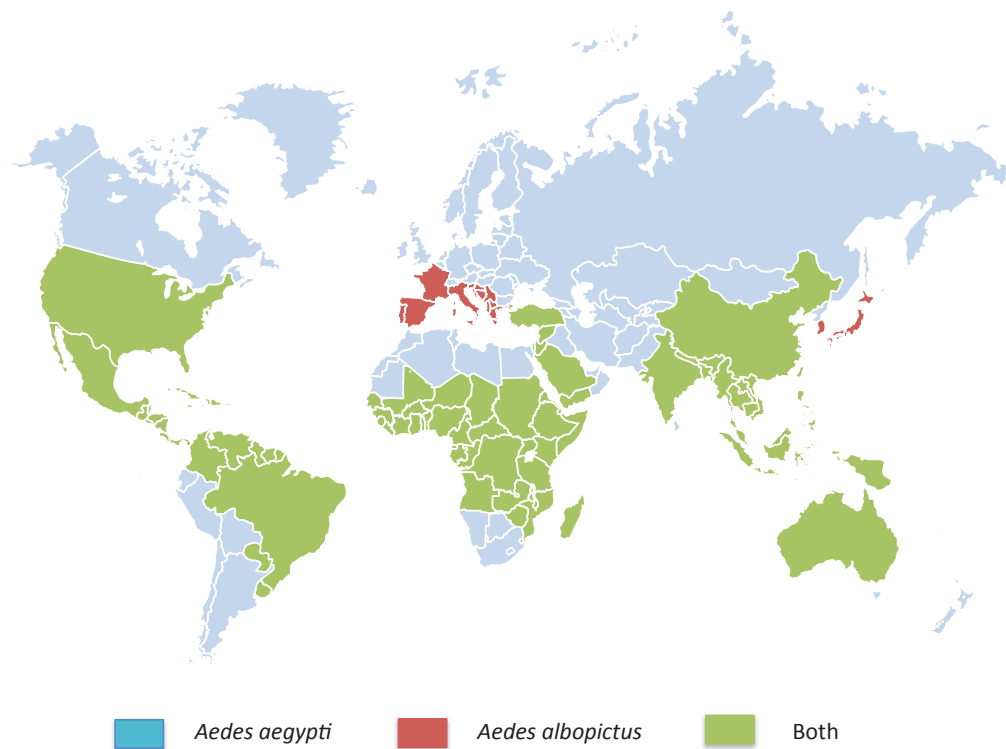
Specie of forest origin in Asia, *Ae. albopictus* spread west to islands in the Indian Ocean and east in the Pacific Ocean in the 19<sup>th</sup> century (Knudsen, 1995; Paupy et al., 2009). Then, it was first reported in Albania in 1979, in Texas in 1985, in Brazil in 1986, and in the Americas in 1991 (Adhami and Reiter, 1998; Forattini, 1986; Rai, 1991; Sprenger and Wuithiranyagool, 1986). The rapid expansion is due to the increase of commercial exchanges and tourism travels but this specie is able to an rapid adaptation to diverse environmental conditions in both tropical and temperate regions, which is unusual for mosquitoes (Rai, 1991).

Recently, the genome of *Aedes albopictus* has been sequenced. The large size of this genome, around 2000 Mb, may account in part to explain the ability of this mosquito to be a successful invasive vector, in contrast to *Aedes aegypti* vector. Important genes have been identified by transcriptome sequencing (RNAseq) to be involved in insecticides resistance, diapause, sex determination, immunity or olfaction, could contribute to the

adaptation to various environment (Chen et al., 2015). Previous studies had suggested that the induction of diapause in temperate areas had a genetic basis in *Ae. albopictus* (Hawley et al. 1987; Hanson & Craig 1994; Urbanski et al. 2010). A genetic background with numerous genes involving metabolic pathways could explain the ability for the susceptible populations to survive at the larval stage into eggs during the winter. In parallel, the high throughput genotyping performed by Goubert *et al.*, demonstrate a high level of differentiation between *Aedes aegypti* population from Viet Nam and temperature invasive population of Europe. They suggest that differences are caused by recent adaptive evolution events in temperate areas.

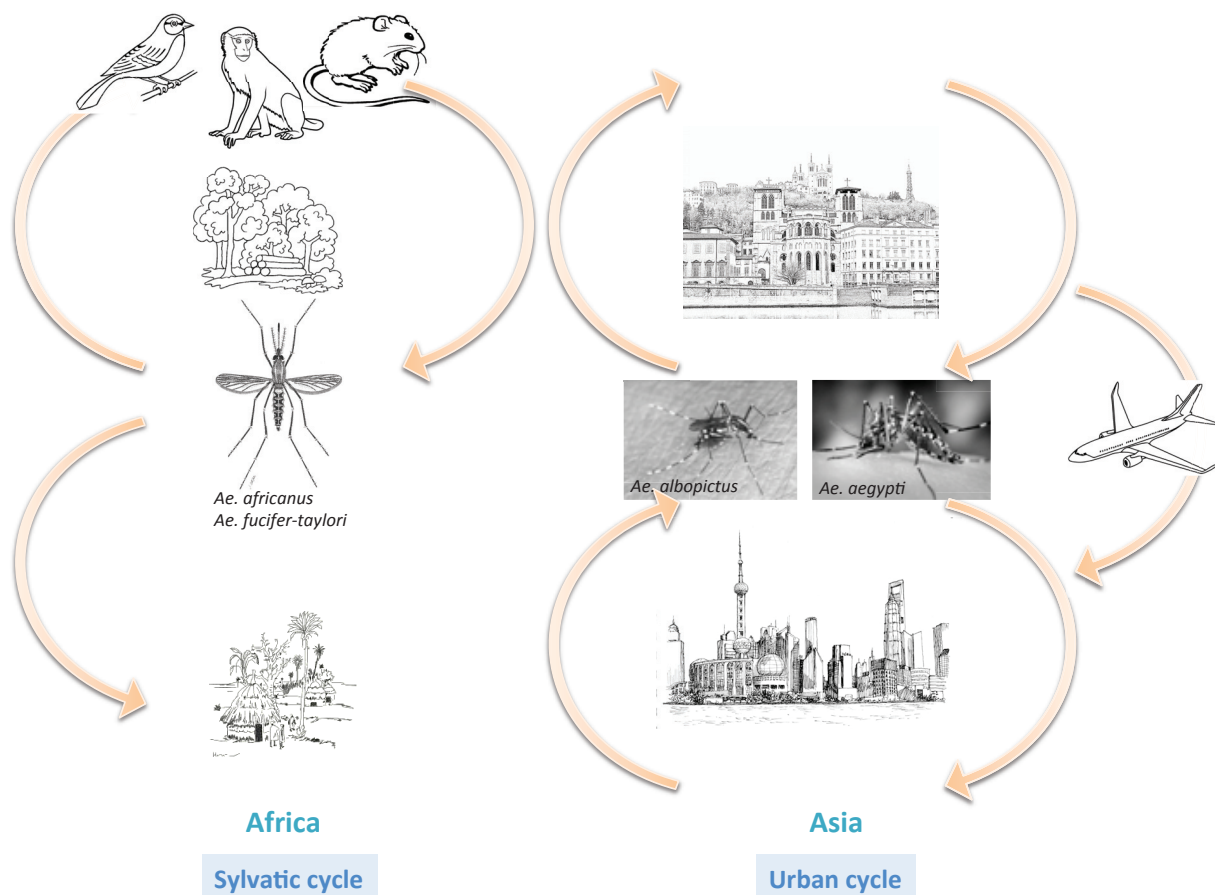
## 2. 2. Currently

Currently, both *Aedes albopictus* and *Aedes aegypti* are present in most Asian countries, large parts of the Americas, and the Mediterranean countries (Lambrechts et al., 2010). The Asian tiger mosquito, *Aedes albopictus* is currently one of the most threatening invasive species. Nowadays, *Ae. albopictus* has settled in every continent except Antarctica and is found in both tropical and temperate climates (Bonizzoni et al. 2013). In Europe, *Ae. albopictus* is also established, in Italy and countries of Adriatic coast, Spain, Portugal but also in France (Schaffner et al., 2013). In the future, the expansion of vector in native environments is not excluding, with *de novo* adaptation. The global distribution of these two species is shown in Figure 2.



**Figure 2. Geographical repartition of *Aedes aegypti* and *Aedes albopictus* mosquitoes**

*Aedes aegypti* is well adapted the urban environment of tropical cities. Because of the proximity with humans, it feed almost exclusively on humans and rest indoors (Scott and Takken, 2012). It is the dominant specie in most Asian cities. *Aedes albopictus* is better adapted to peri-urban or rural areas with vegetation; it bites humans and domestic animals (Paupy et al., 2009). Both cycles are represented in Figure 3.



**Figure 3. Transmission cycles of *Aedes* mosquitoes.**

Two transmission cycles can occur in function of the localization and the environment of mosquito. In Africa, virus is maintained in a sylvatic cycle, between mosquito and reservoir such as birds, rodents and monkeys. Occasionally, the transmission occurs in human, resulting to sporadic outbreak. In Urban cycle, as in Asia, the proximity of mosquitoes and human increase the probability of transmission human-mosquito-human, and outbreak occurs frequently. Adapted from (Thiboutot et al., 2010)

### III. A disturbing vector

#### 1. Definitions

##### 1. 1. Vector and Vector competence

To be an efficient arbovirus vector, three parameters are defined for arthropods. A vector mosquito is able to acquire, maintain and transmit an arbovirus. Various factors define the vector competence, including the ability to infectious agent to survive and to

develop in the mosquito tissues after ingestion, leading to the dissemination of pathogen. The consideration of the minimum level of viral load in blood meal that allows to infect mosquitoes and the percentage of infected mosquito after blood meal are critical factors.

Also important for the vector competence, the innate immunity-related pathways (detailed on the next part), and various tissues barriers that arbovirus need to overcome. Four principals barriers are defined: the midgut infection, the midgut escape, the salivary glands infection and the salivary glands escape (Franz et al., 2015).

Usually, after the ingestion of a sufficient amount of viremic blood meal, the midgut is infected. In this process, the midgut of mosquitoes is the major barrier to pathogen transmission. This tissue is the environment of viral interaction and replication. The infection is spreading to the midgut epithelium. If the replication is sufficient, the virus disseminates to secondary tissues in gut, and then other tissues outside of the midgut via the hemolymph, such as fat body, ovarian tissue, and central nervous chain. The salivary glands are also infected, leading to the release of viral particles into salivary ducts to ensure transmission within the saliva during a mosquito's bite to a vertebrate host (Hardy et al., 1983). The percentage of mosquitoes with infectious viral particles in saliva and the viral load in saliva is also some crucial criteria for the vector competence.

## 1. 2. Vector transmission

The dissemination of the virus inside mosquito depends on different steps (see above §1.1) and therefore takes time. This latency period during which the feeded mosquito is not yet infected is called the extrinsic period. This period depend of environmental conditions, but also of the arthropod and the pathogen (Kramer et al., 1983). Minimal viral particles are required to infect stably the vector. The vector will keep the infectious agent until the end of his life.

During the probing and the blood meal, saliva is injected into the skin and blood vessels. The transmission of arboviruses could be enhanced by mosquito saliva, in association with the modulation of immune response (Schneider and Higgs, 2008; Titus and Ribeiro, 1990; Wichit et al., 2016). The viral load in various organs is also increased. *Aedes*

mosquito saliva plays a crucial role in the vector's capacity to efficiently transmit arboviruses.

Investigations on WNV have shown the enhancement of viral infection in presence of mosquito saliva (Schneider et al., 2006; Styer et al., 2011). Moreover, mosquito saliva seems to alter host immune response during WNV infection, notably the leucocyte recruitment and the cytokine production (Schneider et al., 2010). They have shown with *in vitro* experiments the decrease of the expression of interferon  $\beta$  in macrophages and a transitory enhancement of interleukin 10 (IL-10) expressions. Supplements experiments *in vivo* confirmed previous results and highlighted the dys-regulation of antiviral signaling at the inoculation site, could leading to a possible enhancement of WNV disease.

*Ae. aegypti* saliva increased DENV serum viremia in mice lacking interferon (IFN) regulatory factors 3 and 7 (IRF3/7) (McCracken et al., 2014) and prolonged dengue viremia in "humanized" mice xeno-transplanted with human hematopoietic stem and progenitor cells (Cox et al., 2012). In contrast, Ader et al. found that mosquito saliva inhibits DENV infection of human monocyte-derived DCs (moDCs) *in vitro* (Ader et al., 2004). Recently, one study reveals that mosquito saliva affects dendritic cell migration, increases endothelial permeability, and augments dengue disease severity (Schmid et al., 2016).

On contrary, some virus modulate the protein expression in salivary glands and the protein composition in saliva to optimize the transmission (Tchankouo-Nguetcheu et al., 2012).

### 1. 3. Arboviruses transmitted by *Aedes* mosquito

Arboviruses are defined as all viruses transmitted by arthropod. *Aedes* mosquitoes are able to transmit a large number of arboviruses (Degallier et al., 1988). Mainly, *Aedes aegypti* and *Aedes albopictus* can transmit viruses belonging to Bunyaviridae, Flaviviridae and Togaviridae families. All viruses transmitted by arthropods are listed in Table 3. Some of them cause animal or human diseases, within more or less impact on the health public care.

	<i>Aedes albopictus</i>				<i>Aedes aegypti</i>				<i>Reservoir</i>
	Vector competence		Isolat	Vertical transmission	Vector competence		Vertical transmission		
	Isolat	Infection			Transmission			Infection	Transmission
<i>Togaviridae</i>									
Chikungunya virus	+	+	+	+/-	+	+	+	+/-	Bird, rodent
Eastern Equine Encephalitis virus	+	+	+	ND	-	+	+	ND	Bird, horse
Western Equine Encephalitis virus	-	+	+	ND	-	+	+	ND	Bird, horse
Venezulean Equine Encephalitis virus	-	+	+	ND	-	+	+	ND	Rodent, horse
Sindbis virus	-	+	+	ND	-	+	+	ND	Bird, rodent, horse
O'Nyong Nyong virus	ND	ND	ND	ND	ND	+	ND	ND	unknown
Semliki Forest virus	+	+	+	ND	-	+	+	ND	Bird, rodent
Getah virus	-	+	+	ND	-	+	-	ND	Horse, pig
<i>Flaviviridae</i>									
Dengue viruses	+	+	+	+	+	+	+	+	
Yellow Fever virus	+	+	+	ND	+	+	+	+	ptimates
Japanese Equine Encephalitis virus	+	+	+	+	-	+	+	+	Bird, pig
West Nile virus	+	+	+	ND	-	+	ND	ND	Monkey, rodent
Zika virus	+	+	+	ND	+	+	+	ND	
<i>Bunyaviridae</i>									
La Crosse virus	+	+	+	ND	+	+	+	ND	rodent
Schmallenberg virus	ND	ND	ND	ND	ND	+	ND	ND	ruminants
Rift Valley Fever Virus	ND	ND	ND	ND	ND	ND	ND	ND	Ruminants, rodent

ND: undetermined. Adapted from (Dégallier *et al.*, 1988; Hubálek *et al.*, 2014; Paupy *et al.*, 2009)

**Table 3.** Non exhaustive list of arboviruses.

## 2. Immune system

The mosquito maintains the virus during all its life. Indeed, the mosquito immune system is active and is crucial for the survival during some pathogenic viral infection. Very well documented in *Drosophila* insect, 3 major innate immune pathways have been identified, namely Janus kinase/signal transducer and activator of transcription (JAK/STAT), Toll and Immune deficiency (Imd) (Ramirez and Dimopoulos, 2010; Sanchez-Vargas et al., 2004; Souza-Neto et al., 2009). Pathways are represented in Figure 4, describe in recent review (Cheng et al., 2016). Immune system in mosquito varies according to the localization in organism, and in function of pathogen infection (Figure 4).

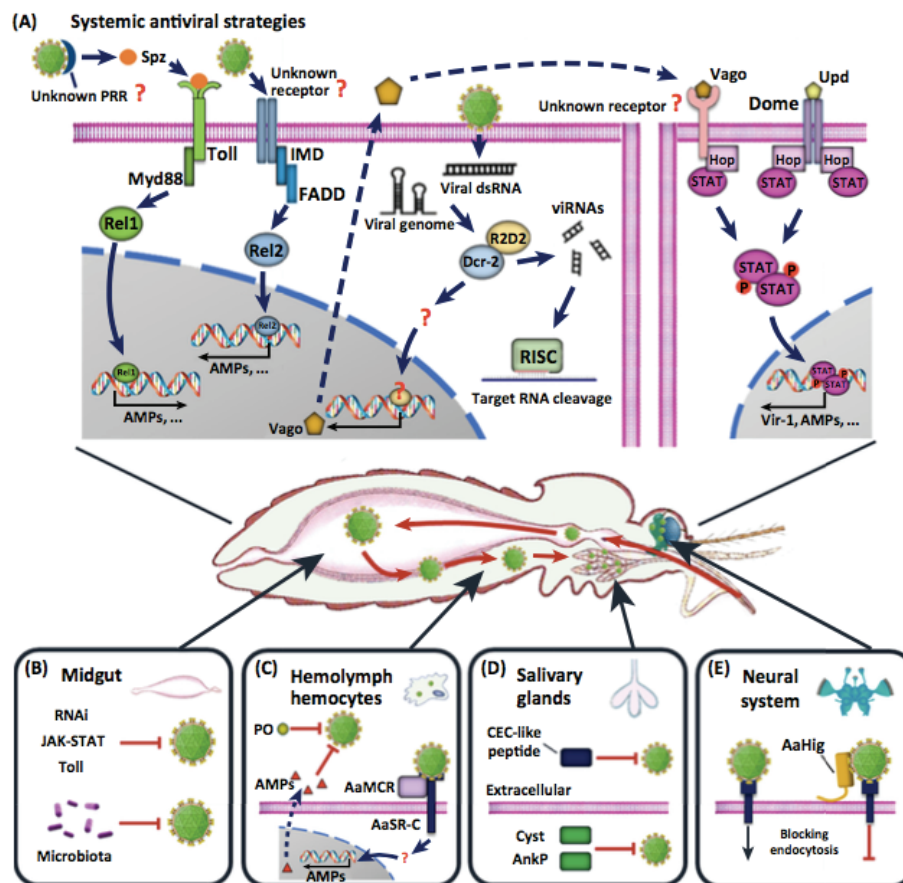


Figure 4. Immune system pathways in mosquitoes.

From (Cheng et al., 2016) (A) Antiviral strategies in insect. 3 major pathways have been identified: RNA interference, Imd and Toll Pathways. All of them lead to the elimination of pathogen. In parallel the JAK/STAT pathway can also contribute to the limitation of pathogen. Insects modulate the immune system in function of pathogen, and organ targeted (B-E). In midgut, RNAi, JAK/STAT and Toll modulate viral infection.

Well described in mammalian model, the autophagy and apoptosis pathways play crucial role in the control of viral infection. Their roles in insect cells are less understood. However, some factors involved in these pathways have been recently described.

## 2. 1. RNAi

Since the characterization of RNA interference in *Anophele gambiae* in 2003 (Hoa et al., 2003; Keene et al., 2004), this immune response is considered to be the most important antiviral mechanism in mosquitoes. Three major types of small RNA systems have been identified: microRNA (miRNA), PIWI-interacting RNA (piRNA) and small interfering RNA (siRNA) (Ghildiyal and Zamore, 2009). They differ in their modes of target regulation and the pathway they regulate. Interconnections are possible between these pathways in order to protect efficiently the genome from external or internal threats. In this part, we will exclusively talk about the small interfering RNA, which is derived from exogenous agents.

Briefly, a long and exogenous dsRNA is detected by a dsRNA-specific RNase III, namely Dicer (Dcr). The exogenous dsRNA is cleaved to form a siRNA duplex of approximately 21nt. The regulation by siRNA is mediated by the RNA-induced silencing complex (RISC), which will cleave target RNA.

Recently, their roles in virus infection have become apparent. RNAi is the major antiviral innate immune pathway in *Ae. aegypti*. This immune defense will be described in next chapters.

## 2. 2. Toll pathway

Unlike the mammalian Toll like receptors (TLRs), arthropod Toll receptors are not pattern recognition receptors (PRRs). The recognition is mediated by extracellular soluble PRRs. Upon recognition of virus debris, the cytokine spaetzle is cleaved into its

active form and binds to the Toll receptor (Arnot et al., 2010). A downstream signaling cascade results in the degradation of the Cactus protein and in the activation of the transcriptional factor REL1. Activated REL1 translocates to the nucleus and induces the expression of antimicrobial peptides (AMPs), such as the defensins.

### 2. 3. Imd pathway

The Imd pathway in mosquitoes is known to be activating by gram-negative bacteria. Binding of peptidoglycan of bacteria to PRGP like receptor activates the cytoplasmic Imd protein. A downstream signaling cascade results in cleavage of complex Caspar-REL2 proteins. REL2 is so translocated to the nucleus and activates transcription of AMPs. Well known for the implication in the antiviral response to insect virus in *Drosophila* with sigma virus (Rhabdoviridae family, *sigmavirus* genus), the only arbovirus known to be controlled with the Imd pathway is Semliki Forest Virus (*alphavirus*) (Avadhanula et al., 2009). However, the role of the pathway is still unclear.

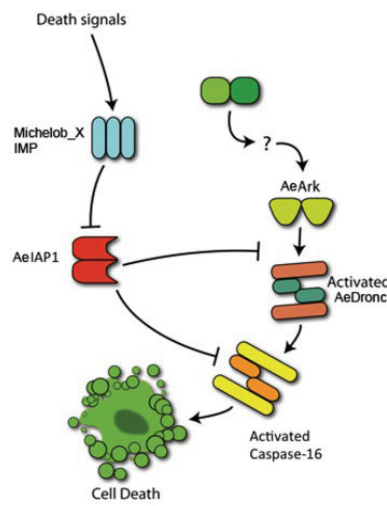
### 2. 4. JAK/STAT pathway

The JAK/STAT pathway has been characterized predominantly in mosquitoes. It plays a role in inhibition of the replication of many viruses (Sim and Dimopoulos, 2010; Souza-Neto et al., 2009). It had shown that the pathway is implied in the inhibition of the replication of *flaviviruses*, dengue virus, and West Nile virus. Upon the viral infection, mosquito Dcr-2 protein recognizes viral dsRNA and initiates a signaling cascade leading to the expression and secretion of Vago protein (Paradkar et al., 2012). The secreted cytokine Vago binds to a receptor on the cell surface, leading to the dimerization of the complex receptor-JAK. The dimerization results to the activation by phosphorylation of STAT protein, and to its translocation. In the nucleus, activated STAT acts as a transcriptional factor inducing expression of AMPs.

## 2. 5. Apoptosis

In mammalian cells, apoptosis pathway is one of the first line defense mechanism following a viral infection, since cell auto-destruction appears to be one of the best way to limit virus production and dissemination. Also named programmed cell death (PCD), this process occurs by morphological modifications including the condensation of the chromatin around the nucleus, a decrease of the cell volume, and the fragmentation of nucleus. Apoptotic bodies (important vesicles) could appear in some cases. PCD is involved in various biological mechanisms, development, regulation of cellular proliferation and the cell senescence, cancer, elimination of infected cells, and others.

Figure 5 shows the different pathways involved in the apoptosis in *Aedes* cells.



**Figure 5. Schematic representation of cell death pathways in *Aedes aegypti*.**

**From (Ocampo et al., 2013).** In insect, the caspase AeDronc interact with the caspase adaptor AeARK. Michelob X and IMP proteins, the IAP antagonist proteins, modulate the activation of apoptotic caspase. Interactions between these proteins and the AeIAP1 protein promote the cell death. AeDronc is cleaved after its activation and activate caspase 16, leading to the cell death.

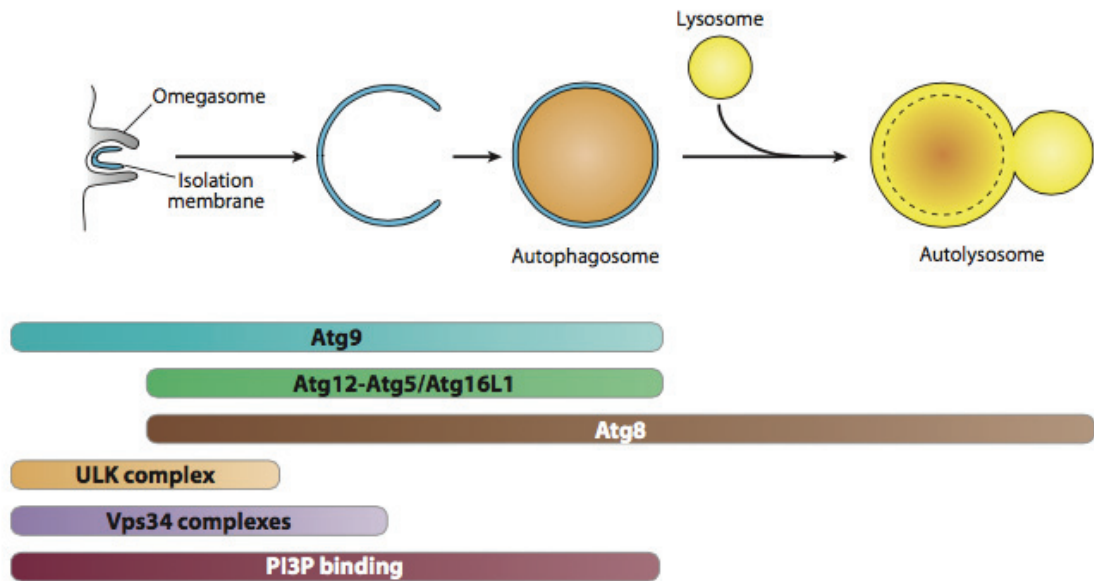
Known to be a crucial key of antiviral defense in mammalian cells, the role of apoptosis is less understood in insect cells. In mosquito, some apoptosis process had been observed after an arboviral infection (Bowers et al., 2003). Mostly doing some parallel with *Drosophila* model, some factors have been identified in *Aedes aegypti* (Bryant et al., 2008; Liu and Clem, 2011; Wang and Clem, 2011). The apoptosis pathway in *A. aegypti* consists of the initiator caspase Dronc (AeDronc) combined with its adaptor protein

AeArk, the effector caspases CASPS7 and CASPS8, the inhibitor-of-apoptosis protein AeIAP1, and two antagonist proteins, AeMichelob and IMP.

AeIAP1, has been identified as the major natural inhibitor of apoptosis in insect cells. It induces strong apoptosis activation when it is silenced by treatment with AeIPA1 double strain RNA in Aag2 cells (Wang and Clem, 2011). On the contrary, the silencing of *Aedronc* gene in Aag2 insect cells has been identified as a suppressor of apoptosis pathway (Liu and Clem, 2011).

## 2. 6. Autophagy

Autophagy is a cellular catabolic process, which sequesters cytosolic components within double-membrane vesicles and targets them for degradation in lysosomes. Further roles of autophagy in immunity include regulation of the inflammasome and cell-specific pattern recognition receptor signaling, as well as clearance of apoptotic bodies, and potentially as a mechanism to control inflammation. Autophagy is a crucial process of the adaptive immune response and is involved in host defense elimination of pathogens.



**Figure 6. Autophagy process induced by chikungunya virus.**

For the majority of proteins, mammalian homologous proteins have been identified. The ULK complex (UNC-51-like Kinase) initiates the process of autophagosome assembly, by translocation to the site of autophagosome formation to recruit and disassemble Atg proteins. Vps34 complex is recruited and assures supply of phosphatidylinositol 3 phosphate (PI3P), which acts as a crucial driving force in the formation of the isolation membrane during the autophagosome assembly. The Atg9 protein shuttles between autophagosome, the trans-Golgi network and late endosomes. During the expansion, PI3P-binding proteins and the Atg12-Atg5 Atg16 L1 complex proteins assist Atg9 protein. Atg8 protein is incorporated into membranes and is required for the fusion and the formation of the autophagosome, composed of a closed double-membrane. Finally, autophagosome fuses with lysosomes to form autolysosome.

In insect, the involvement of the autophagy pathway has been reported in *Drosophila* model, against Vesicular Stomatitis Virus (VSV, *rhabdovirus*) and Rift Valley Fever Virus (RVFV, *bunyavirus*), via the phosphatidylinositol-3-phosphate activation (PI3P) (Moy et al., 2014; Shelly et al., 2009). The Toll like receptor 7 (TLR7) is involved in virus recognition and the activation of autophagy. The PI3P pathway is also known to be activated following the Sindbis infection (Patel and Hardy, 2012). The inhibition of factors of PI3P pathway by drugs lead to the restriction of SINV infection. Although the role of autophagy in the *flavivirus* infection in mammalian cells has already been established, its role in mosquito remains to be determined.

## Chapter 3. Viruses

### I. Chikungunya virus

#### *1. Description*

Chikungunya fever is an arboviral disease caused by Chikungunya virus (CHIKV) and transmitted by mosquitoes. Recognized for the first time in Tanzania in 1956, the name refers to the characteristic posture of patients and means « to walk bent over » in the African dialect Makonde.

#### *1. 1. Geographical distribution – History of an emergent and re-emergent virus*

For several decades, emergences and re emergences of Chikungunya has been documented in Asia and Africa. But in the absence of low cost diagnostic tools, there were no precise surveillance during these outbreaks and the number of cases has been under estimated. Furthermore, diagnosis can be difficult due to the presence to another arbovirus, such as Dengue virus (DENV) responsible of Dengue fever. Signs and symptoms are very closed, and these two viruses circulate in same areas, many cases are probably misdiagnosed and the prevalence of CHIKV infection in some regions is probably higher than reported (Myers and Carey, 1967).

In 2005-2006, CHIKV emerged in the islands of West Indian Ocean, including La Reunion Island (Paquet et al., 2006). It was the first time that CHIKV had infected an Occidental country. The population of La reunion island is estimated at 785 000 peoples, and in few months, approximately one third of inhabitants got infected by CHIKV leading to approximatively 300 deaths. Because of the increase of tourism travels and commercial exchanges, the epidemic also spread in other countries leading to an explosion of cases in 2007. In India, more than 1.5 million people were infected. CHIKV was also detected in Malaysia, Sri Lanka and Indonesia and French Polynesia (AbuBakar et al., 2007; Laras et al., 2005). In Europe, infected travelers returning from epidemic countries were

diagnosed infected (Cordel et al., 2006; Schilling et al., 2009). In July 2007, the virus caused the first autochthonous epidemic outbreak in Italy, with 200 human cases, with the same index cases (Angelini et al., 2008). Another important feature is that CHIK hits for the first time the American continent in October 2013 and spread there since this time. The global distribution of chikungunya virus is reported in Figure 7.

Currently, the disease is present in nearly 40 countries and it is listed as a US National Institute of Allergy and Infectious Diseases (NIAID) category C priority pathogen.

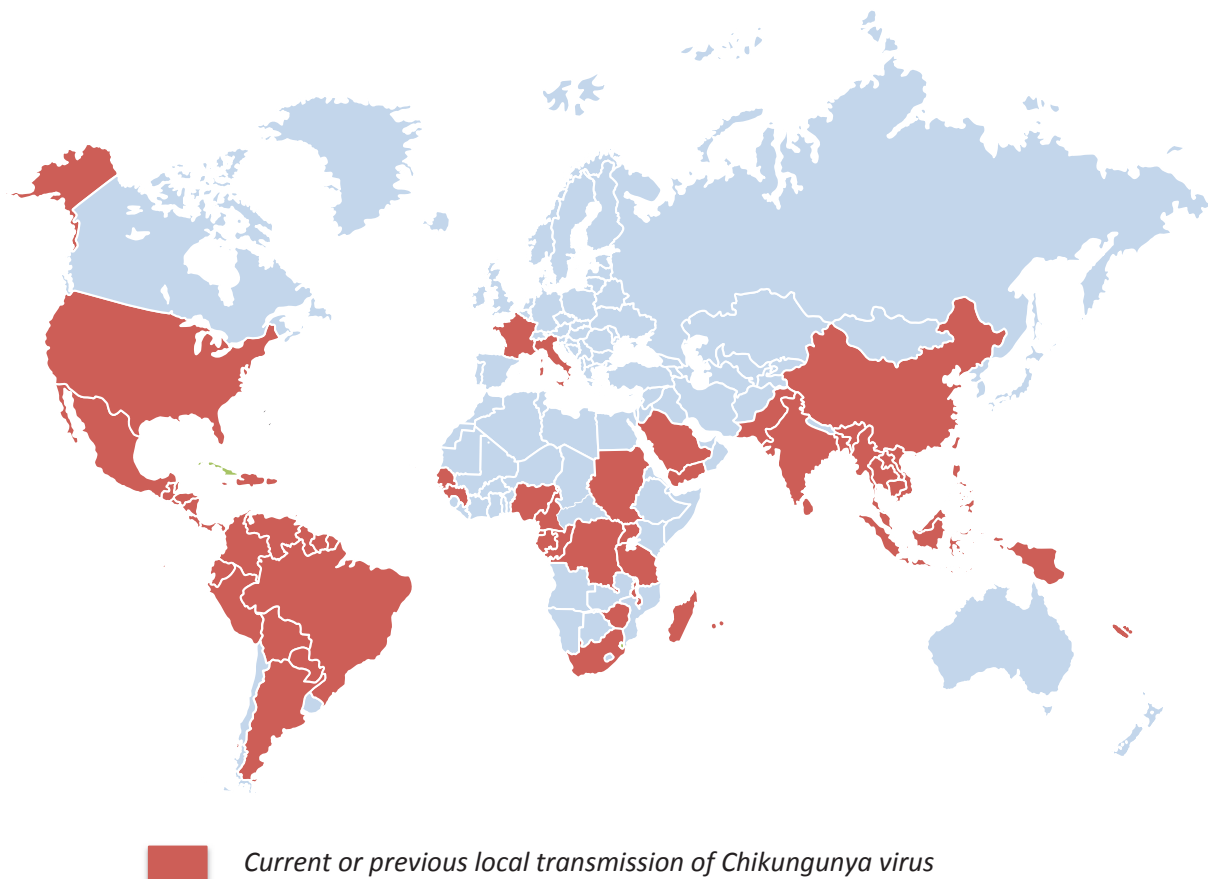


Figure 7. Geographical distribution of chikungunya virus.

## 1. 2. Transmission cycle

### 1.2.a. Vectors and transmission cycle

Chikungunya virus is transmitted via *Aedes* mosquitoes. In Asia, *Aedes* are primarily responsible for the maintenance of an urban cycle, as described in a previous part. The

proximity of humans and mosquitoes in urban cycle led to an increase of transmission human-mosquito-human.

In contrast, in Africa, CHIKV involves a sylvatic cycle in *Aedes furcifer* and *Aedes africanus*. During a sylvatic cycle, the virus resides in wild primates and mosquitoes. Humans may be infected in Africa villages or rural areas. Because of the distance between humans and infected mosquitoes, the circulation periodicity may have a silent interval last approximately 3 years.

Recently, in Europe, *Aedes albopictus* has been reported as vector of CHIKV during the outbreaks occurring in Italy in 2007 (Angelini et al., 2008) and the cases in South of France.

Investigations on vertical transmission in laboratory have shown that the vertical transmission in *Ae. aegypti* larvae and adult is a possible route of transmission. However, this phenomenon occurs rarely, certainly due to the long period needed to infect ovarioles (Agarwal et al., 2014; Bellini et al., 2012). During the “La Reunion island outbreak” in 2007, maternal transmission has been observed occasionally, 10 cases for 84 pregnant women (Robillard et al., 2006a).

### 1.2.b. Reservoirs

During outbreaks, human is the major reservoir for the virus. In Africa, because the transmission human-mosquito-human happens occasionally, virus may circulate into some monkeys, rodents and birds. This transmission occurs principally during non-epidemic period. The existence of this secondary cycle could contribute to the maintaining of virus in endemic areas while humans are immunized. Monkeys can develop viremia without pronounced physical manifestations (Wolfe et al., 2001).

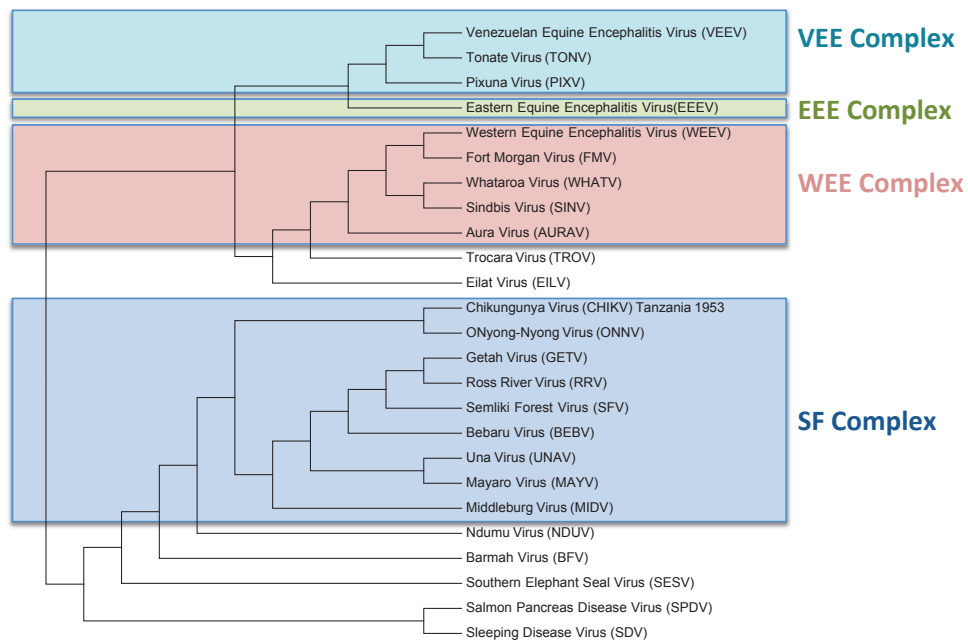
Human seems to be the only one reservoir in Asia. So far, even if controversial, no main seroprevalence has been observed in domestic animals.

## 2. Phylogeny and replication

### 2. 1. Phylogeny

CHIKV has been isolated into three genotypes based on genotypic and antigenic characteristics. Based on sequences of Envelope protein E1, these genotypes are Asian, East/Central/South African and West African. It is estimated that Asian genotype could appear between 50 and 310 years ago, whereas the West and East African genotypes diverged between 100 and 840 years ago (Strauss and Strauss, 1994; Weaver et al., 2012).

*Alphavirus* genus is composed of 28 viruses, and several to them cause disease in human. They are divided in 2 sub groups: the first one mainly found in the “Old World” *alphavirus* that cause severe arthritis and myositis (arthritogenic *alphaviruses*) and includes CHIKV, and Ross River Virus (RRV), O’Nyong Nyong (ONNV), Sindbis Virus (SINV). The second one, mainly found in the “New World” *alphavirus*, can causes encephalitis; it’s include Semliki Forest Virus (SFV), West Equine Encephalitis Virus (WEEV) and Venezuelan Equine Encephalitis Virus (VEEV). *Alphavirus* members are referenced in Figure 8.

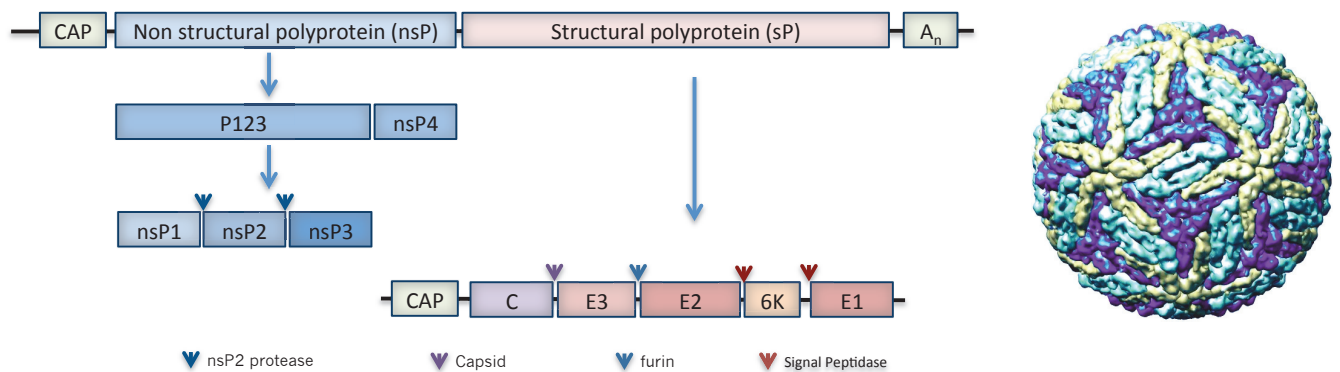


**Figure 8. Phylogeny of *alphaviruses* genus, based on nucleic acid encoding structural proteins.**

Four majors complex composed the *alphavirus* genus: VEE, EEE, WEE and SF complexes.

## 2. 2. Genomic organization and replication

CHIKV belongs to the *alphavirus* genus and *Togaviridae* family. *Alphaviruses* are small (60 nm in diameter), spherical, and enveloped viruses (Simizu et al. 1984; Strauss and Strauss 1994). The genome is composed of one single stranded positive RNA. In addition to genomic length RNA, sub genomic RNA encoding the structural proteins is also synthesized containing a 5'cap and a poly(A) tail. The coding sequence consists of two large open reading frames (ORFs). The 5'ORF encodes the non-structural proteins, nsP1-4, which are involved in RNA replication, and diversion of immune system in cell host. The 3'UTR encodes the capsid and envelope glycoproteins (E1, E2, 6K, E3), responsible of the particle structure. The two polyproteins are cleaved post translationally by viral and host proteases to generate the individual proteins (Figure 9).



**Figure 9. Genomic organization of chikungunya virus and viral particle representation.**

2 Open reading frames composed the alphavirus genome. One encodes non structural proteins (blue colors) and the other one encode structural proteins (red colors). The enveloped viral particle is small and spherical.

Represented in Figure 12, Chikungunya life cycle is similar to other *alphaviruses*. Before CHIKV outbreaks, most of studies of cycle life have been performed on Semliki Forest Virus (SFV) and Sindbis Virus (SINV).

### 2.2.a. Entry – putative Receptors

Every enveloped viruses use membrane receptor to enter into specific target cells (Cheng et al., 1995). The vertebrate host spectrum of *alphaviruses* varies for each virus. The specificity of entry is provided by the *alphaviruses* envelope glycoproteins.

The E1 and E2 glycoproteins form heterodimers on the viral surface. E2 is responsible for virus attachment to cell and E1 is responsible for the membrane fusion of viral membrane with the one of the endosome of target cell (Kielian et al., 2010).

Despite its capability to replicate in both mosquitoes and higher vertebrate cells, no mosquito or human cell surface receptors have been identified to be a clear cell surface receptor responsible of virus entry for CHIKV. However, some receptors have been implicated in this process. Never less, some entry factors have been described. Figure 10 gives an unexhausted list to potential receptors and entry factors for *alphaviruses*.

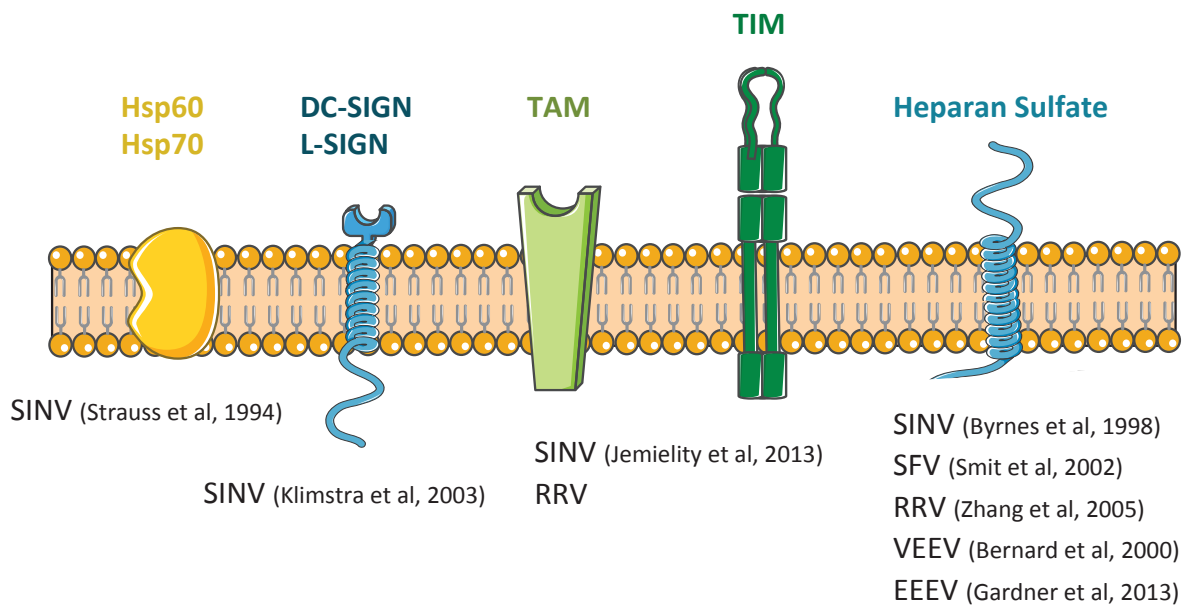


Figure 10. Putative receptors and/or co-factors involved in *alphaviruses* entry.

### 2.2.b. Entry, fusion and release of genome into cells

Cellular requirements for entry in host cells differ among *alphaviruses*.

The first study performed on SFV established the involvement of receptor-mediated endocytosis entry process (Helenius et al., 1980). As demonstrated for VEEV in 2006, CHIKV uses clathrin-dependent endocytosis vesicles to enter into target cells (Bernard et al., 2010; Kolokoltsov et al., 2006). Later, clathrin-coated vesicles are then uncoated and form endosomes. The acidic environment (5.0-6.0) of the endosome triggers conformational changes in the viral envelope that lead to the exposition of the E1 glycoprotein peptide (Kielian, 2006). This process mediates virus-host cell membrane fusion. Due to low pH exposition in endosome, the dimeric interaction of E1 and E2 is destabilized. After the dissociation of the E2 protein, the fusion loop of E1 is exposed and inserts into the target membrane leading to the fusion (Figure 12).

It has been shown that entry process is cholesterol and pH dependent in human cells (Bernard et al., 2010), but also in mosquito *Ae. albopictus* cells (Gay et al., 2012). A cholesterol depletion of human cells (HEK-293T cells) significantly reduced viral infection. Other *alphaviruses*, such as VEEV, are insensitive to membrane cholesterol depletion (Kielian and Helenius, 1984). The variations in E1 sequence at position 226 could explain these differences for cholesterol dependence (Lu et al., 1999).

During the epidemic in La Reunion Island in 2006, the major isolated virus strains presented a mutation in E1 glycoprotein at the same position (A226V). (Schuffenecker et al., 2006). In evidence, this mutation causes a decrease of CHIKV infectivity in *Ae. aegypti* midgut but has no effect on viral dissemination. Following ingestion of the infectious blood-meal, the E1-226V variant was preferentially selected in *Ae. albopictus*, but not after intrathoracic injection (Arias-Goeta et al., 2013). In *Aedes albopictus*, the E1-A226V mutation was proposed to modify of the cholesterol dependence and the increase of the CHIKV fitness, causing an enhancement of dissemination into mosquito secondary organs and to be transmitted to mice (Tsetsarkin et al., 2007; Vazeille et al., 2007). However, this result was not reconfirmed (Tsetsarkin et al., 2011) and the real consequence of the E1-226V still needs to be investigated. This important outbreak in Indian Ocean Islands was certainly due to an adaptation of the CHIKV strain, making *Ae. albopictus* a highly competent vector for CHIKV circulation.

Using a whole genome siRNA screen in U-2 OS human osteosarcoma cells, the cellular tetraspanin membrane protein TSPAN9 has been shown to be critical for the efficient fusion of low pH-triggered *alphaviruses* SINV, SFV and CHIKV with the endosome membrane (Ooi et al., 2013; Panda et al., 2013). The cellular function of this membrane protein is still unclear. Protein is localized at the plasma membrane and in early and late endosomes. The depletion of TSPAN9 decreases SFV membrane fusion in early endosomes. In contrast, infections of viruses using the late endosomes for the fusion are not impacted by TSPAN9 depletion. TSPAN9 modulates the early endosome compartment to make it more permissive for membrane fusion of early-penetrating viruses (Stiles and Kielian, 2016) (Figure 11).

The same genome wide siRNA screen reveals the involvement of Fuzzy protein (FUZ), to the internalization of viral particles via the clathrin-dependent pathway (Ooi et al., 2013). The ion transporter, NRAMP cell surface receptor has been highlighted by another screen in the *Drosophila* model in 2011 (Rose et al., 2011). This protein is required for SINV entry in drosophila and mammalian cells.

To date, no specific receptors for CHIKV had been identified, suggesting that co-receptors and co-factors could be involved in the entry of *alphaviruses*.

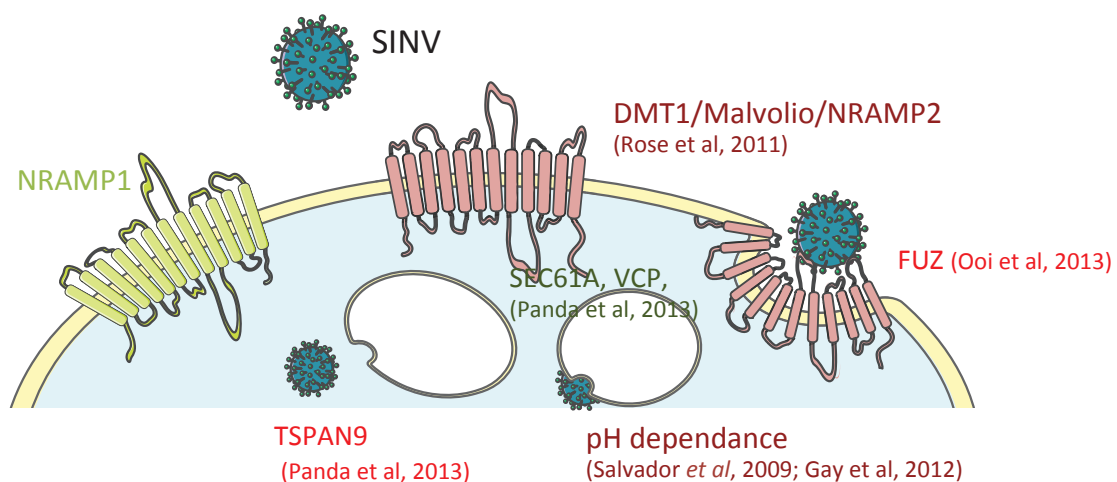


Figure 11. Illustration of identified factors leading to the entry of *alphaviruses* into host cells

### 2.2.c. Genome replication

Entry process allows the penetration of the viral genome into target cell cytoplasm.

In cytoplasm, the viral genome is then translated from two ORFs to generate the nonstructural (P1234) and structural proteins. Early in infection, P1234 is cleaved in *cis* between nsP3 and nsP4 to yield P123 and nsP4 (de Groot et al., 1990; Takkinen et al., 1991). These proteins form an unstable initial replication complex, able to generate negative-strand RNA (Strauss and Strauss 1994). The cleavage of P123 to nsP1 and P23 is occurred in *trans*, with a high concentration of the polyprotein. After complex cleavage to nsP1, nsP2, nsP3 and nsP4, negative-strand synthesis is inactivated and the stable replication complex switches to synthesis of positive-strand genomic and sub-genomic RNA.

Cleavage of the structural polyprotein occurs co-translationally, beginning with the auto-proteolytic cleavage of the capsid protein. The C protein permits the encapsidation of the newly synthesized RNA, recognizing specific signals in the 5' half of the genome (Lee et al., 1996; Weiss et al., 1989).

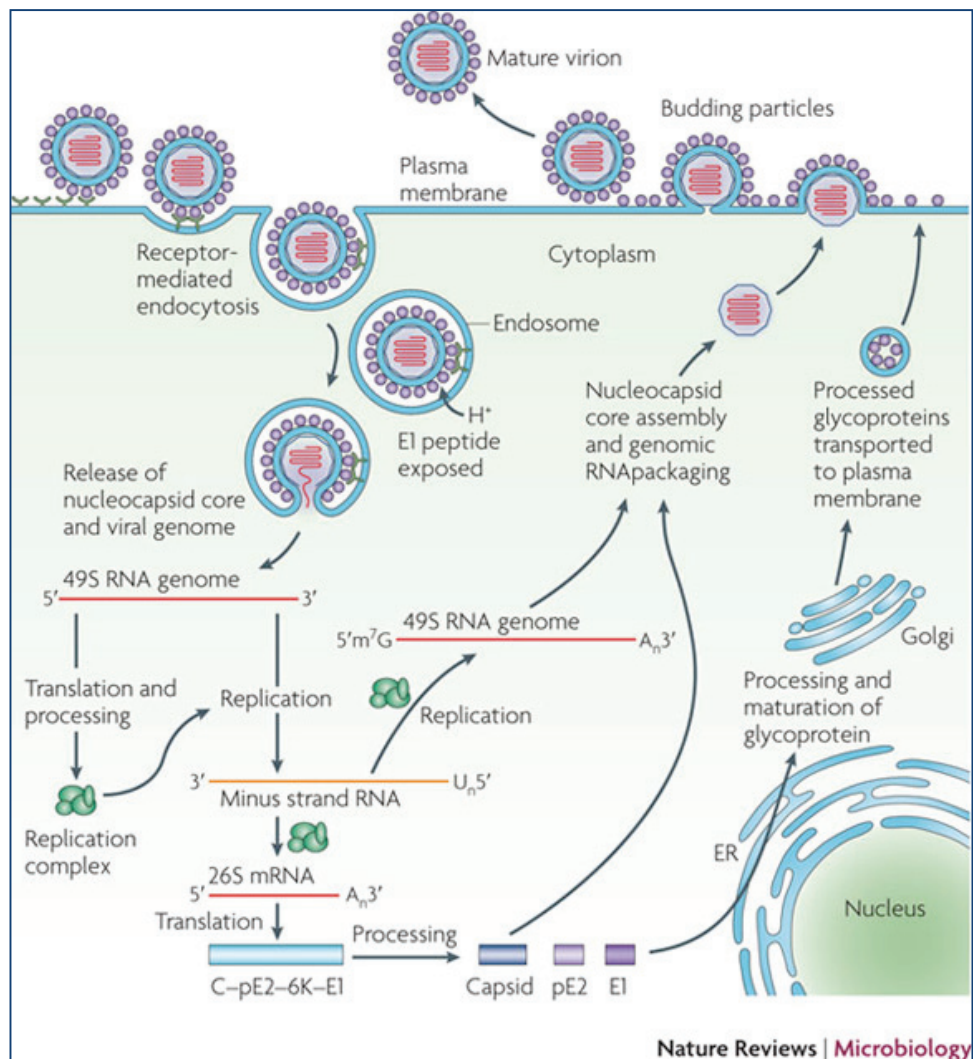
### 2.2.d. Assembly and release

The E3 protein acts as a signal sequence for insertion of the polyprotein into the endoplasmic reticulum, where it is processed by host signal peptidase. The viral 6K protein acts as a signal sequence for the downstream processing of the E1 protein.

The E2 precursor and E1 glycoproteins interacts with each other to form heterodimers. These protein complexes are then transported from the endoplasmic reticulum to the cell surface via the Golgi complex (De Curtis and Simons, 1988; Green et al., 1981; Sariola et al., 1995). The E2 precursor is cleaved at a late stage of transport, by host cellular furin protease, leading to the generation of mature E2 and E3 proteins. This cleavage induces a conformational change that weakens the E1-E2 complex, priming the fusion protein for activation upon exposure to low pH.

The C and E2 proteins interact and drive the budding process, with E1-E2 heterodimers forming an envelope around nucleocapsid-like particles.

Upon release from cells, virion acquires a membrane bilayer derived from the host cell plasma membrane (Figure 12).



**Figure 12. Replication cycle of chikungunya virus.**

From Schwartz et al., 2010

### 3. Pathogenesis and cellular tropism

#### 3. 1. In insect

Virus maintains its replication until the end of mosquito life. Arboviruses cause chronic non-cytopathic infection in mosquito cells.

*In vitro*, every mosquito cells developed are derived from larvae, making difficult the study of tropism. However, we know that viruses do not induce cytopathic effect on every mosquito cells. The most common cell line, C6/36 cells, is derived from *Ae. albopictus* larvae. Study had shown that viruses establish a persistent infection in C6/36 cells that can be seen still after 20 passages (Li et al., 2013). Viral production in insect cells is higher than in mammalian cells.

Since several years, insectarium laboratories have been developed and used to infect mosquito models in order to understand complex process of arboviruses infection and transmission. Mosquitoes ingest an arbovirus-infected blood meal into the midgut.

### 3. 2. In human

CHIKV infects humans through the bite of infected mosquito. Compare to many arborivuses, the CHIKV infection always leads to symptoms. Ninety-five percent of infected people are symptomatic after infection.

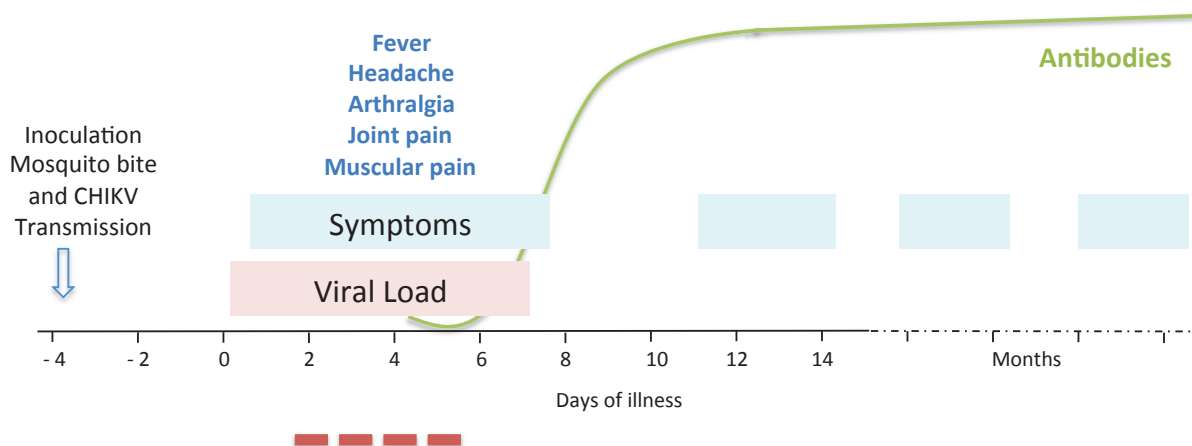
Symptoms appear after only 2-6 days of incubation time (Figure 13). In human, the first stage is acute, which consists to an abrupt fever (more than 38,5°C), intense joint and muscular pains, which are very crippling for patients. Headache, nausea and vomiting have also been described during this stage. The viremia during this phase can reach  $10^{12}$  viral particles per ml of blood, accompanied by a strong immune response with type 1 interferon secretion and induction of inflammatory cytokines and chemokines.

Lymphocytes, T cells, and B cells are refractory for CHIKV infection (Sourisseau et al., 2007). This is probably due to the lack of compatible receptor(s) on these cells as the cells are non-susceptible with the pseudotype system (Salvador et al., 2009).

The acute phase resolves typically within two weeks, but for 30%-40% of patients, a chronic joint pain may persist for weeks to months or years, with possibilities of relapses (Figure 13). This is the persistent phase, resulting of loss of mobility and decreased dexterity. It has been shown that CHIKV is able to infect fibroblast, explaining joint and muscular pain as symptoms. To date, no studies really proved the viral persistence within cells months after disease onset even if some antigens (expressed proteins) are found in these tissues. It has been suggested that the joint pain is immune

mediated, similarly to the disease caused by another member of *alphavirus* genus, Ross River Virus (RRV).

Some severe forms of CHIK disease have been reported since few years, such as hepatitis, or encephalitis. But, these severe cases have been observed in 65-year-old patients, or in patients with underlying medical conditions such as diabetes, impaired renal function or hepatic disease. Children are also concerned by severe CHIKV infection (Haas et al., 2009). Most common of these complications are encephalitis, respiratory failure, acute hepatitis, central nervous system problems and kidney failure.



**Figure 13. Representative symptoms for chikungunya fever.**

After the mosquito bite, first symptoms of chikungunya fever appear in few days, in correlation with viral load in blood. Fever, headache, joint and muscular pain are major symptoms. In response, antibodies are produced by immune cells, and are circulating until few months or years. Some patients develop chronic symptoms, with onset of arthritis. Adapted from Schwartz 2010.

Following transmission, CHIKV replicates locally, in cells of the skin, mainly fibroblast, and then disseminates to the liver, muscle, lymphoid tissue and joints, through the blood. The development of nonhuman primate model (Gardner et al., 2015; Labadie et al., 2010) and mouse models has been helpful to identify the target cells of CHIKV in infected tissues during disease. Using macaques as model allow to be closed of viral, clinical and pathological features of CHIKF.

In skeletal muscles, CHIKV is detected in connective tissue, in the epimysium (muscle fascia), and lesser in the perimysium and endomysium (Couderc et al., 2008). Moreover, CHIKV targets tendinous insertions of muscle, constituted of fibroblasts. The high permissiveness of fibroblasts for CHIKV can also be observed *in vitro* in primary mouse

and human muscle fibroblasts. As some other *alphaviruses*, CHIKV is an arthritogenic virus.

In joints, the virus is present in the connective tissue and in the deep dermis of the skin. Tendons could be sites of replication, even if the cell types targeted in these tissues has not been identified. Long-term infection is observed in joints, muscles, lymphoid organs, and could explain long term symptoms of the disease (Labadie et al., 2010).

Gardner et al., have shown the presence of virus in oral cavity of infected mice due to lesions. The transmission mice-to-mice was observed without any vector action, by cohabitation of infected and uninfected mice (Gardner et al., 2015). The saliva of infected monkey presents also infectious virus, and confirmed the presence of virus in saliva of patients during acute infection. These results are the first proof that oral fluid contains infectious virus.

During the La Reunion outbreak in 2006, some cases of vertical transmission, mother to child (Lenglet et al., 2006; Robillard et al., 2006a; Robillard et al., 2006b). Investigations have been developed in pregnant animals. Although the maternal serum is rich in viral load, the placenta is not a target for CHIKV, and the fetus is uninfected (Gérardin et al., 2008). This observation is in agreement with *in vitro* experiments, using titration after CHIKV infection in placenta cells, BeWo cells. Even if it does not represent of the different cell types of placenta, BeWo cells are non-permissive to CHIKV infection (Couderc et al., 2008). The high viral load detected in placenta probably corresponds to a contamination from infected maternal blood.

#### 4. Immune response

##### 4. 1. In insect

Before CHIK outbreak, the majority of investigations on the immune system in insect in response of *alphavirus* infection have performed on Sindbis (SINV) virus. Experiments performed in *Ae. aegypti* cells had shown that the inhibition of RNAi pathway led to a

more efficient replication of SINV (Cirimotich et al., 2009). Others studies performed on *Ae aegypti* mosquitoes confirmed these results (Khoo et al., 2010; Khoo et al., 2013). It's appearing that RNAi pathway modulates the vector competence in midgut and limit the intensity of infection, crucial for the survey of mosquito. Impairment of RNAi pathway by using transgenic mosquitoes reduce midgut barriers for virus, and it seems to be a specific effect depending of arbovirus infection (Campbell et al., 2008).

Another study had shown the role of the other immune pathways. None of Toll, Imd or JAK/STAT pathways are able to control CHIKV infection in *Aedes aegypti* cells, as well as in mosquitoes (McFarlane et al., 2014). A study performed in *Drosophila* model confirmed this hypothesis (Avadhanula et al., 2009). Sindbis infection is unaffected by mutations in the Toll pathway. However, the implication of Imd pathway in *Aedes* mosquito is still ascertained. Recently, the *diedel* gene of the *Drosophila* was strongly induced by some viral infections, such as SINV or VSV infections, and had been identified to promote the host survival, notably by modulation of the Imd pathway (Lamiable et al., 2016).

Still unknown for CHIKV infection, another *alphavirus*, SINV induces apoptosis during infection (Wang et al., 2012). Previous studies showed the crucial role of *Aeiap1* and *Aedronc* genes, which act as inhibitor and initiator of this pathway, respectively (Liu and Clem, 2011). The silencing of *Aeiap1* induced a high mortality in mosquitoes, and the tissues analyze showed an alteration of midgut morphology. SINV infection was strongly increased with a silencing of *Aeiap1*, whereas the silencing of *Aedronc* induced a reduction of viral infection (Wang et al., 2012). Altogether, this study suggested that there is a correlation between the apoptosis that the virus induces in midgut and the vector competence.

## 4. 2. In human

### 4.2.a. Apoptosis

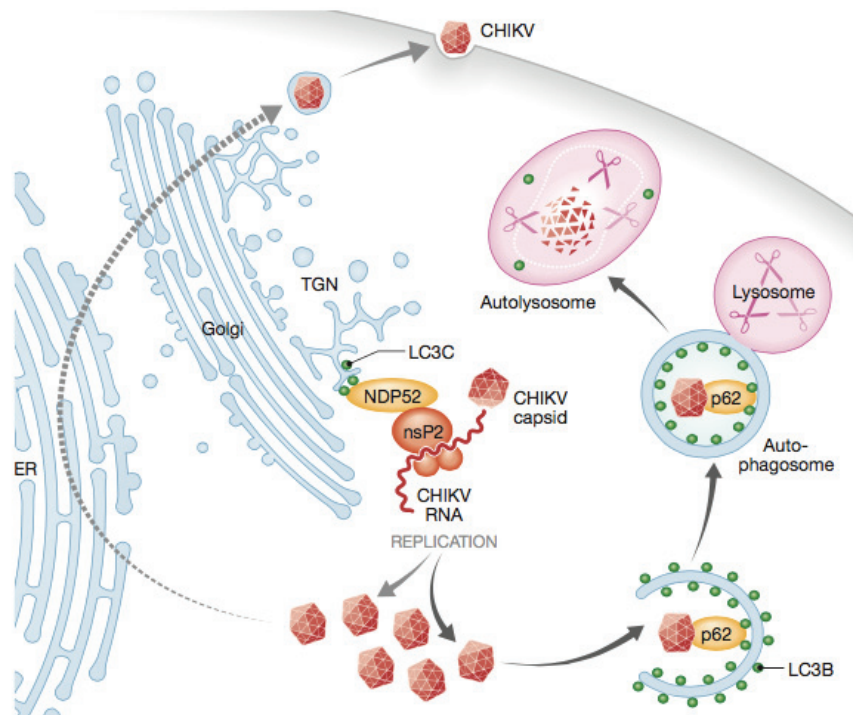
In mammalian hosts, most viruses deal with this immune reaction, and have evolved strategies to avoid apoptosis (Galluzzi et al., 2008; Teodoro and Branton, 1997). Control of the apoptosis is a way to maintain infection and to facilitate the dissemination in organism. CHIKV is known to be a cytopathic virus, with a rapid onset of syncytium formation and activation of apoptosis in infected cells (Sourisseau et al., 2007; Wikan et al., 2012). The induction of apoptosis both occurs by a non-typical intrinsic and extrinsic pathways (Krejbich-Trotot et al., 2010). Interestingly, even if it can seem deleterious for the virus, CHIKV, as other arboviruses (Meertens et al., 2012) developed a strategy to take an advantage of this immune pathway (Jemielity et al., 2013). During the latest stage of apoptosis, apoptotic bodies contain virus (Krejbich-Trotot et al., 2010), and virus use these vesicles as “Trojan horse” to infect neighboring cells, via apoptotic bodies receptors, such as macrophages. This scenario can explain the escape to the immune system by CHIKV, causing the chronic disease.

Others members of *alphavirus* family, SFV and SINV induce p53-independent and Bad-mediated apoptosis, respectively (Glasgow et al., 1998; Moriishi et al., 2002). Moreover, the induction of apoptosis by SINV infection had been correlated with the neurovirulence (Lewis et al., 1996). Experiments based on immunostaining of infected tissue from mouse, revealed the infection of mouse brain with CHIKV (Chatterjee and Sarkar, 1965; Das et al., 2010) and the disease pathogenicity seems to depend of virus-induced apoptosis, with an observation of neuronal cell damage (Dhanwani et al., 2012a).

### 4.2.b. Autophagy

In 2012, it has been shown that CHIKV induces autophagy in mouse model (Joubert et al., 2012a) and promotes viral replication in human kidney cells (Krejbich-Trotot et al., 2011a). Moreover, the autophagy mechanism delays CHIKV-induced cell death and CHIKV interacts with proteins of the autophagy process during its replication. The

human autophagy receptor NDP52 interacts with the non-structural protein nsP2, and co-localized with LC3C protein on trans-Golgi membranes, leading to the promotion of the viral replication. The viral capsid is degraded by lysosomes binds the autophagy receptor p62 through LC3B protein, after recruitment in autophagosome and by this way protects cells from death (Judith et al., 2013). This cellular proteins p62 and NDP52 play anti- and pro-viral roles in human cells, respectively. All interactions between CHIKV and autophagy components are shown in Figure 14.



**Figure 14. CHIKV-induced autophagy in mammalian cells.**

From (Münz, 2013)

The viral capsid degradation may influence the cytokine production, as antigen presentation cells. Autophagy is known to impact cytokine production and can deliver cytosolic antigens for processing and presentation by major histocompatibility complex class II molecules (Randow and Münz, 2012).

#### *4.2.c. Humoral response*

Following the CHIKV infection, a protective adaptive immunity is developed against virus. IgM and IgG antibodies are detected in serum of infected travelers returning from Indian island, and their ability of neutralization have been demonstrated by using sera from convalescent patients (Couderc and Lecuit, 2009; Panning et al., 2008).

The T cells are important effector cells during CHIKV infection, mostly T CD4+ cells (Teo et al., 2013), and play a role in the pathology in mouse model. Cells are involved in the joint swelling without any impact on virus replication.

The activation of humoral response following infection, and the persistence of antibodies in organism could be a factor in the chronic disease. The presence of IgG and IgM was associated with arthritis (Chopra et al., 2008)

However, mechanisms in the establishment of chronic disease induced remains undefined.

### *5. From diagnosis to treatment*

Laboratory confirmation is crucial for a specific diagnosis. The similarities of clinical manifestations with other arboviruses such as dengue virus, or parasites such as malaria, have to be considerate to have a good diagnosis.

The diagnosis is dependent of the kinetic of viremia and antibody response in human. During the acute phase, only viral nucleic acid is useful, when CHIKV infectious particles, and viral RNA reach high levels. Molecular assays permit a rapid and sensitive detection in blood. Retro-transcribed PCR (RT-PCR) were developed using different viral target genes: the envelope E1 gene, the non-structural nsP1 or nsP2 genes (Carletti et al., 2007; Pastorino et al., 2005). In parallel, viral isolation is also useful notably for epidemiology and pathogenesis studies (Figure 15).

After few days, the infectious virus in serum decrease and the diagnosis is mainly based on the detection of humoral immune response, the antibodies, by serological methods. These methods detect antibodies produced in response of viral infection. IgM antibodies are detected 2 days after infection, and may persist until few months. In contrast, IgG may persist for longer periods, until few years (Litzba et al., 2008; Sam and AbuBakar, 2006). Most common technics use Enzyme-linked Assays (ELISA), Immuno-Fluorescent Assay (IFA), hemoagglutination inhibition (HI), or micro-neutralization. Commercial

serological assays are available based on these methods, but new methods are currently developed, in order to increase the efficiency and the specificity of tests (Figure 15).

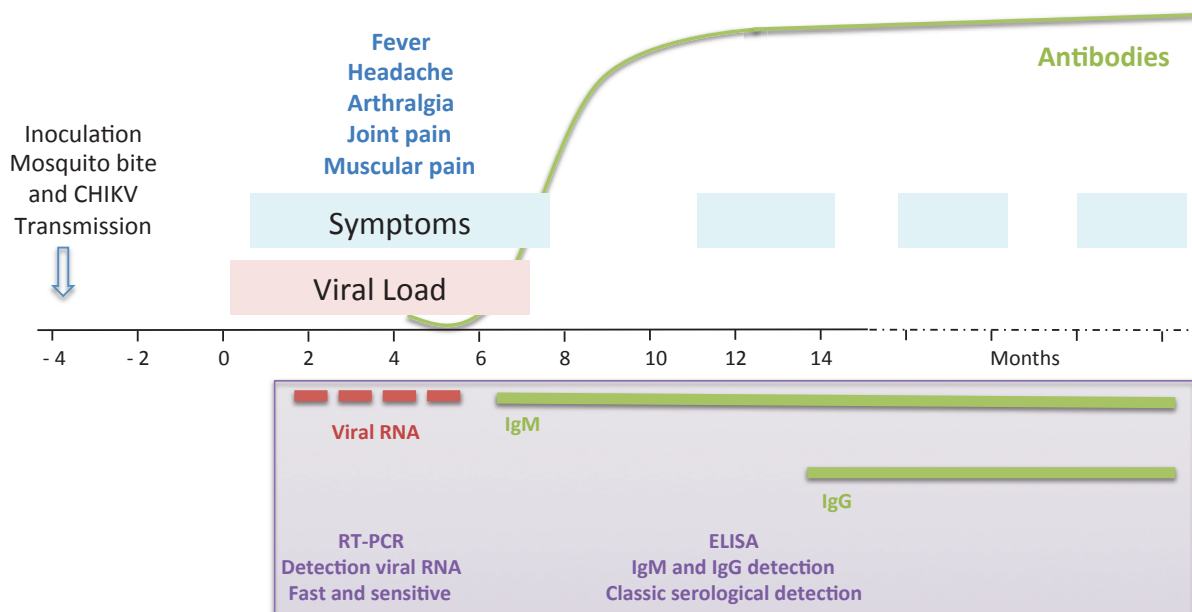


Figure 15. Methods used to detection chikungunya infection according to the time of diagnosis

## II. Dengue Virus

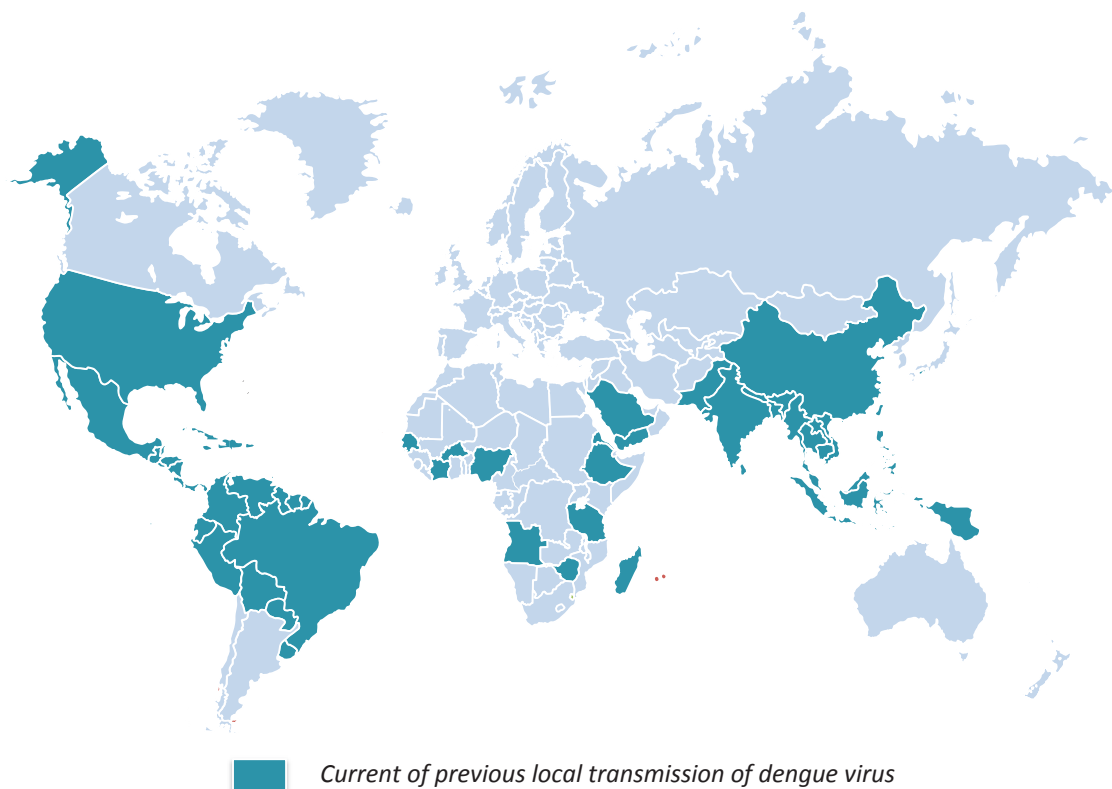
### 1. Description

Dengue fever is probably the most important arthropod borne-viral disease in term of morbidity and mortality. There are between 50 and 100 million infections each year, resulting in 500 000 cases of Dengue hemorrhagic fever (DHF) with 5% of fatality rate. Dengue has been identified by others names such as « coup de barre », « knee fever », or « leg fever » based on symptoms. Dengue fever is endemic in many countries in tropical and subtropical regions.

### 2. Geographical distribution

Since few decades, a dramatic expansion of the DENV occurred due to an increase of international travels, urbanization, and a lack of mosquito control measures in a lot of countries. In last five decades, the reported cases of Dengue have increased by 30-fold,

and currently, dengue is endemic in 128 countries, exposing approximately 4 billion people to infection (Figure 16). In addition, the different serotypes of DENV (1, 2, 3 and 4) previously restricted to different part of the world, are now circulating in most regions.



**Figure 16. Global distribution of dengue serotypes**

### 3. Transmission cycle

#### 3. 1. Vectors and Transmission cycle

Dengue viruses are transmitted to human through the bite of infected *Aedes* mosquitoes, principally *Aedes aegypti*, *Aedes albopictus* or *Aedes polynesiensis*, depending on the geographic areas (Gubler and Rosen, 1976). Enzootic transmission cycle of dengue viruses involves *Aedes* mosquitoes and primates in forest of Asia and Africa. Viruses can occasionally move out into urban areas (Rico-Hesse, 1990) (Figure 17). The sylvatic cycle is the most threatened for public health, because of the proximity between vectors

and hosts. Viruses are maintained in an *Aedes aegypti* – Human – *Aedes aegypti* cycle, with periodic epidemics. In endemic areas, two or more dengue serotypes are present and co-circulate (Pérez-Castro et al., 2016; Pessanha et al., 2011).

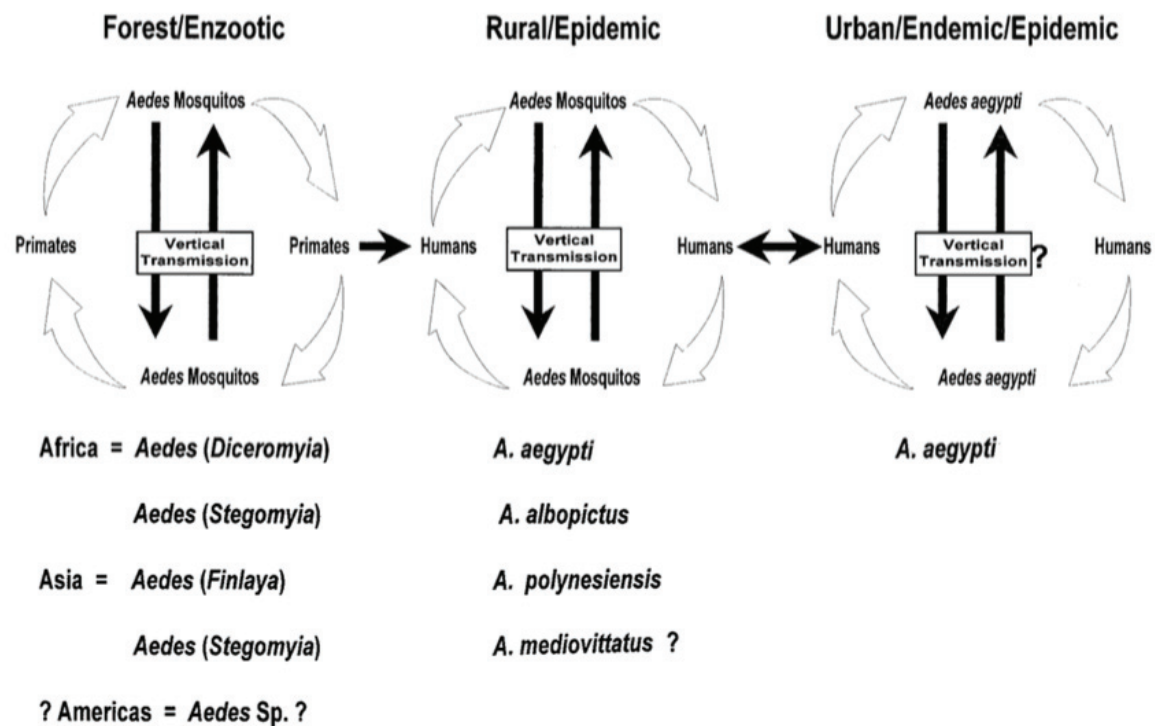


Figure 17. Transmission cycles of dengue viruses and differences of vectors according to geographical areas.

From D. J. Gubler & Rosen, 1976.

Once infected, a mosquito remains infected for life, transmitting the virus to susceptible individuals during probing and feeding. Infected female mosquito may occasionally pass the virus to the next generation by transovarian transmission (Buckner et al., 2013). But this is a rare event and the mechanism is therefore not totally understood. The impact of this transovarian transmission is therefore negligible during human outbreaks.

The mosquito become infected by a blood meal from a viremic person and becomes infectious after an extrinsic period of 8-10 days. Like for CHIKV, the length of time required for this extrinsic incubation depends on environmental conditions, such as temperature, but also the strain of virus and mosquito (Salazar et al., 2007). *Aedes aegypti* can feed on several people during a single blood meal and if infected, may transmit dengue virus to several people in a short time.

After an incubation period of 3 to 14 days, the person may develop acute onset of fever, with several and various symptoms. During this acute febrile period, the viremic period, dengue virus circulates in the peripheral blood (Gubler, 1998). This acute period is crucial for a novel transmission to another uninfected mosquito.

### 3. 2. Reservoirs

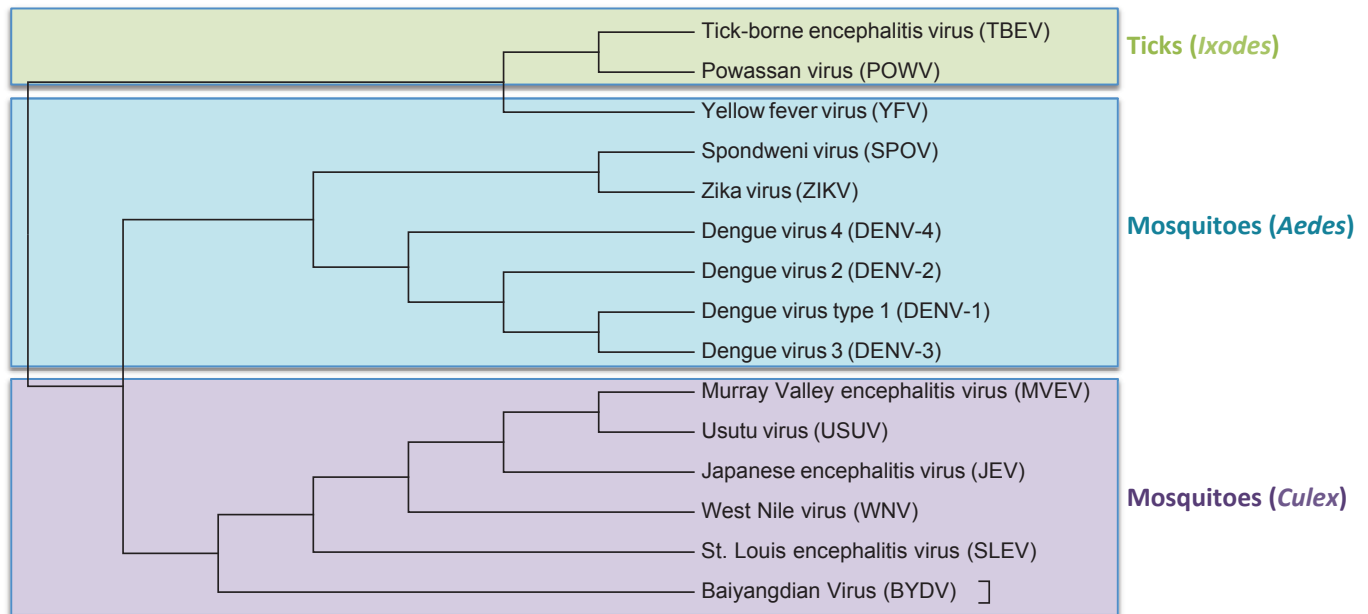
Humans are the main amplifying host of the virus, although studies have shown that monkeys and birds may become infected and serve as a source of virus for feeding mosquitoes in the jungle.

## 4. *Phylogeny and genome*

### 4. 1. Phylogeny

Dengue virus is a member of the genus *flavivirus* and the Flaviviridae family. Currently, four distinct serotypes of the dengue virus exist: dengue-1 (DENV-1), dengue-2 (DENV-2), dengue-3 (DENV-3) and dengue-4 (DENV-4).

The genus *flavivirus* includes 56 species. Viruses are grouped into three groups depending of the vector association and antigenic similarities: (i) tick borne, (ii) mosquito borne, and (iii) viruses with no known arthropod vector. The most important human pathogens in tick borne group are tick-borne Encephalitis Virus (TBEV). *Aedes* spp. or *Culex* spp. mosquito vectors can transmit the mosquito-borne viruses group. A lot of these viruses are associated with severe human diseases, such as Yellow Fever Virus (YFV), Japanese Encephalitis Virus (JEV), St Louis Encephalitis Virus (SLEV), West Nile Virus (WNV) or Zika Virus (ZIKV). The most important members of this family are represented in Figure 18, with vectors associated.

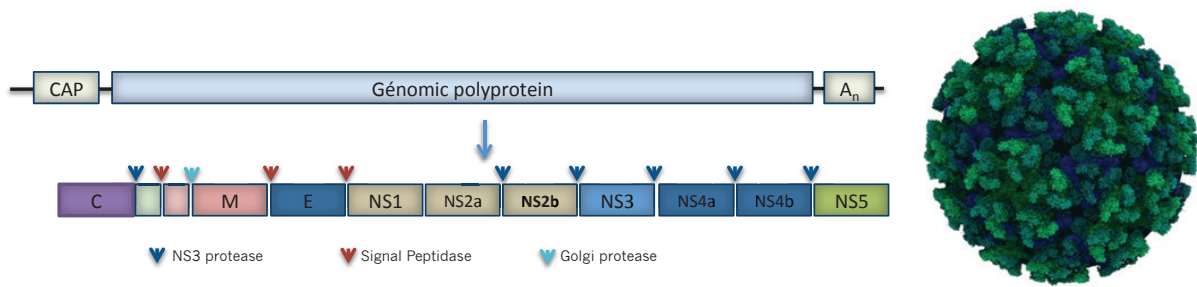


**Figure 18. Phylogeny of Flaviviruses genus.**

Based on nucleic acid coding the E protein.

#### 4. 2. Genome

Like other *flaviviruses*, Dengue virus is an icosahedral particle about 50 nm in diameter with a positive single stranded RNA virus of approximately 11kb. It has a single open reading frame, which encodes a single and long polyprotein, as shown in figure **19**. This polyprotein, after cleavage gives the structural proteins, located at the 5' end, and the non-structural proteins at the 3' end. The mature virion contains three structural proteins: the nucleocapsid (C), a membrane-associated protein (M) and the envelope protein (E). Immature virion bud into the lumen of ER and transport through the secretory pathway. In the trans-Golgi, the prM protein is cleaved by furin or furin-like protease resulting in the formation of mature virion, though the cleavage is often inefficient. Seven non-structural proteins have been identified: NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5.



**Figure 19. Genome organization and representation of viral particle of dengue virus**

The viral replication cycle consists of a sequence of events including the adsorption, endocytosis, uncoating, translation, replication, assembly and budding of viral particles (Figure 21).

#### 4.2.a. Entry process

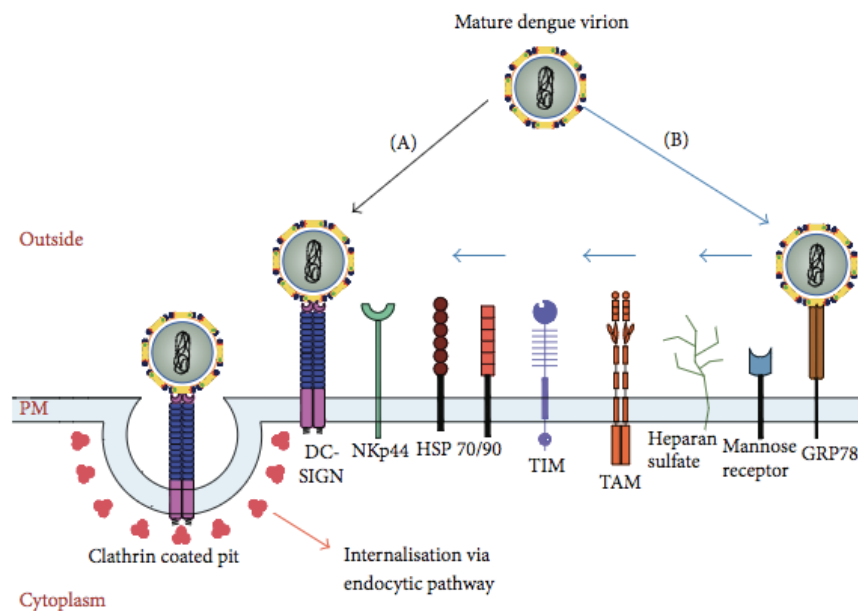
Dengue virus uses two distinct mechanisms to penetrate into host cells: (i) the virion envelope may fuse with the plasma membrane with immediate deposition of the nucleocapsid into the cytoplasm, or (ii) the plasma membrane may invaginate, forming an endocytic vesicle around the viral particle.

Attachment of the viral envelope protein E to the cell target lead to internalization of the virus by receptor-mediated endocytosis and trafficking to endosome (Gollins and Porterfield, 1985). Putative receptors are represented in Figure 20. Similarly to another enveloped virus, fusion of dengue virus with host cell membrane is pH dependent. E protein is a class-II fusion protein carrying both binding and membrane fusion properties. Crystal structure of E protein at different pH allowed to draw a fusion model during which initial E protein dimers change conformation and fold back as trimer structures to induce membrane merging (Cosset and Lavillette, 2011; Modis, 2013).

The acidic environment of the late endosome induces an important and irreversible conformational modification in the viral E protein; that is responsible to induce fusion of loop fusion and endosomal membrane (Guirakhoo et al., 1989). Pores are formed and the viral RNA is released into the cytoplasm.

Interestingly, cells infected with DENV secrete a heterogeneous mixture varying from fully mature (containing M), partially mature virion (containing a mixture of prM and M) to fully immature (containing prM) due to inefficient cleavage of prM to M by furin

during DENV maturation (Junjhon et al., 2008). It has been proved that fully immature virus particles are inherently noninfectious whereas fully mature and partially mature viruses are infectious (Zybert et al., 2008). However, it has been shown that sub-neutralizing antibodies promoted virus uptake into Fc receptor-bearing cells, leading to increase DENV infected cells and eventually a higher virus load (Halstead, 2003; Halstead and O'Rourke, 1977). This antibody-dependent enhancement (ADE) of infection can be observed with antibodies targeting E but also prM. Recent studies on mouse and human antibodies showed that prM antibodies render virtually noninfectious immature DENV particles highly infectious (Dejnirattisai et al., 2010; Rodenhuis-Zybert et al., 2010). This additional entry pathway is thought to increase pathogenesis after infection by different serotypes.



**Figure 20. Schematic representation of dengue entry process and pupative receptors identified.**

From (Khetarpal and Khanna, 2016)

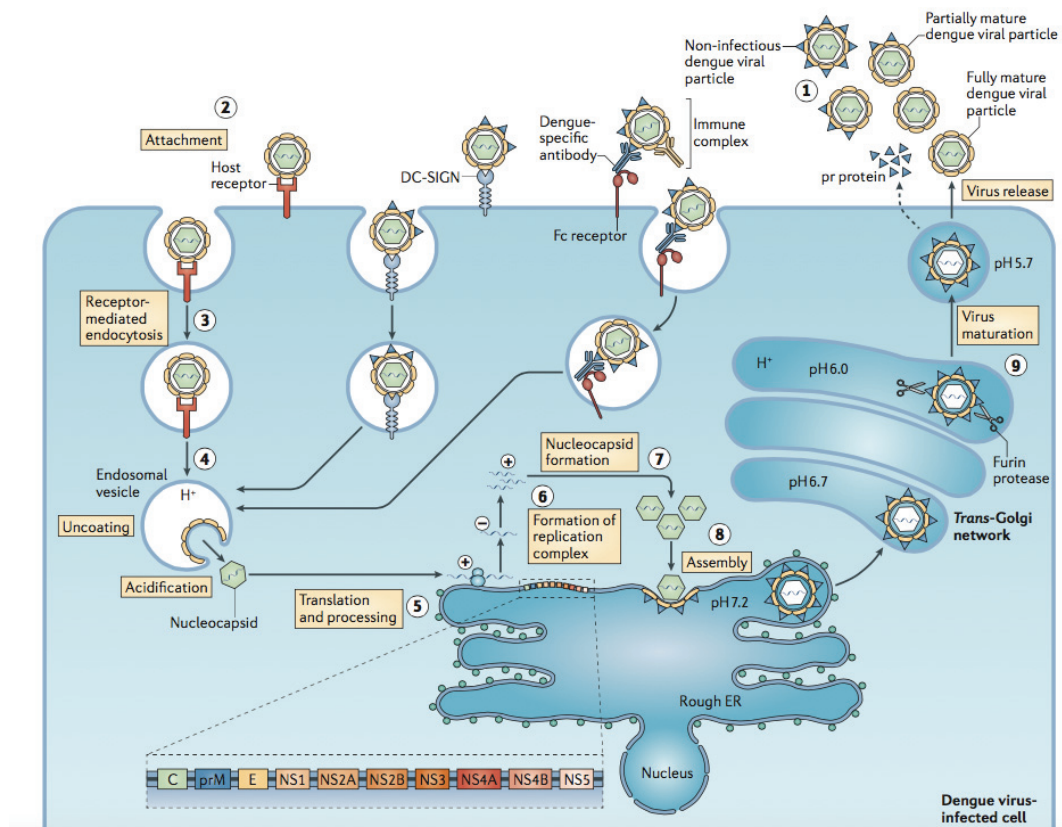
#### *4.2.b. Genome replication*

Very early processes in replication are unknown. The replication can be detected as early as 3h post infection, and it occurs in the perinuclear region of infected cells in association with smooth membranes (Takeda et al., 1978).

The RNA genome is a positive-sense single strand RNA (ssRNA) with 5'Cap and poly A as cellular mRNA. The RNA is therefore translated by cellular PolII polymerase to synthesize the RNA polymerase required for the genome replication. This positive-sense genomic ssRNA is translated into a polyprotein, which is cleaved into five structural and seven non-structural proteins by viral and host proteases. The viral replication is active in association with cytoplasmic membranes of infected cells. A double strand RNA is synthesized from the genomic ssRNA. Positive and negative strands have to make in equal rates to allow RNA amplification (figure 21).

#### *4.2.c. Assembly and release*

Virus assembly occurs at the endoplasmic reticulum. The C viral protein assembles with viral RNA on the endoplasmic reticulum membrane (ER). The virion buds through the ER, it's surrounded by lipid bilayer containing E and prM viral proteins and is transported to the Golgi apparatus. In the trans Golgi network, a cellular protease (Furin) cleaves prM protein into a virion associated M protein and a soluble pr peptide, thereby maturing the virion which is fusion competent. Then, the mature virion is released in the extra cellular environment by exocytosis, and is capable to infect a new cell. As already mentioned, a number of immature or partially mature virion are also released, in which prM proteins are still on the viral surface.



**Figure 21. Replicative cycle of dengue virus.**

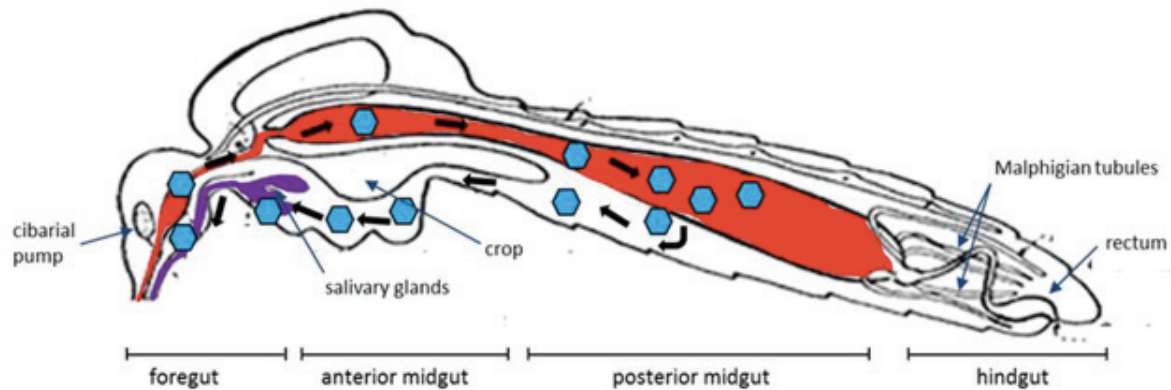
From Screaton et al. 2015.

## 5. Pathogenesis and cellular tropism

### 5. 1. In insect

Mosquitoes can survive to dengue infection. After an infected blood meal, the primary target of the virus is the midgut. If the viral load is sufficient, DENV is able to replicate into the epithelium and produce a high quantity of infectious particles, 4 days after infection (dpi). The entire midgut is differentially infected. The anterior part of midgut is occasionally infected whereas the infection do not occurs in the hindgut (Salazar et al., 2007). Virus will then spread to other organs. It has shown that abdomen is infected at 4 dpi. Virus then disseminate in fat body of abdomen, crucial for viral replication and for the insect immune response. Muscle around the midgut is uninfected with DENV-2 whereas multiple other organs are: trachea, nerve tissue, Malpighian tubules. Head

tissues contain also virus, with salivary glands. Saliva produced by salivary glands could be carrier of infectious particles, and could transmit virus during a novel blood meal on an uninfected host. Figure 22 represents the schematic dengue infection in *Aedes* mosquito



**Figure 22. Mosquito tropism for dengue infection.**

After an infected blood meal, the first step for DENV replication is to colonize the midgut. If the viral load is important, virus is able to infect other organs such as fat body, trachea. Virus reaches salivary glands, where the replication is possible. The presence of viral particles in saliva allows the transmission to a novel host. (Franz et al, 2015)

## 5. 2. In human

### 5.2.a. Pathogenesis

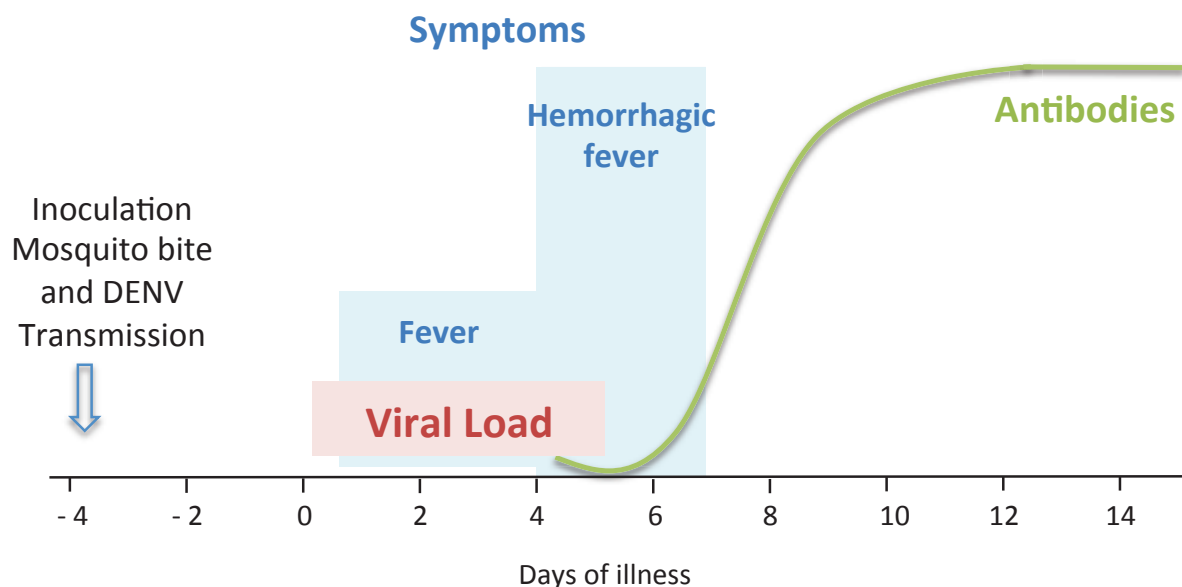
In humans, each of four serotypes has been associated with clinical manifestations (Gubler, 1998). Forty to seventy five percent of infected people are asymptomatic after infection.

Because of varying degrees of pathological conditions, the World Health Organization (WHO) gives some guidelines, with a classification of these clinical manifestations, as symptomatic Dengue Fever (DF), to Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS), which can lead to death. Studies in Cuba and Thailand have shown a consistently high association between DENV-2 infection and DHF/DSS, but a massive outbreak with severe cases in Indonesia and in Thailand was caused by DENV-3

serotype (Nimmannitya et al., 1969). So far it is difficult to associate high pathogenesis to certain serotypes or strains, and pathogenesis is multi-parameters.

The acute phase of infection, following an incubation of 3-14 days, occurs with an abrupt onset of fever (41°C), lasts usually for 5-7 days, and is followed by an immune response (Figure 23). The clinical symptoms could be different according to the virus strain, the age of the patient and its immune status. The classical symptoms are high fever, frontal and retro orbital headache, myalgia, arthralgia, nausea and vomiting.

In some cases, DF may be accentuated by severe complications such as thrombocytopenia, hemorrhagic manifestations and plasma leakage. These symptoms are characteristics of DHF. The plasma leakage determines the severity of DHF. The critical phase is reached at the end of febrile illness, marked by rapid decrease of temperature and associated with plasma leakage. A critical plasma leakage leads to the DSS and may be critical for life. The death can occur rapidly, within 12-24h of going into shock. A volume replacement therapy is the treatment used to recover. Without appropriate hospital care, 40–50% of cases can be fatal; with timely medical care by experienced physicians and nurses the mortality rate can be decreased to 1% or less.



**Figure 23. Timing of dengue disease.**

After the viral inoculation during a mosquito bite, few days are necessary to observe symptoms, such as fever, in correlation with viral load present in infected blood. According to the strain and the patient, severe symptoms can be observed and can lead to death. Specific antibodies are developed to control viral infection, and can be detected until few week or months after the infection.

### 5.2.b. Tropism

During infection in humans, the mosquito delivers the virus in the skin epithelium by the saliva, where it infects and replicates in the cells in presence, such as monocytes, dendritic cells, macrophages and Langerhans cells. The endothelium is the primary fluid barrier during dengue virus infection. The infection alters capillary permeability, permits virus replication and induces the recruitment of immune cells to the endothelium (Dalrymple and Mackow, 2012).

The cells migration brings virus until lymph nodes, and the replication results to the viremia. Then, a systemic infection of liver, lungs and spleen is observed by immunohistochemistry and *in situ* hybridization (Jessie et al., 2004). The liver infection induces jaundice or an elevation of liver enzymes (Jagadishkumar et al., 2013). It has been reported that hepatomegaly is more frequent in patients with DHF than in those with DF.

Studies suggest that cardiac complications in dengue illness are not uncommon. Severe disease is characterized by cardiovascular compromise from capillary leakage.

The tropism of DENV for endothelial cells (EC) lining the blood vessels remains controversial. Not all studies have shown the presence of DENV antigens in EC from liver or lung using autopsy samples from deceased patients or monkey's studies (Jessie et al., 2004). Also mouse models of DHF suggest that EC are target cells for DENV *in vivo*. It cannot be excluded, however, that EC are infected early in infection, during the peak viremia period, that tropism for EC differs per virus strain, or that EC of different vascular beds have differential susceptibilities to DENV infection.

Ocular complication can be diagnosed, resulting of inflammatory changes in vascular endothelium and a vascular leakage. Infection of brain result of various neurological symptoms, such as convulsions, headache or neck rigidity.

## 6. Immune response

### 6. 1. In insect

Even if the mosquito does not develop symptoms, a complex and interconnected network of effectors and inhibitors composed the immune system in mosquito and is activated after dengue infection. Among the immune pathways known in arthropods, almost all are identified to control dengue infection. 10 days after infection, genes involved in the immunity are modulated, such as the Toll, JAK/STAT pathways or apoptosis (Xi et al., 2008). Immune pathways are represented in the figure 24.

Firstly, the Toll pathway is activated in the midgut during the first step of viral replication and plays a crucial role in controlling infection in mosquito and in insect cells (Ramirez and Dimopoulos, 2010; Sim and Dimopoulos, 2010; Xi et al., 2008). During infection, it has shown that effector components of this pathway, such as cactus protein, inhibitor of Toll pathway, are down regulated. The down regulation leads to the activation of the pathway. Another components are impacted after infection; the REL1 effector is up regulated, leading to the activation of pathway (Xi et al., 2008). To confirm these results, transitory and experimental activation of REL1 have been realized and lead to the decrease of DENV infection (Ramirez and Dimopoulos, 2010), suggesting the involvement of Toll pathway during DENV serotypes infections. Moreover, the key roles of fat body in the immune response against a DENV infection are highlighted by the demonstration of the important regulation of immune genes in this part of mosquito.

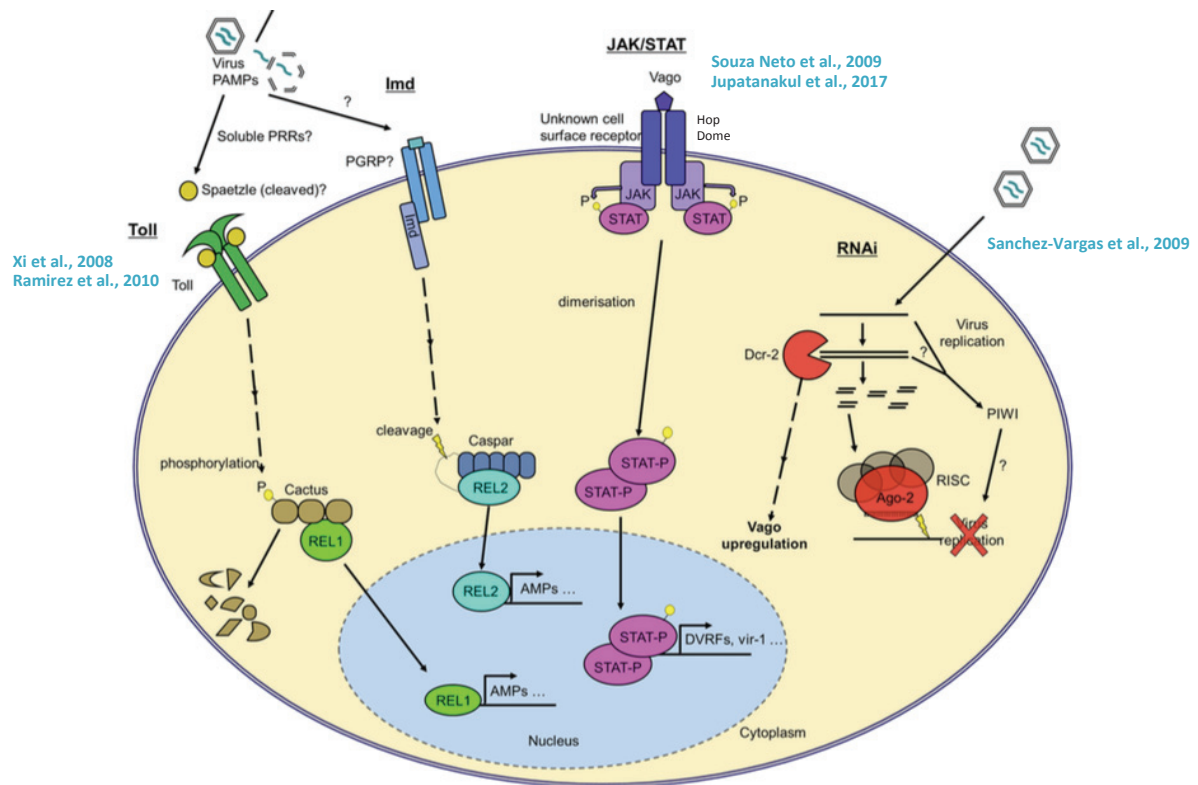
The JAK-STAT is also recruited during the viral infection. Suppression of the Dome and Hop receptors by RNA interference result to the increase of DENV infection, and the PIAS silencing, major inhibitor of pathway, allows a resistance of mosquito to infection (Souza-Neto et al., 2009).

More recently, Dimopoulos's team, used *Aedes aegypti* genetically modified, in order to activate the JAK/STAT pathway after a blood meal (Jupatanakul et al., 2017). It reveals that this pathway is able to control the dengue infection in mosquito, but not necessary the chikungunya or zika infection. It suggests that this pathway is a specific antiviral response against dengue viruses.

The RNAi pathway is also involved in the control of DENV infection. The silencing of *Dcr2* and *r2d2* genes, which play an important role in this pathway, reveals an increase of viral replication in *Aedes aegypti* cells and in mosquito (Sanchez-Vargas et al., 2004). Moreover, the extrinsic period in mosquito is significantly decreased, allowing a rapid viral transmission.

Recently, investigations on miRNA expressed following the DENV infection in C6/36 cells have shown the over expressed of 6 of them, and 9 of them are down expressed (Avila-Bonilla et al., 2017). The putative targets of these miRNA are involved in the antiviral response, such as the autophagy, the JAK-STAT or the Toll pathways, and confirmed previous studies. Vesicle-mediated transport and protease are also highlighted in this study.

Altogether, dengue virus infects efficiently insect cells, and the modulation of cellular pathways allows the maintaining of the equilibrium between viral replication and antiviral response during a chronic infection in insect cells.



**Figure 24. Immune pathways in *Aedes* mosquitoes.**

Adapted from Ruckert C et al., 2014

## 6. 2. In human

### 6.2.a. Autophagy

Autophagy is key mechanism for limit viral infection in mammalian cells.

Dengue infection also triggers autophagy, a general homeostatic response that helps the infected cell survive and produce virus. Dengue virus induces autophagy through activation of endoplasmic reticulum (ER) stress and ataxia telangiectasia mutated (ATM) signaling and the production of reactive oxygen species (ROS), enhancing its ability to reproduce (Datan et al., 2016). Although many autophagy-inducing pathways are activated in dengue-infected cells, inhibition of ER stress signaling limits the ability of dengue-2 virus to induce autophagy and protect infected cells. In contrast, protection of virus-laden cells from inducers of apoptosis by increasing autophagy increases the potential of dengue to replicate within cells and establish successful infections.

In 2008, the first characterization of autophagy during a DENV infection was shown in infected hepatocytes cells (Lee et al., 2008). The inhibition of pathway with drugs (rapamycin) or silencing of target genes demonstrates a diminution of viral infection. The following year, the NS1 viral protein was identified to co-localize with a marker of lysosomal fusion (LAMP1) (Panyasrivanit et al., 2009), they hypothesized that fusion between endosomes and autophagosomes allows a prolonged viral replication of DENV. The expression of the other non-structural protein NS4A seems to contribute to the autophagosome formation, and thus to contribute to viral replication (McLean et al., 2011).

Molecular mechanisms of the contribution of the viral replication still remain to be determined, but the autophagy may promote replication through repressing innate immunity. Connections and involvement of autophagy during DENV infection differ between cell type and organs.

### *6.2.b. Apoptosis*

In 2004, researchers showed that the DENV infection induced changes in cells, such as alteration of morphology or cellular detachment, typical to apoptosis processes. However, investigations didn't show the up-regulation of p53 protein, major factor for activation of apoptosis pathway, or complex with dengue proteins (Thongtan et al., 2004).

More recently, another study showed that a deficiency of p53 in hepatocytes (HepG2) allowed to an higher viral production than control HepG2 cells (Hu et al., 2017). They

also showed a correlation between infection and the increase of the amount of interferon production. Authors suggest that production of interferon mediate activation of transcriptional factor p53, which promote the cell apoptosis.

It has been shown recently in insects that apoptosis-related genes are also involved in regulating autophagy, and that *Aedronc* factor may play an important role in DENV-2 infection success in *Ae. aegypti* mosquito, possibly through its regulation of autophagy (Eng et al., 2016). Previously, the evaluation of differential expression of factors involved in the pathway (*Aedronc*, AelAP1 or caspase 16) after DENV-2 infection in one permissive *Aedes aegypti* strain and another one refractory to infection. Midgut and fat body reveals differences (Ocampo et al., 2013). The level of caspase in the refractory strain of mosquito is increased following DENV-2 infection. Moreover, the knockdown *Aedronc* gene by RNAi induces the enhancement of the infectivity in the refractory strain. Altogether, apoptosis pathway in *Aedes* mosquito contributes in the innate immune response against DENV infection.

#### 6.2.c. Humoral immune response

During a first exposure to any dengue virus serotypes, an innate immune response is activated after the acute phase.

Rapidly after the infection, T lymphocyte cells produce pro-inflammatory cytokines. A cocktail of molecules is secreted in order to control the viral infection. Type I interferon has been shown to limit DENV replication (Kou et al., 2011). Moreover, independently of interferon response, a cytosolic sensor, RIG-I (retinoic acid-inducible gene – I) has been demonstrated to play a role in the antiviral response in monocytes and dendritic cells, by blocking the infection (Olagnier et al., 2014).

Antibodies produced play a crucial role in the immune defense against the dengue serotype implied in the infection. Although the first infection produces life-long immunity to the infecting serotype, it is only temporary and a partial protection against the three other serotypes, and secondary or sequential infections are possible after a short time.

In endemic areas, people are susceptible to be infected many times in their life, and co circulation in some countries of 2 or more serotypes is not rare (Pérez-Castro et al., 2016; Pessanha et al., 2011).

A second infection may occur with a different serotype. Antibodies developed for the first infection are not enough to limit the second one. A cross-reactivation of antibodies occurs with the second infecting virus, to form infectious complex with Fc-gamma-Receptor, present in hematopoietic cells (Littau et al., 1990). This immune complex facilitate entry into monocytes and dendritic cells, suggesting that different mechanisms occur during internalization of receptors and dengue viruses (Rodrigo et al., 2006). Called antibody-dependent enhancement (ADE), this pathway of infection could be attributed to severe cases of dengue illness (Halstead and O'Rourke, 1977). Indeed, the production of IgG is highly increased, triggered a platelet reduction, and could lead to a thrombocytopenia (Wang et al., 2017). Moreover, in late illness stage, cytokines are produced in abundance, resulting to an abnormal vascular permeability, leading to sock and death (Guzman et al., 2013). Various factors are associated to the dramatic issue of the second infection. The age of the outcome of the second infection has an effect. Young infants and elderly individuals are more susceptible affected to a second infection, and an interval of 20 years could increase the severity of symptoms (Guzmán et al., 2002a; Guzmán et al., 2002b).

### *7. From diagnosis to treatment*

The two basic methods for establishing a diagnosis of dengue infection are detection of virus in cell culture or by serology with the detection of anti-dengue antibodies.

By the time a person infected with dengue virus develops fever, the virus is found in serum and plasma, in circulating blood cells, and in targeted tissues for 2-7 days, and the antibodies can be detected 4-5 days after the onset of fever. It has been observed high titers of immunoglobulin M (IgM) and immunoglobulin G (IgG) antibodies produced by immune cells, appearing respectively after 3-5 days and 6-10 days, respectively. IgM antibodies disappear 2-3 months after the onset of infection, whereas IgG antibodies persist for life.

The employed commercial tests use anti-dengue IgM and IgG detectable by IgG and IgM antibody-capture enzyme-linked immune-adsorbent assay (ELISA). The sensitivity of

this method is around 95%. Detection by reverse transcriptase PCR is also employed since several years. It permits a rapid, sensitive and specific detection of the viral RNA. Currently, there is no specific antiviral treatment available for Dengue fever. To treat fever and other symptoms, Aspirin and no steroidal anti-inflammatory drugs are avoided. Intensive care is required for severe cases, including intravenous fluids, blood or plasma transfusion, medicines. Consequently, there are costs for each dengue patient, including the cost of hospitalization. In addition, the local municipalities pay for the vector control policy, may reduce the tourism in touristic areas. The first dengue vaccine (CYD-TDV, Dengvaxia®), produced by Sanofi-Pasteur, has been licensed by several dengue-endemic countries in Asia and Latin America for use in populations above 9 years of age.

### III. Comparative table between CHIKV and DENV

As described previously, chikungunya and dengue viruses have many differences but they present some similarities. The table 4 compiles the characteristics in term of genomic organization, replication cycle, tropism, symptoms and immune response induced in mammalian and insect model.

		Chikungunya virus	Dengue virus
<b>Genome</b>		2 Open Reading Frames (ORFs)	1 Open Reading Frames (ORFs)
	Entry	Receptor-mediated endocytosis Clathrin-dependent Cholesterol and pH dependent	Receptor-mediated endocytosis Clathrin-dependent Cholesterol and pH dependent
<b>Replication</b>	Replication	Cytoplasmic Maturation of glycoproteins in ER and Golgi network Viral polymerase nsP4 Viral protease nsP2	Cytoplasmic Maturation of glycoproteins in the trans-Golgi network Viral polymerase NS5 Viral protease NS3
	Assembly and exit	Assembly in cytoplasm Budding of particles to the plasma membrane Furin maturation Exocytosis	Assembly in the ER Furin maturation Exocytosis in the plasma membrane
<b>Tropism</b>	Mammalian	Liver Muscle Joints Brain	Liver Lungs Spleen Brain
	Insect	Gut Salivary glands Saliva Legs Fat body	Gut Salivary glands Saliva Legs Fat body
<b>Symptoms</b>	Mammalian	Fever Headache Join and muscular pain Arthralgia	Fever Headache Hemorrhagic fever nausea
	Insect	ND	ND
<b>Immune response</b>	Mammalian	Apoptosis Autophagy	Apoptosis Autophagy
	Insect	RNA interference Imd pathway Apoptosis	Toll pathway JAK-STAT pathway Imd pathway RNA interference

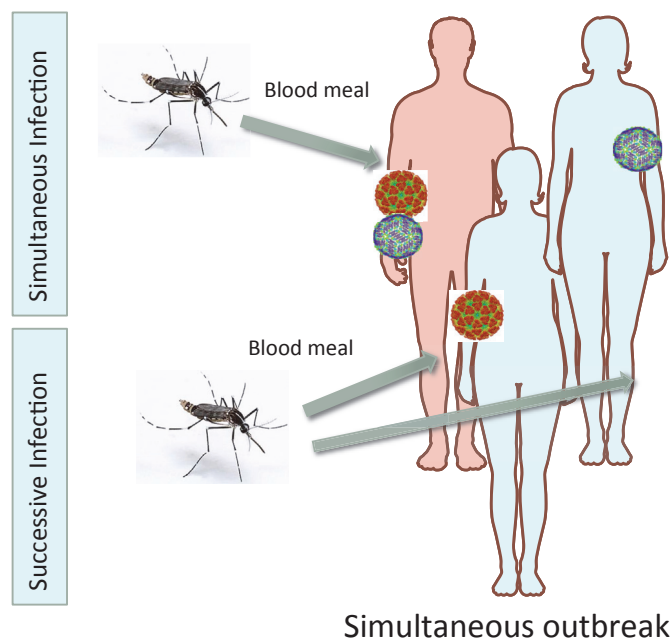
**Table 4.** Comparative table between chikungunya and dengue viruses.

## Chapter 4. Interaction between Chikungunya and Dengue viruses

Up to recently, co infections were rare and due to a persistent virus, providing a background onto which future viral infection is superimposed. They have rarely been studied for possible effects of one virus on the other one. Because of the transmission by the same vector *Ae* spp. chikungunya and dengue viruses are sharing more and more common geographical area around the world (Furuya-Kanamori et al., 2016).

### I. Co-infection models

During a simultaneous outbreak, both viruses are co-circulating. Patients can be carrier of one or more viruses. The possibility of co-infection model in patients is variable, and shown in Figure 25. Co-infection of a mosquito can occur by a sequential infectious blood meal taken on two different infected and viremic individuals, with an interval of few hours or days. It can also occur by one blood meal taken on a co-infected host.



**Figure 25. Schematic co-infection process during simultaneous outbreak.**

During co-circulation of both viruses, co-infections in mosquito and in human are possible. A uninfected mosquito can take a blood meal on a co-infected individual. After the extrinsic period, mosquito became co-infected. This is a simultaneous infection. But co-infection can also occur during a subsequent blood meal taken on two independent individuals mono-infected. This is a sequential infection.

Although in theory, these models of co infection are possible, very few studies established a real impact of co-infection between chikungunya and dengue viruses during simultaneous outbreaks. Moreover, the impact of co-infection on mosquito survival characteristic, on the viral replication of each virus, or on symptoms in humans is not yet determined.

## II. Epidemiology on the field

For several years now, reports of co-infected cases clearly depict the spread of both viruses across countries over time. The first cases of chikungunya-dengue co-infection were reported in Thailand in 1962 where different patients were precisely diagnosed co-infected by chikungunya and dengue viruses. The percentage of co-infected cases reached 3,6% in 1964 (Nimmannitya et al., 1969). During the same year, co-infected cases were also reported in India (Myers and Carey, 1967).

In 2004, an outbreak of a new strain of chikungunya occurred in Est of Africa. Through international travel and transport of goods, the chikungunya outbreak rapidly spread to islands of the Indian Ocean, India and Asia (Cordel and Investigation Group, 2006). Consequently, with an increase in diagnostics and improve specificity, co-infected cases were diagnosed in Sri-Lanka, Madagascar, India and Malaysia (Baba et al., 2013; Caron et al., 2012; Chahar et al., 2009; Rezza et al., 2014).

Nigeria is known to be an endemic country for various arboviruses, such as WNV, YFV, CHIKV and DENV. Overall, the prevalence of DENV is 67% and 50% for CHIKV. This country was the location of an exhaustive study where 310 individuals were recruited with arboviruses symptoms. Precise and specific serological methods (neutralization tests) were used to detect viruses and to analyze the prevalence of co infection in patients. The results indicated that co-infections are not rare as 76,8% of sera samples were positive for more than one arbovirus. Co-infection between CHIKV and DENV is the most commonly observed, with 17,8%. Co-infections of CHIKV with others *flaviviruses*, (YFV and WNV) are also observed, with 5,9% and 3,2% of prevalence. Co-infection between two *flaviviruses* is observed and DENV and WNV represent 4,1% of co infected patients. Only one patient was co-infection with WNV and YFV (Baba et al., 2013). In addition to these viruses, Malaria and Typhoid are also detected which complicate the

analysis of the outcome of the multiple infections on human pathogenesis. It should be noted however that sero-neutralization assays were used which can detect sequential infections and not only simultaneous infection.

In 2009, a clinical study was conducted in India on 69 patients with clinically suspicious arbovirolosis using specific PCR to detect and identify acute infections. Chikungunya and dengue outbreaks are not rare, and sporadic cases of co-infection are frequently reported. Only 6 co-infected individuals were diagnosed. Even if all the patients carried a CHIKV strain similar to the strain of the outbreak in La Reunion Island in 2006, different DENV serotypes were detected that prevent comparison of the pathogenesis.

More recently, in 2014 in Yemen, a study analyzed both viral RNA by PCR and the antigen presence by using ELISA methods on 400 patients. Unfortunately, no co-infections were detected by PCR. However, 13 patients had IgM antibodies against both DENV and CHIKV from a recent infection. One patient had a positive PCR result for DENV diagnosis, and antibodies IgM against CHIKV, which suggested a close sequential infection in this patient (Rezsa et al., 2014). Overall, it is difficult so far to have a clear study on human co infection by DENV and CHIKV.

All these studies were performed on humans, and do not indicate the potential of co infection in mosquito vector.

The originality of the study performed in Gabon in 2012 was to show the presence of both viruses in mosquitoes. Between 2007 and 2010, an active surveillance of acute febrile syndromes and a collection of mosquitoes were established in different cities. Although the number of co-infected patients was low (less than 1%) pooled abdomen and individual carcass from collected *Aedes albopictus* mosquitoes indicated the presence of both viruses (Caron et al., 2012). This result was the first observation of co-infection in mosquito in nature, and indicates the potential transmission of both viruses by a same vector.

Up to now, 13 out of the 98 countries/territories where both chikungunya and dengue epidemic or endemic transmission have been reported, demonstrating a possible increase of co-infected cases in last few years. A chronology of chikungunya-dengue co-infection reports with prevalence is shown in Table 5.

Location	Year	DENV+/CHIKV+	Co infection cases	Co-infection prevalence (%)	Strain CHIKV/DENV	Vector	Reference
<b>Africa</b>							
<b>Angola</b>	2014	NA	1	NA	CEA/4	NR	(Parreira <i>et al.</i> , 2014)
<b>Gabon</b>	2007	337	8	2,4	NR/2	<i>Ae. albopictus</i>	(Leroy <i>et al.</i> , 2009)
	2007	374	9	2,4	WA/2	<i>Ae. albopictus</i>	(Leroy <i>et al.</i> , 2009)
	2008	164	0	0	WA/2	<i>Ae. albopictus</i>	(Leroy <i>et al.</i> , 2009)
	2009	14	0	0	WA/2	<i>Ae. albopictus</i>	(Leroy <i>et al.</i> , 2009)
	2010	1400	28	2	WA/2	<i>Ae. albopictus</i>	(Leroy <i>et al.</i> , 2009)
<b>Madagascar</b>	2006	38	10	26,3	CEA/1	<i>Ae. albopictus</i>	(Ratsitorahina <i>et al.</i> , 2008)
<b>Nigeria</b>	2008	183	63	34,4	NR/NR	NR	(Baba <i>et al.</i> , 2013)
	2014	NA	1	NA	NR/NR	NR	(Raut <i>et al.</i> , 2015)
<b>Tanzania</b>	2013	93	4	4,3	NR/NR	NR	(Chipwaza <i>et al.</i> , 2014)
<b>Americas</b>							
<b>Saint Martin</b>	2013	651	16	2,5	Asian/1,2,3	NR	(Omarjee <i>et al.</i> , 2014)
<b>Asia</b>							
<b>India</b>	1964	332	7	2,1	NR/2	NR	(Myers & Carey, 1967)
	1964	294	8	2,7	Asian/2	<i>Ae. aegypti</i>	(Carey, 1971)
	2006	65	6	9,2	CEA/1,2,3,4	NR	(Chahar <i>et al.</i> , 2009)
	2007	387	8	2,1	NR/3,4	NR	(Neeraja, 2013)
	2009	44	16	36,4	NR/NR	NR	(Schilling <i>et al.</i> , 2009)
	2010	51	5	9,8	CEA/1	NR	(Singh <i>et al.</i> , 2012)
	2010	303	68	22,4	NR/2,3	NR	(Taraphdar <i>et al.</i> , 2012)
	2011	21	2	9,5	NR/NR	NR	(Kalawat <i>et al.</i> , 2011)
	2011	68	9	13,2	NR/NR	NR	(Afreen <i>et al.</i> , 2014)
	2012	191	2	1	NR/NR	NR	(Mohanty <i>et al.</i> , 2013)
	2012	NA	1	NA	NR/NR	NR	(Kumar <i>et al.</i> , 2012)
<b>Myanmar</b>	1970	539	36	6,7	NR/NR	<i>Ae. aegypti</i>	(Ming <i>et al.</i> , 1974)
	1971	129	8	6,2	NR/NR	<i>Ae. aegypti</i>	(Ming <i>et al.</i> , 1974)
							(Tun <i>et al.</i> , 2014)
•	1972	244	11	4,5	NR/NR	<i>Ae. aegypti</i>	(Ming <i>et al.</i> , 1974)
	2010	60	7	11,7	CEA/NR	NR	(Tun <i>et al.</i> , 2014)

Table 5. Occuring of co-infection during simultaneous outbreaks of chikungunya and dengue virus.

From Furuya et al, 2017

### III. Impact of co-infection

Given that the initial symptoms associated with dengue mono-infection are often indistinguishable from those presented by patients with chikungunya infection, confirmatory laboratory diagnosis of co-infection is required.

In term of clinical outcome, very few studies have described the severity of dengue-chikungunya co-infection. Most of them described no significant novel clinical manifestations during co-infection.

A study was performed on imported case in Germany in 2009 (Schilling et al., 2009). A 25 years-old female who work as volunteer in India developed clinical symptoms. After her back in Germany, ELISA and IFA methods confirmed CHIKV and DENV infection, however, PCR were negative. The history of the patients and the fever peaks observed, suggested subsequent infection with the 2 viruses, or a simultaneous infection. The patient did not develop novel clinical symptoms; distinguishable from mono-infection, even if a prolonged metrorrhagia may have indicated a more severe clinical manifestation. Another Indian study described symptoms during simultaneous outbreak in 2012 (Taraphdar et al., 2012). It is reported that young adults were principally co infected (91%) after the detection of Immunoglobulin IgM antibodies against both CHIKV and DENV and the prevalence of co-infection increase with age. Concerning clinical manifestations, no significant differences are observed between mono- and co-infections. However, only co-infected patients suffer of diarrhea in 16% of cases.

In Gabon, although no evidence of differences between mono and co-infections was shown, Caron et al, highlighted the fact that viral load in co-infected patients was always lower for both virus than those in mono-infected patients (Caron et al., 2012). This result could suggest a mutually negative modulation in case of co-infection in human.

The unique publication showing an exacerbation of symptoms during co-infection was performed in India in 2009. Even if the number of cases diagnosed in this study is small, among the 6 co-infected patients, 2 developed a hemorrhagic fever and one patient died. However, there is no statistical comparison with mono-infection patient at the same time. In addition, the severity of DENV outbreaks are different depending on the circulating strain and it is difficult to make any correlation with co circulation of CHIKV. So far, no clear specific clinical entity has been described for co-infected patients.

Recently, an *in vitro* study of co-infection performed on human peripheral blood mononuclear cells (PBMCs) have shown a reduction of CHIKV replication and an enhancement of DENV production during co-infection condition (Ruiz Silva et al., 2017). The change of cytokines production in co-infection condition (IFN, IL-6, IL-8 or TNFalpha) could play a critical role in pathogenesis of viral diseases.

To understand the real impact of co-infection on clinical manifestations and the severity of them, more study is needed.

#### IV. Laboratory experiments

As there is an increase in co circulation and in co-infected patients, many questions remain to be clarified. For this, researchers try to develop *in vitro* and *in vivo* models to answer to these problematics.

In order to experiment co-infections with CHIKV and DENV, investigations were performed at Pasteur Institute of Paris by the group of Anna-Bella Failloux in a BSL-3 laboratory equipped with an insectarium (Vazeille et al., 2010). In a first approach, *Aedes albopictus* from La Reunion Island were successively exposed with blood meal carrying DENV-1 then CHIKV isolated in the same areas during the 2004 and 2005 outbreaks, respectively. During this subsequent co-infection, even if few mosquitoes took the second blood meal, dissemination of both viruses has been detected. In a second setting, mosquitoes were simultaneously co-infected with both viruses. Viral dissemination of both viruses was confirmed by using Immuno-Fluorescent Assay and the titration of viruses in saliva allowed to compare viral transmission. The dissemination rate of DENV-1 was found to be higher when DENV-1 is co-inoculated with CHIKV than only DENV-1. In addition, the infectious particles of DENV-1 in saliva of mosquito after 14 days post-inoculation are also increased in co-infection conditions. Altogether, this group showed for the first time that both viruses could replicate and disseminate in the same mosquito, and may probably transmit viruses by the presence of them in the saliva.

However, this result of simultaneous co-infection was not validated in *Aedes aegypti*. Analyzing by PCR on pooled or single mosquitoes, the absence of co-infection with both viruses suggested an interference problem (Rohani et al., 2009). However, the

replication of DENV was found to be lower than CHIKV, and depend of mosquito species and this experiment needs to be tested again.

Recently, *Aedes aegypti* were exposed to different combinations of CHIKV, DENV-2 and ZIKV. The RNA level of each virus in saliva by RT-qPCR indicated that the three arboviruses can be potentially transmitted by a unique bite (Ruckert et al., 2017). Interestingly, the infection, and the dissemination of all viruses are similar and the co-infection conditions do not seem to affect the vector competence for any viruses. In the same time, another study confirm the potential co-transmission of CHIKV and ZIKV after simultaneous inoculation in *Aedes aegypti* mosquitoes (Göertz et al., 2017) and no difference were detected whether the virus were alone or in combination.

## V. Thesis objectives

With the enhancement of co circulation of DENV and CHIKV in different territories, the increased detection of simultaneous infection in humans, mosquitoes have more chances to be co infected after one or two blood meals. Resulting from simultaneous or sequential infections, the infectivity, the dissemination and the transmission of each virus may be altered. In the field, multiple viral infections in natural populations may have significant biological and epidemiological consequences.

When we started this work, at the exception of the successful co infection of *Aedes albopictus* by Failloux's group, and the none infection of *Aedes aegypti* by Rohani's group, no studies focused on the consequences of *alphavirus* and *flavivirus* co-infection.

My first objective in my PhD thesis was to develop a sequential infection model in *Aedes* mosquito cells. Firstly used in C6/36 cells, the model was extended to other *Aedes albopictus* cells (U4.4) and *Aedes aegypti* cells (Aag2). Viruses belonging to *alphavirus* and *flavivirus* genus were used to performed infection. Results obtained include infection in BSL-3 laboratory, staining of infected cells (flow cytometry), RNA quantification (RT-qPCR) and confocal microscopy. We showed that the permissiveness and production of DENV-2 is enhanced in presence of CHIKV. We also generalized this phenotype to YFV, Zika *flaviviruses* and SINV and SFV *alphaviruses*.

Then, we developed an *in vivo* model of sequential infection in *Aedes aegypti* mosquitoes in collaboration with Valérie Choumet. Mosquitoes were firstly infected with an infectious blood meal containing CHIKV and 4 days later, with a blood meal with DENV-2. Two distinct organs crucial for the vector competence, the midgut and the salivary glands were extracted and viral RNAs were analyzed by RT-qPCR. The saliva was also analyzed. The first results obtained, even if not statistically significant due to the low numbers of mosquitoes, seems to indicate an increase of DENV-2 proliferation in salivary gland.

Finally, in a third part, experiments aiming to identify the molecular mechanisms involved in the co-infection phenotype are presented. Although unachieved, experiments consisted to express different viral proteins in C6/36 cells. Techniques of transfection, electroporation and infection were principally used in this part.

# Experimental Report

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## Chapter 1. Interaction between *flaviviruses* and *alphaviruses* during co-infection at the cell level

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## Abstract

One consequence of the re-emergence and rapid spreading of Chikungunya virus (CHIKV) is an increased overlapping distribution with Dengue virus (DENV), whose levels and geographical range also have been increasing since the 1990's. Simultaneous outbreaks and diagnostic progresses lead to higher detection of co-infections in humans with a percentage that can reach more than 36%. In parallel, in the field, co-infected mosquitoes have been isolated, confirming laboratory data indicating that mosquitoes could in theory carry and transmit simultaneously CHIKV and DENV. However, the pathology, as well as the epidemiology of a pathogen can be influenced by the interactions between several infectious agents present within an organism. Even if DENV and CHIKV share common cellular tropism and exploit similar cell pathways in mosquitoes, very little is known about the impact of CHIKV and DENV co-infection on the outcome of these viruses. To analyze this overlooked phenomenon, we measured any interference between DENV and CHIKV during sequential infection at the cell level *in vitro*. We first chronically infected *Aedes albopictus* C6/36 cell line by CHIKV and we then infected these cells by DENV-2 virus. Interestingly the permissiveness and production of DENV-2 is enhanced in presence of CHIKV. On the contrary, in the reverse order, there was no effect of DENV-2 pre-infection on subsequent CHIKV infection. We generalized the synergistic phenomena and we showed that CHIKV pre-infection also increased the infection by DENV-1, DENV-3 and DENV-4, but also more important, the infection by another *flavivirus*, the Yellow Fever Virus (YFV), which causes recent outbreak in South America. The synergistic interaction between CHIKV and DENV-2 viruses is not restricted to *Ae. albopictus* C6/36 cell line as it was similar in U4.4 (*Ae. albopictus*) and Aag2 cell lines (*Ae. aegypti*). Finally, we showed that a chronic infection by another *alphavirus*, the Semliki Forest Virus (SFV), also increased a subsequent DENV-2 infection. Altogether, our results pave the way for the characterization of molecular interaction between DENV and CHIKV in mosquito cells, which may provide clue for the propagation of viruses in one organism and the epidemiology of simultaneous outbreaks.

Key words: Anergy/synergy, Chikungunya, Dengue, co-infection

## I. Introduction

Pathology associated to infection is often considered as resulting from the reciprocal interaction of a given pathogen with a given host. This paradigm looks however oversimplistic since the pathology, as well as the epidemiology of a pathogen, relies also on the interactions between several infectious agents present within an organism. It is therefore crucial to consider to which extent a host infected by a first microorganism is modified and whether its reaction to the infection by a second microorganism is consequently altered. This consideration is especially relevant for Arthropod-borne viruses (arboviruses) that are the causative agents of some of the most important emerging and re-emerging infectious diseases. Among these viruses, the genus *flavivirus* (i.e. Dengue DENV and Zika ZIKV viruses) and *alphavirus* (i.e. Chikungunya, CHIKV virus), belonging to the Flaviviridae and Togaviridae family respectively, are responsible for significant growing global public health problems with tremendous impact on the economy worldwide.

Dengue is regarded as the greatest threat to global public health caused by arboviruses. According to WHO over 50 or according to a recent study even up to 400 million people (Bhatt et al. 2013) are infected with Dengue virus (DENV) annually. DENV is endemic in several countries in Africa, Asia, Caribbean, and Latin America. However due to changing climate conditions, DENV is spreading since 1990's to more temperate climates. There are four antigenically different serotypes of the virus (DENV-1 to DENV-4). An infection with any of four serotypes result in a wide clinical presentation, ranging from asymptomatic, flu-like disease dengue fever (DF), to dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Only one licensed DENV vaccine is available for specific conditions and no effective antiviral treatment is currently available. In parallel to DENV spreading, CHIKV has recently induced epidemic outbreaks of unprecedented magnitude with more severe symptoms (chronic joint and muscle pains) than previous contagions, with reports of severe neurologic complications and deaths. Since 2005, the East Central South African (ECSA) genotype of CHIKV has been responsible for more than 1 million human cases in the Indian Ocean islands, the Indian subcontinent and southeast Asia where outbreaks had been absent for 32 years (Santhosh et al., 2008). Outbreaks also occurred in Africa (Leroy et al., 2009) and for the first time in Europe

(Rezsa, 2008). However, the latest large-scale outbreak is currently occurring for the first time in the American continent following introduction of a CHIKV Asian genotype, related to strains identified in Asia (in Indonesia in 2007, in China in 2012, and in the Philippines in 2013) (Omarjee et al., 2014). With no approved vaccine or antiviral treatment, millions of people risk to be infected.

DENV is spread by the bite of the highly anthropophilic *Aedes aegypti* mosquito, and to a lesser extent, by *Ae. albopictus*. *Aedes* spp. mosquitoes also transmit CHIKV. Although *Ae. aegypti* constituted the main vector species for CHIKV in Kenya during the major 2004 outbreak, *Ae. albopictus* was the principal vector in succeeding epidemics in Gabon, Madagascar, La Reunion and probably Caribbean (Kariuki Njenga et al., 2008; Leroy et al., 2009; Omarjee et al., 2014; Sang et al., 2008). As the same *Aedes* spp. mosquitoes transmit both pathogens, there is a reasonable expectation that the epidemiology of DENV and CHIKV infections is temporally and spatially related. These overlapping leads to a higher detection of co-circulation and co-infection in humans and mosquitoes. Co-infections are increasingly being recognized, in travelers coming from Southern Asia or Africa (Chang et al., 2010; Nayar et al., 2007; Tappe et al., 2010), well as in inhabitants in India or Africa (Chahar et al., 2009; Leroy et al., 2009; Parreira et al., 2014) and more recently in South America, confirming previous data (Myers and Carey, 1967). It is not clear if co-infection leads to a novel clinical entity with specific or severe symptoms (Omarjee et al., 2014). However, hemorrhagic complications in co-infected patient had been reported (Tappe et al., 2010). Based on the latest epidemics, the percentage of co-infection when viruses co-circulate is between 2-30% and all DENV serotypes were involved (Furuya-Kanamori et al., 2016; Kalawat et al., 2011; Omarjee et al., 2014; Taraphdar et al., 2012). Furthermore, the mosquito *Aedes albopictus* could carry simultaneously CHIKV and DENV in the field (Caron et al., 2012). Oral co-infection of *Aedes albopictus* has been shown to be possible experimentally using strains of DENV-1 and CHIKV provided in the same blood-meal (Vazeille et al., 2010). These co-infected mosquitoes present both viruses in their saliva and can potentially transmit both viruses together. More recently, successful dual-infections with CHIKV, DENV or ZIKV have confirmed previous work in *Aedes aegypti* mosquito (Göertz et al., 2017; Ruckert et al., 2017).

At the cellular scale, CHIKV and DENV viruses share a common cellular tropism in mosquito. Arboviruses infect the mosquito midgut, replicate and disseminate in the whole organism, until the salivary glands, for the transmission by the saliva. The midgut and salivary glands act as barriers to virus infection and escape. However, no tissue tropism difference in insects has been reported, except for germinal cells that are not always infected. However, nothing is known about the interaction between CHIKV and DENV during co infection of the same insect cell. The interaction of both viruses with common pathways can potentially mediate an interference, anergy or synergy between CHIKV and DENV.

In the following study, we therefore first assessed the interference between DENV-2 and CHIKV during the sequential co-infection of different mosquito cells. This experimental strategy allowed us to study CHIKV and DENV co-infection mimicking the super infection of a previously infected mosquito during a second blood meal during simultaneous outbreaks. By using *Aedes albopictus* and *Aedes aegypti* cell lines, we showed that pre-infection by CHIKV can promote infection by DENV-2 virus. We showed that this phenomenon is relatively broad as CHIKV promote infection of DENV-1, DENV-3 and DENV-4, as well as infection by Yellow Fever Virus and Zika Virus. Finally, we also showed that another *alphavirus*, the Semliki Forest virus, can also increase infection by DENV-2. Altogether, our data demonstrate that pre-infection of mosquito by *alphaviruses* can increase infection by *flaviviruses*.

## II. Materials & methods

### Cell lines and reagents

African Green Monkey kidney epithelial cells (VeroE6), Baby Hamster Kidney 21 (BHK-21) cells were grown in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal bovine serum (FBS). *Aedes albopictus* larval C6/36 cells were grown in Schneider's (Gibco, Invitrogen) and Mitsuhashi-Maramorosh medium (Bioconcept, Allschwil, Switzerland) (50%-50%) supplemented with 10% FBS and *Aedes albopictus* U4.4 cells were grown in Mitsuhashi-Maramorosh medium supplemented with 20% FBS. *Aedes aegypti* larval Aag2 cells were grown in Schneider's (Gibco, Invitrogen) supplemented with 10% Tryptose phosphate buffer (pH 7.3) and 10% FBS. All culture media were supplemented with 10U/ml of Penicillin and 10µg/ml of Streptomycin (Gibco®, Invitrogen). The DENV-2 was detected by the anti-E, clone 3H5 (1:500, clone 3H5, mouse, Millipore, Billerica, MA, USA). For other DENV serotypes and Yellow Fever Virus, the anti-Flavivirus Group antibody 4G2 and 5C12 were used, respectively. The anti-SFV C-protein was used to detect *alphaviruses* (1:800, Kind gift from Pr Beatrice Grummer, University of Veterinary Medicine, Hannover, Greisser Wilke Moraes JGenVirol 1989). Alexa Fluor® 488 Goat Anti-Mouse IgG (1:1000, Invitrogen) was used for flow cytometry method and a secondary horseradish peroxidase (HRP) conjugated goat anti-mouse IgG antibodies (1:1000, Sigma-Aldrich) was required for immunostaining.

**Virus production** DENV-1 strain PU0359 (isolated in 1980 in Thailand) was kindly provided by Duane J. Gubler, (Centers for Disease Control and Prevention, Fort Collins, USA and and Robert E. Shope, University of Texas Medical Branch, Galveston). DENV-2 strain 16681 isolated in 1964 from a DHF/DSS patient in Bangkok was used (Halstead et al., 1970), DENV-3 strain PaH881 (isolated in 1988 from a patient with classical dengue fever by and kindly provided by Vincent Deubel, Pasteur Institute, Paris, France). DENV-4 strain 1228/TVP980 (isolated in 1978 from a classical dengue fever case in Indonesia) was a gift from Duane J. Gubler and Robert E. Shope. Plasmids encoding Chikungunya LRic strain LR2006 OPY1 (European Virus Archive; Marseille), Chik-GFP and Chik-mcherry (constructed in the laboratory) were used to generate viruses after transcription (according to manufacturer recommendation; Ambion, ThermoFisher) and

electroporation of BHK-21 cells. Cells were trypsinized, pooled, and washed twice in Opti-MEM medium (Sigma-Aldrich) and resuspended at  $1.10^7$  cells/ml in Opti-MEM.  $5.10^6$  cells were electroporated in 0.4 cm width cuvette with a Gene Pulser apparatus (Bio Rad) at 270 V and 975uF. The cells were plated in DMEM-FBS 10% and incubated at 37°C, before changing the medium after 18h. After 2 days, the supernatants were harvested, filtered through 0.45 µm filters and kept at -80°C. The viral stocks were titrated by plaque assay on a VeroE6 cells in 96-well plate.

**Viral infection.** *Aedes albopictus* larval cells (C6/36 and U4.4) and *Aedes aegypti* (Aag2) cells were infected by the different viruses at a multiplicity of infection (M.O.I.) of 0.1 and 1, respectively, in serum-free media supplemented with 10% Hepes for 4-6 hours at 28°C. Cells were then kept to establish chronically infected cells. The full infections of all cells were monitored every day by measuring the percentage of infected cells by FACS or the viral titer by IFA.

**To measure the CHIKV or DENV permissiveness,** a cytoplasmic immunostaining was performed. Cells were fixated for 10 min with 4% paraformaldehyde and then resuspended in a staining buffer (PBS, 0,1% saponine, and 10% FBS) after centrifugation at 800g for 2min. Cells were stained with specific primary and second antibodies for 1 hour at 4°C. The staining of *flaviviruses* was observed with a panFlavivirus antibody (2H2) and the staining of Capsid protein expression lead to CHIKV detection. Percentage of DENV or CHIKV positive cells at each time point were determined by flow cytometry using a FACS LSRII flow cytometer (BD Biosciences, San Diego, CA). Cell aggregates were excluded on the basis of forward and side light scatter. Data were analyzed using FlowJo software. Each experiment was performed at least three times in duplicate independently.

Total RNA was extracted from infected cells with MOI 1 or 0,1 using RNeasy Mini Kit® (Qiagen) and 250ng total RNA was reverse transcribed using hexamers with PrimeScript Reverse Transcriptase (Takara). Viral RNAs were quantified by qPCR amplification, using a set of primers targeting E protein (DENV-2) and nsP1 protein (CHIKV) and normalized with RpL27 housekeeping gene. Results are reported as RNA copies compare to RpL27 gene.

**For titration of viruses produced by infected mosquito cells,** infections were conducted on mammalian cells with different dilutions of cell culture supernatant of infected cells. After washing, cells were grown in DMEM 10% FBS medium supplemented with 10% Carboxymethylcellulose (CMC; Invitrogen). Two days post infection; cells were fixed for 10 min with 4% paraformaldehyde in PBS 1X. Cells were permeabilized with 0,1% Triton X-100 in PBS for 10 min and then blocked with 3% BSA in PBS for 30 min. After one wash with PBS, cells were incubated with monoclonal anti-DENV-2 E protein antibody at 1:300 dilution in PBS + 1% BSA for 1 hour at room temperature. The cells were then washed twice in PBS-BSA 1% and incubated with secondary horseradish peroxidase (HRP) conjugated goat anti-mouse IgG antibodies (1:1000, Sigma-Aldrich) for 1 hour at room temperature. Infected cells were detected following reaction with AEC (3-amino-9-ethyl carbazole) reagent containing 950  $\mu$ L NaOAc (0,05M pH = 5), 50  $\mu$ L AEC (prepared by diluting solid commercial pill into 5 mL of DMF, both from Sigma-Aldrich, St-Louis, MO, USA) and 5  $\mu$ L 30%-H<sub>2</sub>O<sub>2</sub> (Sigma-Aldrich) for 1 hour. DENV titers were reported as foci forming unit per ml (FFU/ml). CHIKV titers in supernatant of mosquito-infected cells were determined by plaque assay, and reported as plaque forming unit per ml (PFU/ml) by using epifluorescent microscopy (Axiovert 135 microscope, Carl Zeiss SAS, Le Pecq, France) with a 10X objective. Each experiment was performed at least three times in duplicate independently.

Viral RNA was extracted from cellular supernatant using NucleoSpin®RNA Virus (Macherey Nagel). Total RNA of rat was added in supernatant before extraction, for further normalization. Viral RNAs were quantified by qPCR amplification, using a set of primers targeting E protein (DENV-2) and nsP1 protein (CHIKV) and normalized with GAPDH housekeeping gene. Results are reported as RNA copies compare to GAPDH gene.

### **Immunofluorescence microscopy**

Insect cells were placed on chamber slides (Lab-Tek, Sigma Aldrich) in order to be simultaneous infected by CHIKV-mcherry and DENV-2 at M.O.I 0,1 and M.O.I 1 respectively. Cells were fixed in 4% paraformaldehyde at 4°C for 10 min and permeabilized in PBS with 0,1% saponine and 10% FBS. After being incubated with anti-DENV-2 E protein and anti-mouse IgG Alexa fluor 488, the slides were imaged with

the Leica Zeiss confocal microscope.

### **RNA quantification by RTqPCR.**

RT-PCR reactions were performed with PrimeScript Reverse Transcriptase (Takara): 250ng total RNA was reverse-transcribed using hexamers. The target transcripts were amplified in Stratagene Mx3000P apparatus (Agilent Technologies), using the KAPA SYBR Fast Universal Kit (KAPA). The CHIKV primers were selected in the non-structural protein 1 regions: sense (AGAGCAGGAAATTGACCCCG); anti-sense (TTTTCCTGCGGCAGATGCTA). The DENV-2 primers were selected as described in Raquin et al., 2015: sense (GAGAAACCGCGTGTCAACTG), antisense (GGAAACGAAGGAATGCCACC). The housekeeping gene used in cells and in supernatants are Rat GAPDH (sens: CTCTCTGCTCCTCCCTGTTCT; antisens: CGACCTTCACCATCTTGTCTATGA); RpL27 (sens TCATCAAGATGCTGAACTACAACC; antisens TTTATTACACAGCACTCTCCCTC).

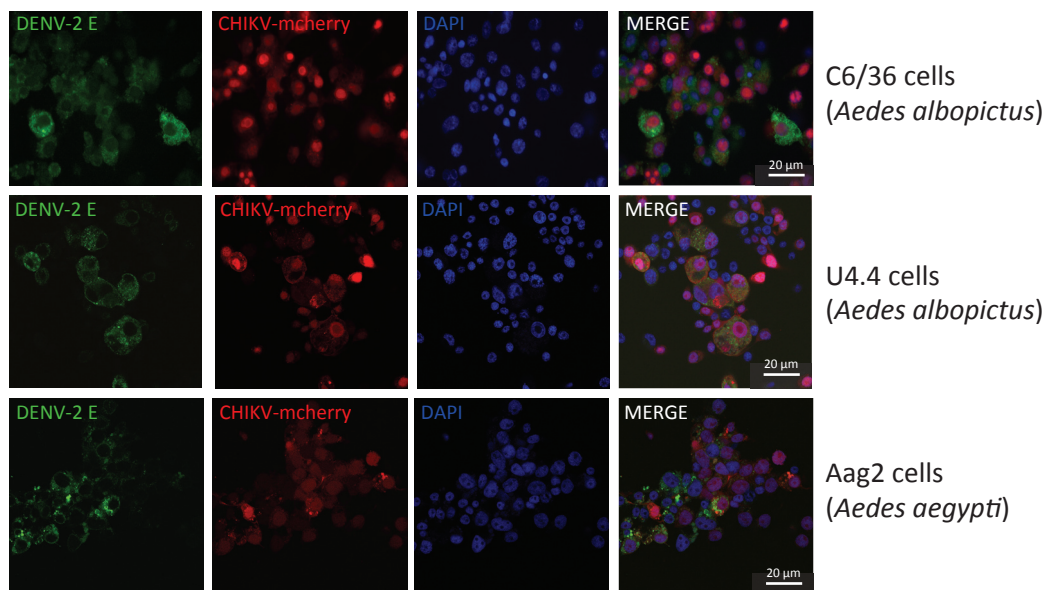
### **Statistics**

Statistical analyses were performed using GraphPad Prism version 5.02 for Windows, GraphPad Software (San Diego California, USA). The Mann-Whitney or Wilcoxon tests were used for statistical comparisons. A p-value of 0.05 or less was considered as significant. When applicable, data are presented as mean  $\pm$  standard deviation and results of the statistical analysis are shown as follows: ns, nonsignificant ( $P > 0.05$ ); \*, significant ( $P < 0.05$ ); \*\*, very significant ( $P < 0.01$ ) and \*\*\* high significant ( $P < 0.001$ ).

### III. Results

#### 1. Possible simultaneous co infection in mosquito cells.

In order to verify the possible co-infection of insect cells that may lead to interaction, we first simultaneously infected *Ae. albopictus* (C6/36 and U4.4) and *Ae. aegypti* (Aag2) cells by CHIKV and DENV. After 3 days, viruses were stained and analyzed by confocal microscope. The DENV E protein was detected in cytoplasm, probably in endoplasmic reticulum. CHIKV was detected by the presence of mcherry fluorescent protein, diffuse in the entire infected cell. As shown in Figure 26, both viruses can infect a cell at the same time. There is no major difference in the presence of both viruses whatever the cell types. It is believed that the innate response of C6/36 cells is defective due to ineffective “siRNA response” but the U4.4 or Aag2 cells are also co-infected (Brackney et al., 2010, 3).

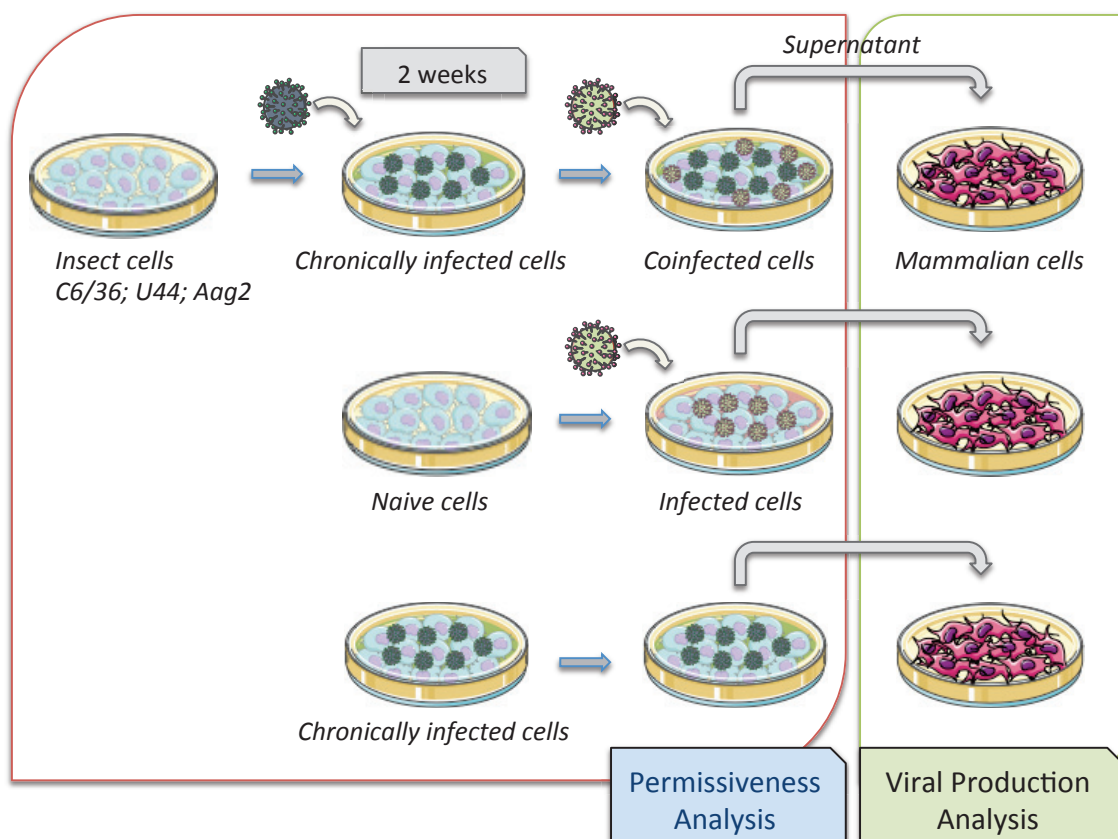


**Figure 26. Co-infection in insect cells.**

Different mosquito cell lines were grown on plastic cover slips and either incubated with  $1.10^4$  pfu of CHIKV and  $1.10^5$  ffu of DENV-2 for 2h. Cells were subsequently fixed and incubated with antibodies directed against DENV-2 E protein (4G2) and appropriate secondary antibody, before visualization under a confocal microscope. Representative single-channel (green: DENV-2 E; red: CHIKV-mcherry; blue: DAPI), and merged images are shown.

In order to assess the interaction between DENV-2 and CHIKV during the co-infection, we set up an experimental strategy mimicking the super infection of a previously infected mosquito during a second blood meal during simultaneous outbreaks.

Chronically infected *Aedes spp* insect cells were generated using a "first" virus (Figure 27). After several days, naive and chronic insect cells were infected by serial dilutions of a "second" virus in parallel to allow direct comparison. Staining of the infection on insect cells allows assessing the influence of the first virus on the permissiveness of the cells for the second virus (Figure 27). Until 6 days, the supernatants of each infection were transferred onto VeroE6 cells. The titrations of the supernatant on VeroE6 allow the quantification and the comparison of the viral production between naive and chronic infected cells (Figure 27). This comparison shows the influence of the presence of the first virus in the insect cells on the production of the second virus.



**Figure 27. Experimental strategy of sequential infection.**

Insect cells are chronically infected with a first virus. Plated in parallel with naive cells, these chronic infected cells are infected with a second virus. Several days post infection, cells are harvested and stained to reveal the number of infected cells by flow cytometry and quantify the infectivity for the second virus (called permissiveness ») in presence or absence of the first virus. In the same time, supernatants are harvested and are transferred to infect mammalian cells in order to quantify the viral production of both viruses (called « Viral production »).

## ***2. CHIKV chronically infected C6/36 cells increase permissiveness and production of DENV-2***

The first experiment studies aim to determine the effect of CHIKV on the DENV-2 permissiveness and the viral production as illustrated in Figure **27**. CHIKV chronically infected C6/36 cells were established after 10 days post infection and used for DENV-2 infection. Until 6 days p.i., CHIKV chronic and naïve C6/36 cells were fixed and stained to reveal DENV-2 infected cells. The percentage of DENV-2 infected cells in CHIKV chronically infected C6/36 cells is significantly higher (2,6 Fold at Day 3,  $p < 0,01$ ) than the infection on naïve C6/36 cells, showing an enhancement of DENV-2 permissiveness in CHIKV chronically infected C6/36 cells at M.O.I. 1 (Figure **28A**). This increased permissiveness is true every day until the 6<sup>th</sup> day, and also observed with lower M.O.I. (Figure **28B**; M.O.I. 0,1,  $p < 0,001$ ). Strikingly, DENV-2 virus infection appears after one day in the presence of CHIKV whereas it appears after 2 days in naïve cells. The latency of DENV-2 replication that is reduced in presence of CHIKV in C6/36 cells for both DENV-2 infectious doses that suggest that the kinetic of replication is increased. At day 3 p.i., the increase is the most important, from 10 to 40% of DENV-2 infected cells in presence of CHIKV in cells at MOI 1 ( $p$ -value  $p < 0,01$ ). Quantify by RT-qPCR, level of DENV-2 RNA is higher in co-infection condition in compare of mono-infection, in both MOI used for infection (**28C** and **28D**). This result suggests the induction of a strongest replication of DENV-2 by the pre-infection with CHIKV in C6/36 cells.

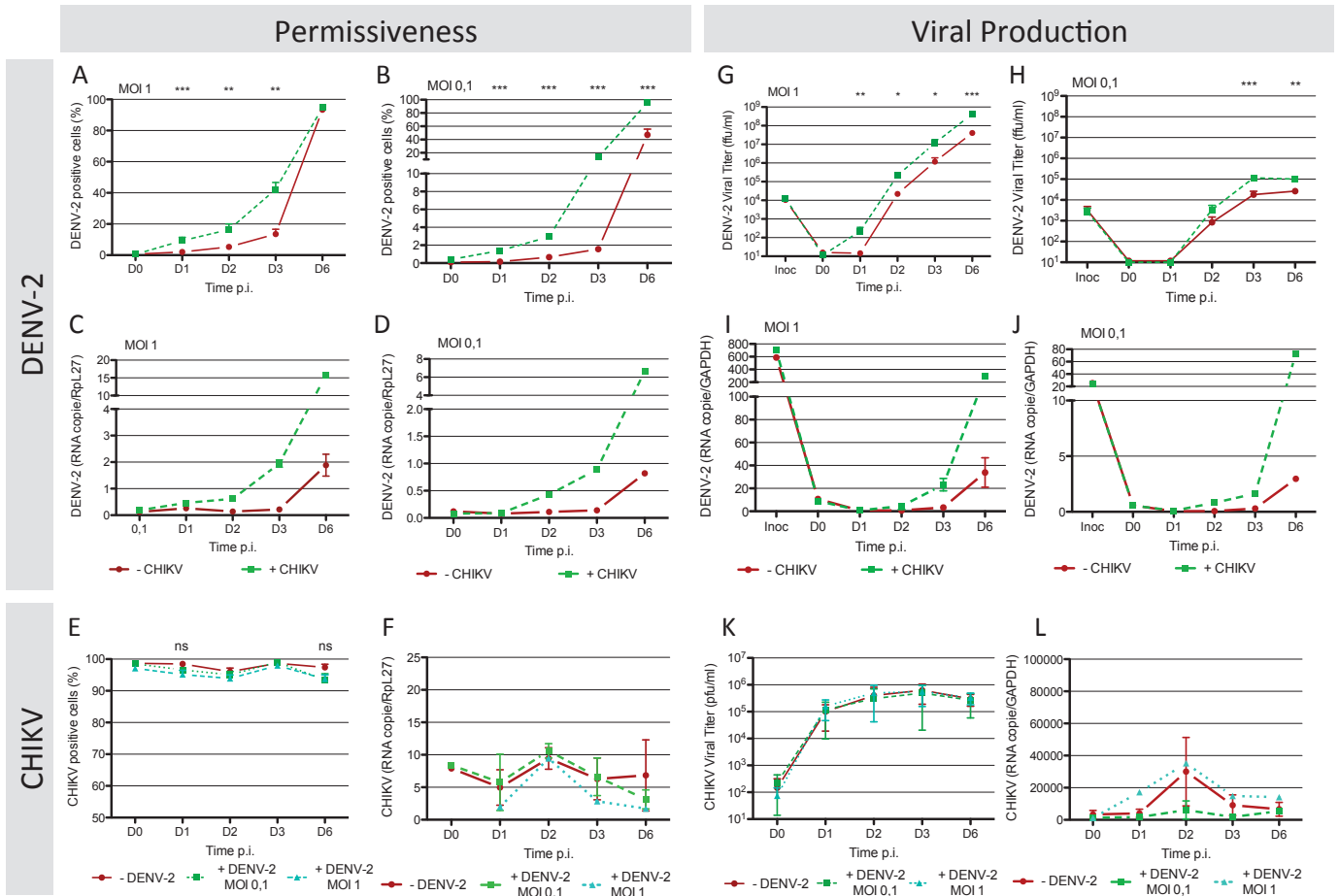
In the meantime, the percentage of CHIKV chronically infected cells is stable whatever the DENV dose (Figure **28E**). However, the quantity of CHIKV RNA into cells reveals a stable replication of CHIKV after the addition of DENV-2 (Figure **28F**). The cellular factors activated during the CHIKV infection may be directly useful the DENV-2 replication, without drastically impact the CHIKV replication. This result does not indicate the interference between both viruses at cellular scale.

To assess viral production, DENV-2 released in the supernatant of C6/36 cells were measured from day 1 to 6. After DENV-2 infection, the supernatants of both naïve and CHIKV chronic infected cells were transferred onto VeroE6 cells. After 48 hours, VeroE6 cells were fixed and stained to reveal DENV-2 titers. Until 6 days p.i., the DENV-2 titers produced by CHIKV chronic C6/36 cells are significantly higher than the production by naïve cells (9 Fold at day 3,  $p = 0,0156$ ), whatever the M.O.I. (Figures **28G** and **28H**). At MOI 1, similarly to the permissiveness, the time of latency of DENV-2 production by

CHIKV chronic infected C6/36 cells is also reduced compare to naive cells (Figure **28G**). Indeed DENV-2 appear in cells supernatant at day 1 for CHIK infected C6/36 whereas it appears only at day 2 for naive C6/36 cells (p-value  $p < 0,01$ ). RNA level in supernatant were also detected by RT-qPCR. As observed with flow cytometer, the DENV-2 RNA quantity in supernatant is drastically increased in presence of CHIKV into cells. The result is observed at MOI 1 (Figure **28I**, 15-Fold at day 6) and MOI 0,1 (Figure **28J**, 26-Fold at day 6), and suggests that CHIKV chronic C6/36 cells release some more of DENV-2 particle than C6/36 cells.

In parallel, the analysis of CHIKV production by titration is not impacted by the addition of DENV-2, whatever the DENV-2 dose (Figure **28K**). However, the analysis performed by RT-qPCR indicate that CHIKV RNA level in supernatant is similar in any conditions of co-infection. At day 2, there is a peak of RNA quantity, followed by a decrease (Figure **28L**).

All these results indicate an enhancement of DENV-2 infection into C6/36 cells. The number of infected cells and the RNA quantity of DENV-2 into is enhanced in co-infection condition. However, this enhancement seems to be lightly at the expense of CHIKV replication. The ratio of infectious titer per detected genome for the DENV-2 viral particles is better in supernatant of CHIKV chronic infected C6/36 cells.



**Figure 28. Interactions during co-infection of CHIKV chronically infected C6/36 cells.**

The permissiveness of DENV-2 in CHIKV Chronic infected C6/36 cells are analyzed by using two different MOI 1 (A) and 0,1 (B). C6/36 naïve cells (red) and CHIKV chronic infected C6/36 cells (green) were harvested, permeabilized and stained of E DENV-2 protein expression at day 0, 1, 2, 3 and 6 post infection. Percentages of DENV-2 positive cells at each time point were determined by flow cytometry. Data represent medium values of three independent experiments. Viral RNAs from infected cells with MOI 1 (C) and 0,1 (D) were quantified and normalized with RpL27 housekeeping gene. Chronicity of CHIKV in cells is also observed by the staining of Capsid protein expression. Percentages of CHIKV positive cells at each time point were determined by flow cytometry (E), and viral RNA were quantified and normalized with RpL27 housekeeping gene (F). Results are reported as RNA copies compare to RpL27 gene. Supernatants were transferred onto VeroE6 cells in order to quantify the viral production of DENV-2 at MOI 1 (G) and 0,1 (H), from CHIKV chronic infected C6/36 cells. CHIKV production in chronic infected C6/36 cells was also titrated (K). Viral titers were determined 48h post infection and results are reported as foci forming units per ml (ffu/ml). Viral RNAs were quantified and normalized with GAPDH housekeeping gene. Results are reported as RNA copies compare to GAPDH gene (I, J and L).

### 3. DENV-2 chronic infection doesn't affect the permissiveness nor the production of CHIKV

We conducted the reverse experiments to test the influence of a DENV-2 chronic infection on the permissiveness and production of wild type CHIKV by C6/36 cells. DENV-2 chronically infected C6/36 cells were obtained after 10 days post infection. Then, naïve and chronic infected C6/36 cells were infected with the CHIKV at different

doses, and growth kinetics was performed until 2 days p.i. Cells were fixed and stained to determine percentage of infected cells by flow cytometer. As shown in Figure **29**, there is no significant difference in percentage of CHIKV infected cells between DENV-2 chronic or naïve C6/36 cells, indicating that the permissiveness for CHIKV in both setting is the same (Figure **29A** and **29B** p-value  $p > 0,05$ ), whatever the CHIKV infectious dose (Figure **29A** and **29B**). The same experiment was performed in another mosquito cell type, U4.4 cell line. The chronic DENV-2 infection didn't influence the permissiveness in these cells (supplementary data, **S1**).

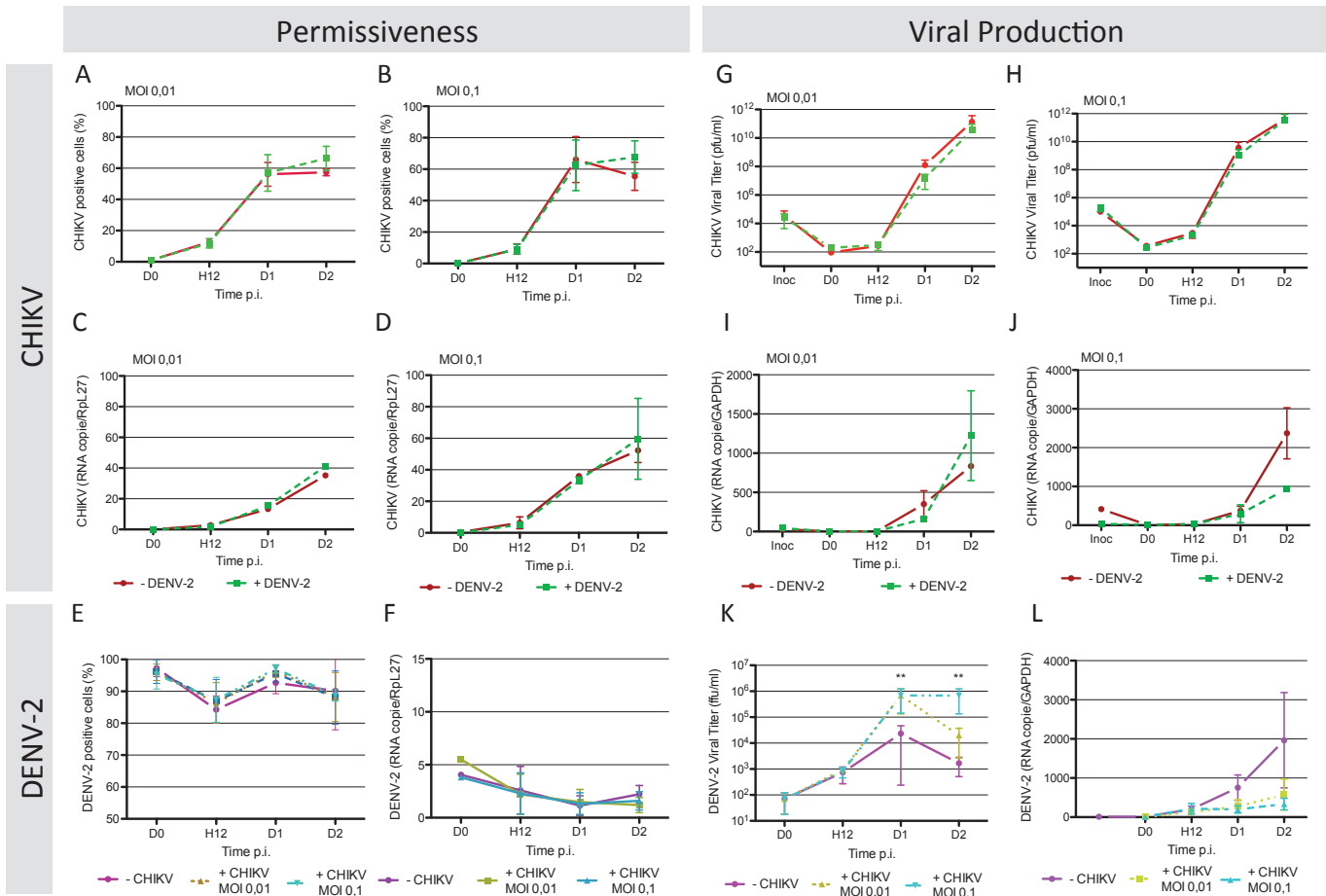
Detected by RTqPCR, the RNA level of CHIKV after the infection is similar in presence or absence of DENV-2 into cells, at MOI 0,01 (Figure **29C**) and MOI 0,1 (Figure **29D**). The DENV-2 infection does not impact the CHIKV replication in C6/36 cells.

In parallel, the percentage of chronically DENV-2 infected cells is also analyzed, and there is no significant difference of DENV chronicity in C6/36 cells (Figure **29E**). The DENV-2 RNA quantity is also identical in both infection conditions (Figure **29F**). These results suggest an absence of impact of CHIKV on the DENV-2 replication into cells. As mentioned previously, this result does not indicate the interference between both viruses at cellular scale

Until 2 days post infection, supernatants of each infection were collected in order to infect VeroE6 and analyze the viral production. Similarly to CHIKV permissiveness, there is no difference in the production of CHIKV by either naïve or DENV-2 chronically infected C6/36 cells, whatever the CHIKV infectious doses. These data are confirmed both by virus titration calculated by counting foci (Figure **29G** and **29H**) or by measuring released genomes by RTqPCR at the lowest MOI 0,01 (Figure **29I**). However, at MOI 0,1, the released particles are less important in presence of DENV-2 at day 2 of the analysis (Figure **29J**).

In contrast, when we observe the DENV-2 production, there is a significant increase of DENV-2 with a high dose, observed by titration onto VeroE6 (Figure **29K**,  $p < 0,01$ ), but the analyze of release genomes by RTqPCR show a dose-dependent decrease in the DENV-2 RNA level in supernatant with CHIKV (2,5 Fold at day 2, Figure **29L**). All these results indicate the absence of impact of the replication of DENV-2 in C6/36 cells on the permissiveness or the production of CHIKV. However, the diminution of DENV-2 genomes in supernatant suggest that the CHIKV infection reduce the DENV-2 release in

supernatant, but the infectivity of DENV-2 particles released in supernatant is enhanced by the addition of CHIKV into cells. Although no significant difference of DENV-2 replication had been observed in cellular scale, the presence of CHIKV modulate the DENV-2 viral production, to reduce the number of released genomes, and in improve the infectivity.

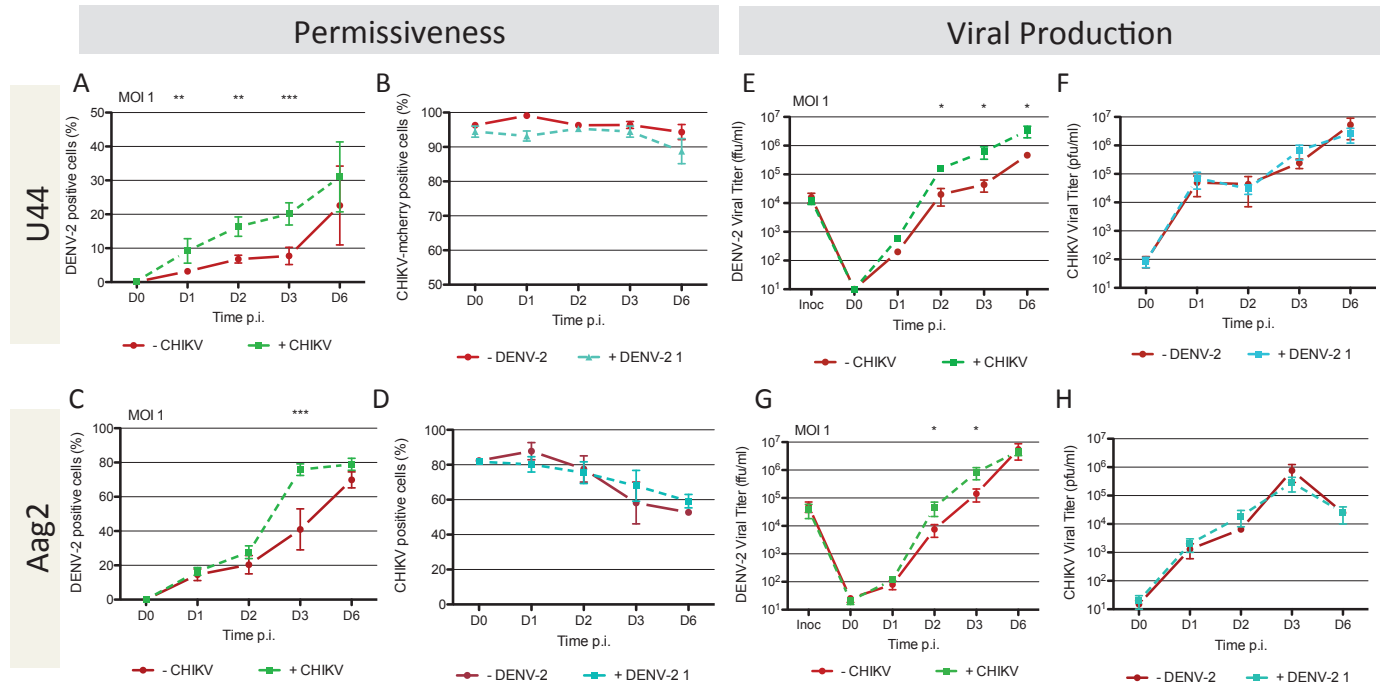


**Figure 29. Interactions during co-infection of DENV-2 chronically infected C6/36 cells.**

Infections with two different MOI of CHIKV in DENV-2 chronic infected C6/36 cells are investigated (A and B). C6/36 naïve cells (red) and DENV-2 chronic infected C6/36 cells (green) were harvested, permeabilized and stained with capsid protein expression at 0, 12 hours, 1, and 2 days post infection. Percentages of CHIKV positive cells at each time point were determined by flow cytometry. Data represent medium values of three independent experiments. Viral RNA from infected cells with CHIKV at MOI 0,1 (C) and 0,01 (D) were quantified and were reported as RNA copies compare to RpL27 housekeeping gene. Data represent medium values of two independent experiments. DENV-2 chronic infection of C6/36 cells was determined by flow cytometry after a staining using the E protein (E), and viral RNA was quantified by RT-qPCR (F). Results are reported as percentages of CHIKV positive cells and RNA copies compare to RpL27 housekeeping gene. Quantification of viral production after super-infection with CHIKV onto DENV-2 chronic infected cells is analyzed by titration of supernatants in VeroE6 cells. At MOI 0,01 (G) and 0,1 (H), viral titers were determined 48h post infection. DENV-2 production in chronic infected C6/36 cells was also titrated (K). Results are reported as plaque forming units per ml (pfu/ml). Viral RNAs in supernatant were quantified by RT-qPCR and results are reported as RNA copies compare to GAPDH housekeeping gene (I, J and L), and by RT-qPCR after RNA extraction (L).

#### 4. Evolution of DENV-2 permissiveness and production in CHIKV chronically infected U4.4 (*Ae. albopictus*) and Aag2 (*Ae. aegypti*) cells

To determine whether we can generalize these results to other mosquito cell lines, U4.4 (*Ae. albopictus*) and Aag2 (*Ae. aegypti*) mosquitoes cell lines were used for co infection. CHIKV-mcherry chronically infected U4.4 and Aag2 cells were generated and then infected with DENV-2 at MOI 1. At day 3-post infection, the presence of CHIKV increases the permissiveness of DENV-2 in U4.4 (Figure **30A**,  $p < 0,001$ ) and Aag2 cells (Figure **30C**,  $p < 0,001$ ). The CHIKV chronicity was also analyzed for each cell line. The percentage of infected cells is stable whatever the DENV-2 infectious dose for U4.4 (Figure **30B**). In the long term, we observed a decrease of the percentage of CHIKV infected Aag2 cells, suggesting that the innate answer is stronger than in others insect cells, thus causing the viral elimination after many days (Figure **30D**). Next, the supernatants were collected and transferred onto VeroE6 cells to determine the viral production. Figures **30E** and **30G** show an increase of DENV-2 production when DENV-2 is produce in CHIKV chronic cells in U4.4 and Aag2 cells, respectively. The increase is lower in the other cell lines, Aag2 cells. As the CHIKV LRic strain seems well adapted to *Ae. albopictus* (Tsetsarkin et al., 2006), we decided to test another CHIKV strain derived from Asian lineage more fit to *Ae. aegypti* .and that co-circulated with DENV. We generated chronically infected Aag2 cells with a CHIKV strain from a human isolate from Saint Martin Island, where *Ae. aegypti* is endemic. This CHIKV strain seems to have the same impact on DENV-2 permissiveness and viral production in Aag2 cells (supplementary data, **S2A** and **S2B**).



**Figure 30. Interactions during co-infection of CHIKV chronically infected Aag2 (*Ae. aegypti*) and U4.4 (*Ae. albopictus*) cells.**

Permissiveness and viral production of DENV-2 were analyzed in naive (red) or CHIKV-mcherry chronic infected (green) U4.4 and Aag2 insect cells. DENV-2 infection had been determined by flow cytometry after a staining using E protein at day 0, 1, 2, 3 and 6 post infection, and reported as percentages of positive cells, in U4.4 cells (A) and Aag2 cells (C). The chronicity of CHIKV-mcherry in both cells are also observed (B and D), using mcherry reported expression. Percentage of CHIKV-mcherry positive cells at each time point was determined by flow cytometry. The viral production is analyzed by the titration of supernatants onto VeroE6 cells. Infectious titers were determined 48h post infection a staining using IFA revelation, and reported as foci forming units per ml (ffu/ml). Titrations were performed in supernatants from U4.4 cells (E) and in Aag2 cells (G). In parallel, the chronic CHIKV production was analyzed in same supernatants (F and H). Data represent medium values of three independent experiments.

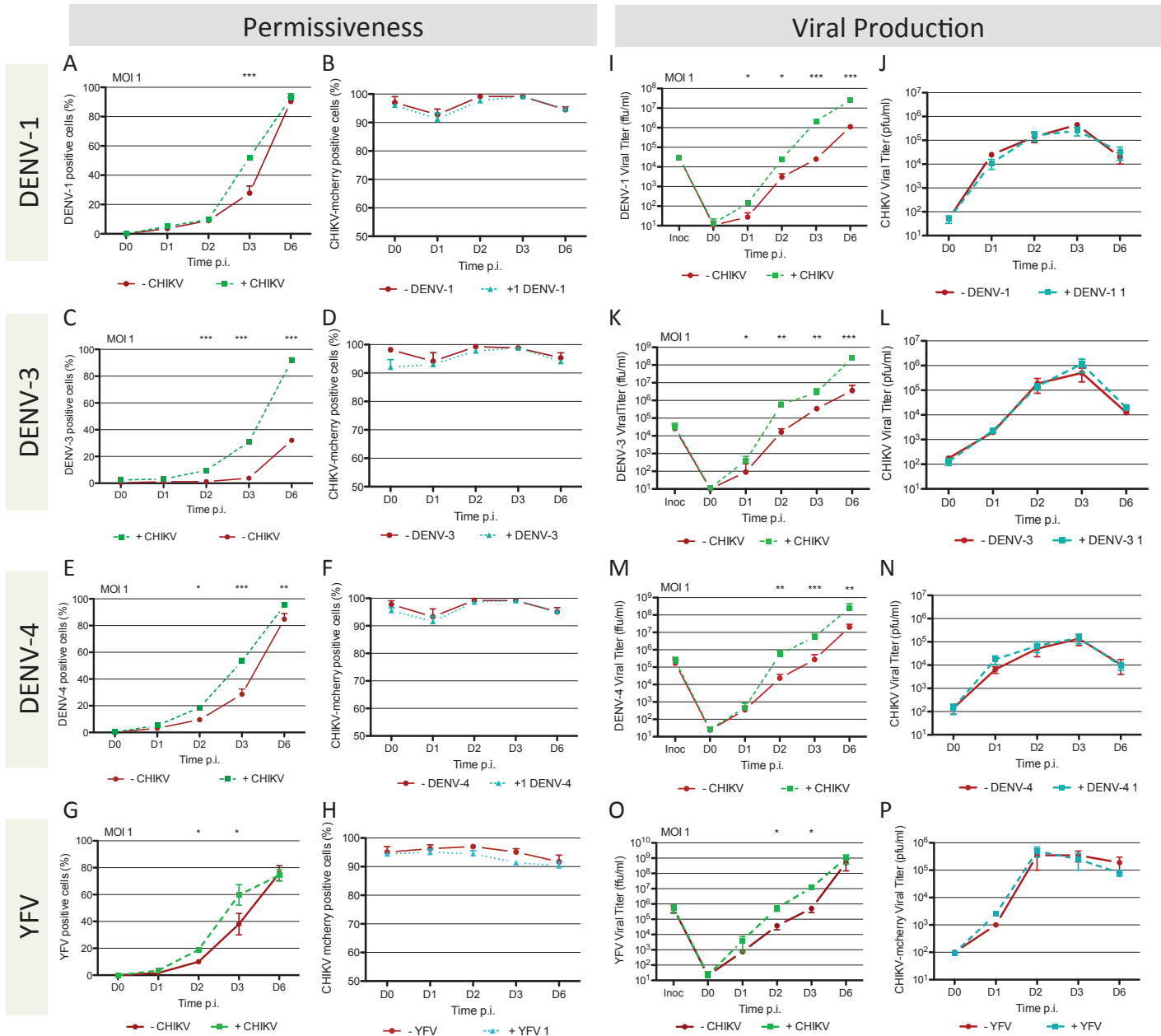
### 5. Enhancement of others Flavivirus in CHIKV chronic C6/36 cells

To go further, we next address if CHIKV is able to enhance all DENV serotypes (DENV-1 - 3 and -4) infections. Antibody specific for *flavivirus* was used for staining in C6/36 cell line. As shown in figure 31A-C-E, the presence of CHIKV in C6/36 cells increase each serotype with different intensity. Strikingly, the presence of CHIKV increase the permissiveness of DENV-3 and the kinetic is accelerates by 1 day (Figure 31C). The increase of DENV-1 and DENV-4 infection is similar to DENV-2 (Figure 31A and 31E). As we have shown for DENV-2, the percentage of CHIKV chronic infected cells is stable, in presence or not of the different DENV serotypes. (Figure 31E-G). All supernatants were used to infect VeroE6 cells to determine the viral production. As expected from the data of permissiveness, for each DENV serotype, viral production is increased in the presence

of CHIKV in chronic cells. (Figure **31I-K-M**). Once again, the DENV-3 infectivity is increase the more compare to DENV-1 and DENV-4 (Figure **31K**). The addition of each serotype has no impact on the CHIKV production (Figure **31J-L-N**).

To go further, we want to know if CHIKV is able to enhance another *flavivirus* infection. We infected naïve and CHIKV chronically infected C6/36 cells with Yellow Fever Virus (YFV). Interestingly, we were able to detect an increase of permissiveness and production of YFV in presence of CHIKV in C6/36 cells (Figure **6G** and **6O**), whereas the percentage of CHIKV chronic infected cells and the CHIKV production in supernatant is stable (Figure **6H** and **6P**).

Also transmitted by *Aedes* mosquitoes, Zika virus (ZIKV), a *flavivirus* member, was used to infect chronic CHIKV or SINV infected C6/36 cells. Detected by RTqPCR in cells and in supernatants, the number of ZIKV RNA copy per ml was higher ( $1,5\log^{10}$ ) in presence of SINV in cells and supernatants (Figure **S3A** and **S3B**). In chronic CHIKV infected cells, the ZIKV RNA copies per ml were higher ( $1,5\log^{10}$ ) than the mono-infection (Figure **S3C**). Altogether, these results show that CHIKV is able to facilitate permissiveness and production of all DENV serotype, and other *flaviviruses*, also vectorized by the same *Aedes* mosquito.

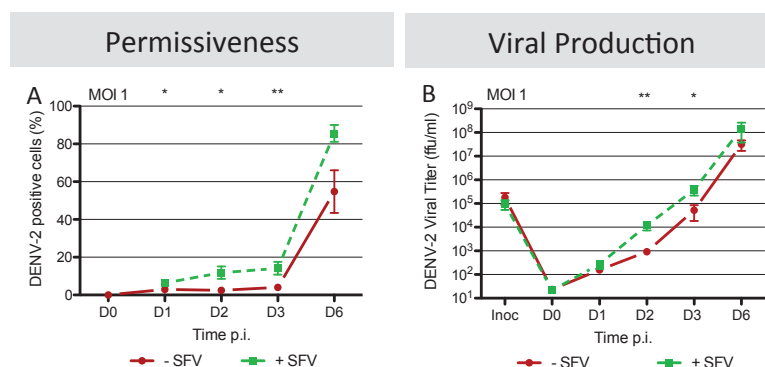


**Figure 31 Enhancement of flaviviruses members in presence of CHIKV in C6/36 cells.**

Naive (red) or chronic CHIKV infected (green) C6/36 cells were infected with DENV serotypes and YFV at MOI 1. Permissiveness in these cells were firstly analyzed for DENV-1 (A), DENV-3 (C), DENV-4 (E) and YFV (G) after a staining using E protein at day 0, 1, 2, 3 and 6 post infection. In parallel, the CHIKV-mcherry chronicity was analyzed after DENV infections (B-D-F-H). Percentages of DENV-2 and CHIKV positive cells at each time point were determined by flow cytometry using the mcherry protein expression. Transferred onto VeroE6, supernatants are titrated to determine the viral production following the DENV infection. The staining was performed 48h post infection and infectious titers were reported as foci forming units per ml (ffu/ml). The chronic CHIKV production was also analyzed in same supernatants, and was reported as plaque forming unit (pfu/ml). Experiments were performed for DENV-1 (I and J), DENV-3 (K and L), DENV-4 (M and N) and YFV (O and P) for DENV and CHIKV detection, respectively. Data represent medium values of three independent experiments.

### 6. Co-infection in SFV chronic C6/36 cells

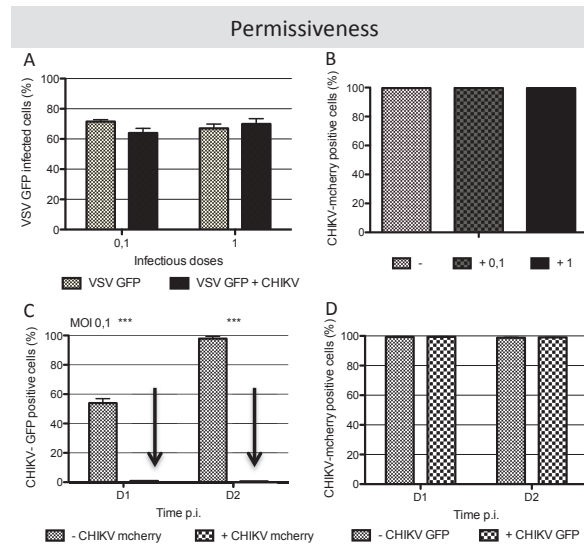
To determine to specificity of the increased DENV infectivity by CHIKV, we have chronically infected C6/36 cells by another *alphavirus*, the Semliki Forest Virus (SFV). As shown by Figure 32A and 32B, DENV-2 permissiveness and production are increased in presence of SFV in C6/36 cells at different time points (p-value  $p < 0,05$ ). The increase DENV-2 infection by SFV suggests that the promotion of *flavivirus* infection is maybe a more conserved process that we previously thought.



**Figure 32. The chronic Semliki Forest Virus infection in C6/36 cells enhances the DENV-2 infection.**

Naive (red) or chronic SFV infected (green) C6/36 cells were infected with DENV-2 at MOI 1. Permissiveness (A) of DENV-2 in cells is determined by flow cytometry reported as percentage of positive cells. The viral production (B) of DENV-2 in supernatant were determined by titration and staining onto VeroE6 after 48h post infection. Infectious titers are reported as foci forming units per ml (ffu/ml). Data represent medium values of three independent experiments.

To test the extent of such a phenomenon, we tested the infection of CHIKV chronic infected cells onto infectivity of the vesicular stomatitis virus (VSV), a *rhabdovirus* not related to *flavivirus* (Figure 33A). At day 2-post infection, the permissiveness of C6/36 cells for VSV is similar whether the cells are pre-infected or not by CHIKV, and the CHIKV chronicity is not impact by the addition of VSV with different M.O.I. (Figure 33B). These data suggest that the promotion of *flavivirus* infection in mosquito cells is a conserved process for at least two *alphaviruses*, CHIKV and SFV, but not for other arboviruses able to infect the same cells (VSV).



**Figure 33 Chronic CHIKV infection does not impact the permissiveness of Vesicular Stomatitis Virus (VSV) and promotes super-infection exclusion process for a second infection with CHIKV.**

Infection with VSV-GFP in naive or chronic CHIKV-mcherry infected C6/36 cells was analyzed at two different infectious doses MOI 0,1 and MOI 1 (A). In parallel, the chronicity of CHIKV-mcherry cells was analyzed after VSV infection (B). The percentage of infected cells was determined by flow cytometry using the GFP (VSV) and mcherry (CHIKV) reporter expressions. Super-infection exclusion was performed on chronic CHIKV-mcherry infected C6/36 cells, by a second CHIKV-GFP infection at MOI 0,1. The second infection was not detected in chronic CHIKV-mcherry infected cells (C), after 1 or 2 days post infection. The second infection does not impact the chronicity of CHIKV-mcherry in infected cells (D). Results are reported as percentage of positive cells and Data represent medium values of three independent experiments.

### 7. CHIKV-mcherry mediate homologous super-infection exclusion on CHIKV-GFP

As a control to study the specificity of the effect we reported earlier of CHIKV on DENV, we decided to study the CHIKV/CHIKV super-infection. For this purpose, we used two replicative CHIKV viruses expressing two different fluorochromes, the CHIKV-mcherry and the CHIKV-GFP, producing a red and green fluorescence respectively. These two viruses allowed us to distinguish the CHIKV-mcherry in chronically infected C6/36 cells and the CHIKV-GFP superinfecting the cells. The experiment was conducted like previously (Figure 2). At day 1 and 2 post infection, the cells were fixed and analyzed by flow cytometer. According to this experiment, the naive C6/36 cells are clearly more permissive for CHIKV-GFP than the chronic CHIKV-mcherry C6/36 cells (Figure 33C, p-value  $p < 0,001$ ). As shown in Figure 8D, the addition of the second virus, CHIKV-GFP, has no impact on the CHIKV-mcherry chronicity in C6/36 cells. Experiments were performed with two different M.O.I. (data not shown). These data indicate that a previous infection with CHIKV protect the cells for a second infection by the same virus. These results are in accordance with an homologous super-infection exclusion already described for other *alphaviruses*, Sindbis virus and Ross River Virus (Karpf et al., 1997).

## IV. Discussion

One consequence of the rapid spreading of CHIKV in the tropical as well as in the temperate regions via *Aedes albopictus*, is an increased overlapping distribution with DENV, whose levels and geographical range also have been increasing. Overlapping in the distribution of the two arboviral diseases DEN and CHIK leads to a higher detection of co-infections in humans, as recently re-observed in Sri Lanka and Africa (Hapuarachchi et al., 2008) and in travelers (Schilling et al., 2009). Clearly, the mosquito *Aedes albopictus* and *Aedes aegypti* could carry and transmit simultaneously CHIKV and DENV (Caron et al., 2012; Ruckert et al., 2017; Vazeille et al., 2010). However, nothing is known so far about the molecular and cellular basis of co-infection between CHIKV and DENV. The interaction may be influenced by the immune response to the pathogens, but also on interactions inside one cell. So far, the study of the co-infection by DENV and CHIKV at the cellular scale is relevant due to common cellular tropism of the two viruses in mosquitoes as well as in humans. Moreover, at the cellular level, DENV and CHIKV share interactions with the innate immune system, autophagy and apoptosis pathways (Dhanwani et al., 2012b; Fang et al., 2014; Joubert et al., 2012a). These analogous host-virus interactions probably impact the outcome of one virus during co-infection as they may interact with similar host proteins. Our project studied the co-infection of the same cell by CHIKV and DENV, which should help to anticipate any influences of this phenomenon on the propagation of the viruses in one organism or on the epidemiology of the diseases. To characterize any interference or synergy between DENV and CHIKV during replication, we performed sequential co-infection in the *Aedes albopictus* C6/36 and U4.4 and *Aedes aegypti* Aag2 cell lines. We found that the permissiveness and production of *flaviviruses* (DENV serotypes, YFV and ZIKV) are enhanced in presence of CHIKV. We showed that a chronic infection by CHIKV does not impact the second infection with VSV (*rhabdovirus*) and the chronic infection with Semliki Forest Virus (*alphavirus*) also increased a subsequent DENV-2 infection. All these results suggest that the enhancement result to a specific and conserved mechanism between *alphaviruses* and *flaviviruses*.

On the contrary, the DENV-2 pre-infection has no effect on subsequent CHIKV co-infection, both in cells and in supernatants. However, the addition of CHIKV seems to limit the release of DENV-2 particles in the supernatants, while viruses are more

infectious. The increased permissiveness and production of DENV could be caused by various factors.

Firstly, one of these could be due to an inhibition of the cellular innate immune response. Indeed, CHIKV replicates faster and at higher titer compared to DENV strains. During early part of infection, CHIKV bypass the antiviral strategies to an efficient replication. While the CHIKV infection induces cytopathic effects in mammalian cells, and mediate the autophagy pathway (Joubert et al., 2012a; Krejbich-Trotot et al., 2011b; Lee et al., 2008) and the apoptosis (Krebich-Trotot et al., 2010), the arboviruses infection in insect cells induces a persistence through suppression and/or by evading immune defense (RNA interference, Toll, Jak/STAT and Imd/Jnk) (McFarlane et al., 2014). The CHIKV infected cells can therefore be more sensitive to DENV as no or little innate response counteract DENV infection. However, little is known about the interactions between CHIKV or DENV and the immune cellular pathways in mosquito vectors. Recently, the Toll and JAK-STAT pathways have been involved in the control of DENV-2 in *Aedes* mosquitoes, but the CHIKV replication is not impacted by these pathways (Jupatanakul et al., 2017). RNAi drives another important innate response in insect. Although a part of this study was performed on C6/36 cells, results were confirmed with two other cell types. C6/36 are deficient in RNA interference protein due to mutation in DICER protein. However, the whole RNAi pathway is not entirely suppressed, and could be involved in the increase of DENV-2 replication after the first infection with CHIKV. Indeed, both viruses are known to be targeted by the RNAi pathway for their replication into insect cells.

The chronic infection by CHIKV could lead to a repression of the immune response but it can also be due to activation of a cellular pathway crucial for DENV replication. Viral proteins, which play a role of inhibitors, mediate interactions with antiviral responses but some are involved in enhancement or activation of cellular pathways. It is known that both DENV and CHIKV interact with the immune autophagy pathway (Joubert et al., 2012a; Krejbich-Trotot et al., 2011b; Lee et al., 2008) and they both rely on it for their replication. We can hypothesize that the chronic presence and replication of CHIKV in the cells could induce autophagy that will enhance the replication of DENV-2, especially during the early times of infection of this “slow” virus. As CHIKV is more rapid and replicates at a higher titer, these kinetic differences may also explain why DENV does not impact neither the permissiveness, nor the production of CHIKV in DENV-2 chronic

C6/36 cells.

The main innate response pathway that is counteracted needs still to be determined. We can make hypothesis involving CHIKV nsP and this will help us to target one viral protein and a cellular pathway. Interestingly, the nonstructural protein 2 (nsP2) of *alphaviruses* interplay in the inhibition of JAK-STAT pathway (Breakwell et al., 2007; Frolova et al., 2002), but also in the reduction of cellular transcription by a rapid degradation of RPB1, a catalytic subunit of the RNA polymerase II (Akhrymuk et al., 2012). NsP2 has also been involved in autophagy and it is able to interact with the human autophagy receptor NDP52 but not its mouse orthologous (Judith et al., 2013). Even if there is no evidence of interaction of nsP2 with the mosquito NDP52 ortholog, activation of autophagy by nsP2 can prime the cells for DENV infection and accelerate its replication. The nonstructural protein 3 (nsP3) of CHIKV is involved in the inhibition of induction of stress granules (McInerney et al., 2005). The following step will be to overexpress nsP from CHIKV in mosquito cells using replicon, and to observe an enhancement of DENV infection.

The enhancement of DENV-2 particles released in the supernatants can be a resulting to the enhancement of the viral replication in cells. However, other explanations can accentuate the observation. During the reverse experiment, the chronicity of DENV-2 in cells is not impacted by the second infection with CHIKV. However, the DENV-2 RNA copies in supernatant are decreased by the addition of CHIKV, while the number of DENV-2 infectious particles is enhanced. This result suggests that the secretion of DENV-2 is reduced, but the fraction of mature and infectious particles is more important. A competition for the exocytosis of both viruses could explain a decrease of DENV-2 release. CHIKV, with a stronger and faster replication, mobilize cellular partners involved in the exocytosis process. The requirement of beta-1 tubulin involved in the endocytosis/exocytosis pathways has been described for CHIKV and DENV release in *Aedes aegypti* mosquitoes and into mosquito cells (Paingankar et al., 2010; Tchankouo-Nguetchou et al., 2010). A competition for cytoskeleton proteins requires for the exit of virion is possible.

However, CHIKV could influence the maturation of DENV-2 particles to enhance the number of mature virion. During the processing and the secretion of DENV in insect cells, the maturation of DENV virion is mediated by Furin protein (Junjhon et al., 2008).

The cleavage of precursor membrane (prM) is required for rearrangement of E glycoprotein at the surface and for the infectivity. Mutations in basic residues involved in the recognition for the furin protease reduce the exit and the infectivity of virion (Keelapang et al., 2004). For *alphaviruses*, it has been shown that Furin was involved in the maturation, by the cleavage of glycoprotein PE2 into E3 and E2 (Heidner et al., 1996; Metz et al., 2011). During the chronic infection with CHIKV, the protein synthesis such as furin may be enhanced and promote the maturation of DENV-2 after the particles formation of the ER and trans-Golgi Network. A quantification of furin at the RNA and protein level in mono and co-infection conditions may confirm this hypothesis. Similarly, it will be interesting to quantify the ratio between prM and M quantity at the virion surface in both conditions. In parallel the titration of viral particles retained in cells, and in the supernatants, as performed in the publication of Junjhon et al., in 2008 may bring some indications.

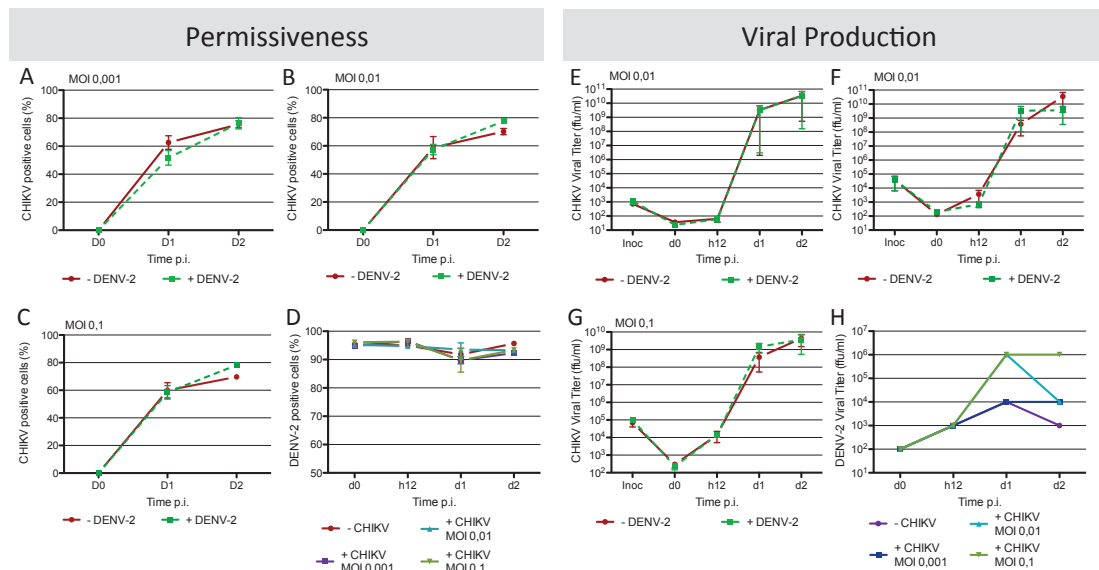
Another potential and unknown process during co-infection is the formation of chimeric particles in co-infected cells, by the incorporation of viral proteins into the other virion. A DENV particle with CHIKV glycoproteins, or CHIKV with DENV proteins may modulate the infection and the tropism for cells and tissues. To date, none of studies have been performed to demonstrate interactions between viral proteins of both viruses. These experiments are challenging to design, as it needs a precise purification step of one population of virus. However, the expression of individual proteins or part of them may help the test heterologous incorporation. For example, we can express nsP from CHIKV with SP from DENV and test the formation of reporter virus particles (and vice and versa). However, the differences in the replication cycle may limit interaction between viral proteins of both viruses. Indeed, while the budding of CHIKV is at the plasma membrane, the DENV particles bud between the ER and the Golgi network. The ER and the trans-Golgi network are used by both viruses for the formation and maturation of viral proteins. Interactions between viral proteins are possible at this stage of cycles. Experiments consisting to retained DENV particles and stained with specific CHIKV antibodies against glycoproteins could be developed.

*In vivo* experiments of *Aedes aegypti* mosquitoes need to be done (and has been done in the second part) to confirm our *in vitro* results. Oral co-infection of *Aedes albopictus* has

been shown to be possible experimentally using strains of DENV-1 and CHIKV provided in the same blood-meal (Vazeille et al., 2010). These co-infected mosquitoes present both viruses in their saliva and can potentially transmit both viruses together. Recently, simultaneous exposure to CHIKV, ZIKV and DENV-2 in *Ae. aegypti* mosquito shows the co-infection and the transmission of all combinations of viruses (Göertz et al., 2017; Ruckert et al., 2017) confirming possible co-infection of *Ae aegypti*, contrary to previous results (Rohani et al., 2005).

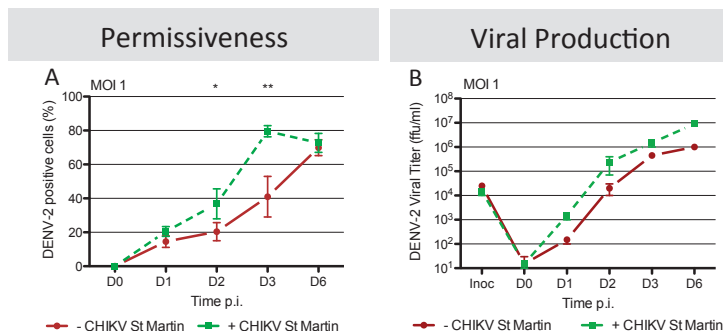
In all these studies above, simultaneous exposure to different combination of viruses have been performed and no significant variations in the outcome to viruses was observed. In our condition of sequential infection, various criteria could be impacted, as well as the viral dissemination or the transmission. The potential transmission in the field is largely random and models of co-infection can be multiple. The vector competence could be modified during simultaneous outbreak in favor of one virus, or could induce important severe cases due to the infection enhancement. We will assess the effects of CHIKV and DENV-2 co infection and we could generalize the effect seen in insect cells. The increase of the DENV replication could potentially facilitate the transmission to patients. It will be interesting to explore co-infection in mammal cells, and our results may have implication on the pathology. Indeed, in-patient, infected by CHIKV can last for month and in some tissues, antigens have been observed in muscle or joints a long time after infection (Labadie et al., 2010). If resident macrophages can be infected in the long term, they may be co-infected by DENV during secondary infection and DENV infection will be increase. The secondary DENV infection may lead to an increase of severity. These data would allow estimating the consequences of interactions during simultaneous outbreaks. An increased prevention should be necessary in areas with co-circulation of both viruses, to prevent severe cases and death.

## Supplementary datas.



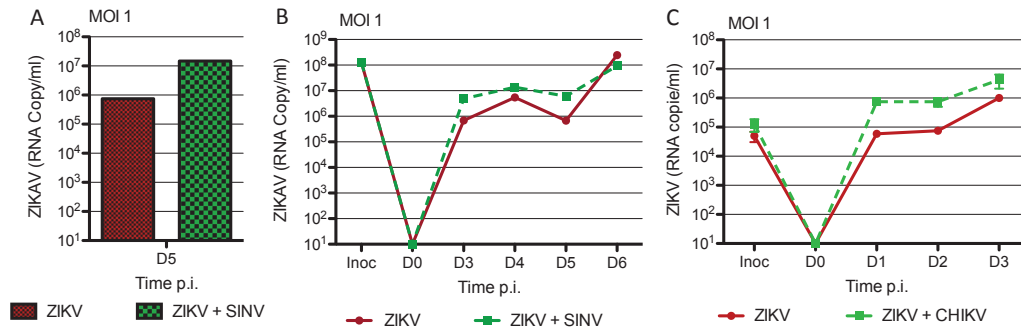
## Supp 1. Interactions during co-infection of DENV chronically infected U4.4 cells.

Naïve (red) and DENV-2 chronic (green) insect cells were infected with CHIKV at MOI 0,001 (A) and MOI 0,01 (B), and MOI 0,1 (C) according to the experimental strategy described in Fig.2. CHIKV infection had been determined by flow cytometry, and reported as percentage of positive cells. The chronicity of DENV-2 in cells is analyzed after CHIKV infection (D). Supernatants were transferred onto VeroE6 cells in order to quantify the viral production following the infection. Infectious titers have been determined by counting foci under microscopy after a staining using IFA revelation, and reported as foci forming units per ml (ffu/ml). Three infectious doses of CHIKV were used, at MOI 0,001, MOI 0,01 and MOI 0,1 (E-G). The chronic production of DENV-2 was also titrated (H).



## Supp 2. Enhancement of DENV-2 virus infection and production by CHIKV chronic infected Aag2 cells using Saint Martin strain.

Naïve (red) and CHIKV chronic (green) Aag2 cells were infected with DENV-2 at MOI 1 (A). DENV-2 infection had been determined by flow cytometry, and reported as percentage of positive cells. Supernatants of infected cells were collected at each time points and were transferred onto VeroE6 in order to quantify the viral production following the infection (B). Infectious titers have been determined by counting foci under microscopy after a staining using IFA revelation, and reported as foci forming units per ml (ffu/ml).



### Supp 3. Impact of chronic alphaviruses infection in C6/36 on ZIKV infection.

Naive (red) and Sindbis virus chronic (green) C6/36 cells were infected with ZIKV at MOI 1 (A). ZIKV infection had been determined by RT-qPCR, and reported as RNA copies per ml. Supernatants of infected cells were collected at each time points and were transferred onto VeroE6 in order to quantify the viral production following the infection (B). Infectious titers have been determined by counting foci under microscopy after a staining using IFA revelation, and reported as foci forming units per ml (ffu/ml). Naive (red) and CHIKV virus chronic (green) C6/36 cells were infected with ZIKV at MOI 1 and supernatants were transferred onto VeroE6 (C). Infectious titers have been determined by counting foci under microscopy after a staining using IFA revelation, and reported as foci forming units per ml (ffu/ml).

# Experimental Report

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## Chapter 2. Sequential infection *in insecta: Aedes aegypti*

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## Abstract

Dengue (DENV) and Chikungunya (CHIKV) viruses are two mosquito-borne arboviruses that cause human epidemics of several million serious clinical cases over the last decade. *Aedes albopictus* and *Aedes aegypti* are main vectors of these two arboviruses, causing a similar geographical area of distribution particularly in sub-Saharan Africa and Southern Asia. Recently, with the re-emergence of the CHIKV, the number of reported cases of patients infected by both DENV and CHIKV is increasing. In theory, the co-infection in mosquitoes is possible and may be responsible of the transmission of both diseases simultaneously. To characterize any interference or synergy between DENV and CHIKV during replication in mosquitoes, we performed a sequential co-infection in *Aedes aegypti* mosquitoes. 4 days after a first CHIKV infectious blood meal, female mosquitoes received the second DENV-2 infectious blood meal. Several days after infectious, midgut and salivary glands, two organs, which play crucial roles for the vector competence, were analyzed by RT-qPCR to detect viral RNA. Saliva was also collected 10 days post infection to perform analyzes. Our preliminary results showed that the second infection with DENV-2 did not impact the viral replication of CHIKV in midgut and in salivary glands. In the same way, the CHIKV did not seem to influence the DENV-2 replication in midgut. On the contrary, the level of DENV-2 RNA is higher in co-infection condition in salivary glands. When the saliva is analyzed after 10 days, DENV-2 was not detected, but CHIKV was detected well as in mono and co-infection conditions. Altogether, our *in vivo* results suggest an interaction between CHIKV and DENV-2 in mosquito, also previously observed with an *in vitro* model. This interaction could lead to a change of transmission of arboviruses during a simultaneous outbreak and may modify the epidemiology.

Key words: *Aedes aegypti*, co-infection, Chikungunya, Dengue, Arboviruses

## I. Introduction

Arthropods born viruses (arboviruses) are the causative agents of important emerging infectious diseases. Particularly endemic in some geographical areas and since several decades in expansion in native countries, these viruses are considered as growing global public health problems with a potential impact on the economy. The geographical distribution of many viruses continues to increase, and 40% of world population lives at present in high-risk areas of arboviroses. Outbreaks involving two arboviruses, the chikungunya (*alphavirus*) and dengue (*flavivirus*) occur frequently in Asia, Sub-Saharan regions and in South of America. With 2,5 billion of individuals threatened, DENV is considered as one of the most important arbovirosis in the world. Various symptoms are associated to infection with this virus, from flu-like syndrome, to hemorrhagic fever, and choc syndrome in the most severe cases. Each year, 400 million patients are diagnosed, with 700 000 severe cases. CHIKV is also responsible of almost millions of infections, with symptoms characterized by important joint and muscular pains. Recently, encephalitis and abortion are attributed to CHIKV infection (Thiberville et al., 2013).

Transmitted by *Aedes aegypti* mosquito, CHIKV and DENV are also associated with the invasive vector, *Aedes albopictus*, which the expansion was rapidly increased since several decades. So capable of co-circulate during outbreak, both viruses may share same vectors and same hosts. Cases of humans co-infection with both viruses are recently observed, in habitants or in travelers, confirming former data (Chahar et al., 2009; Chang et al., 2010; Myers and Carey, 1967; Parreira et al., 2014). Moreover, mosquitoes sampling in the field showed the simultaneous presence of CHIKV and DENV in *Ae. albopictus* mosquito (Caron et al., 2012), suggesting the potential co-transmission of viruses to a novel host. Performed on laboratory, study confirmed this data by the demonstration of both viruses in mosquito saliva after orally simultaneous co-infection (Vazeille et al., 2010). More recently, investigations in *Aedes aegypti* confirmed the simultaneous co-infection with three distinct arboviruses (CHIKV, DENV and ZIKV) (Göertz et al., 2017; Ruckert et al., 2017).

Even if CHIKV and DENV are able to infect same organs and cells in insects and humans (Gasque et al., 2015; Gregson and Edelman, 2003), none of study explain cellular interactions possible between them during co-infection. Previously, we have shown the

impact of co-infection in insect cells. We have demonstrated that the permissiveness and the viral production of DENV-2 virus are increased on *Aedes albopictus* C6/36 cells previously infected by CHIKV and still chronically infected. On the contrary, the chronic infection by DENV-2 in the same cells, does not impact CHIKV permissiveness of production. To go further, these results were confirmed by using several mosquitoes cell types, the *Ae. albopictus* U4.4 but also the Aag2 derived from *Ae. aegypti*. Although the molecular mechanism is still unknown and have to be determining, our results need to be confirmed with *in vivo* experiments.

Previous work had already shown the presence of both viruses in mosquito organs after a simultaneous co-infection, and the dissemination up to salivary glands, which allows the presence of both viruses in saliva, crucial for transmission (Vazeille et al., 2010). In our laboratory, experimentations on simultaneous infection reveal a facilitation of the viral replication in salivary glands of co-infected mosquitoes (Le Coupanec et al., 2017, Appendix 1).

However, the model of simultaneous co-infection is not the only one that happen during simultaneous outbreaks. A pre-infection by a first virus may influence the vector competence, the viral replication, or the dissemination for the second virus. Such modification may impact the transmission, and the pattern of the current outbreak, with frequent severe cases observations.

In this study, we determined the kinetic of DENV-2 infection in orally infected *Aedes aegypti* mosquito in mono and co-infection conditions. We analyzed replication and dissemination after the blood meal in the midgut and the salivary glands, and also analyzed the saliva. If we detected no impact in midgut virus replication, DENV-2 RNA level was higher in the presence of CHIKV in salivary glands than without CHIKV. Our *in vivo* results suggest that interactions between CHIKV and DENV viruses influence DENV replication in specific organs of *Aedes aegypti* mosquito.

## II. Methods

### Mosquito rearing

The *Aedes aegypti* Paea strain given in 1994 by Louis Malardé Institute (Tahiti, French Polynesia) has been characterized for its competence for dengue serotypes and chikungunya viruses. Adult mosquitoes are maintained at 25°C, 80% relative humidity and with 12-12h light-dark photoperiod. Adults were provided with 10% sucrose solution ad libitum. For maintained the population, the blood meal is given to females and the egg laying occurs on wet papers. 24h after the impounding of wet paper, larvae hatched, and are fed with food complement with brewer's yeast (Gayelord Hauser) and with dry cat food (Beef and Chicken) in 1 liter of tap water. Pupae are separated and collected in plastic cups into the cage and allowed to emerge.

### Virus production

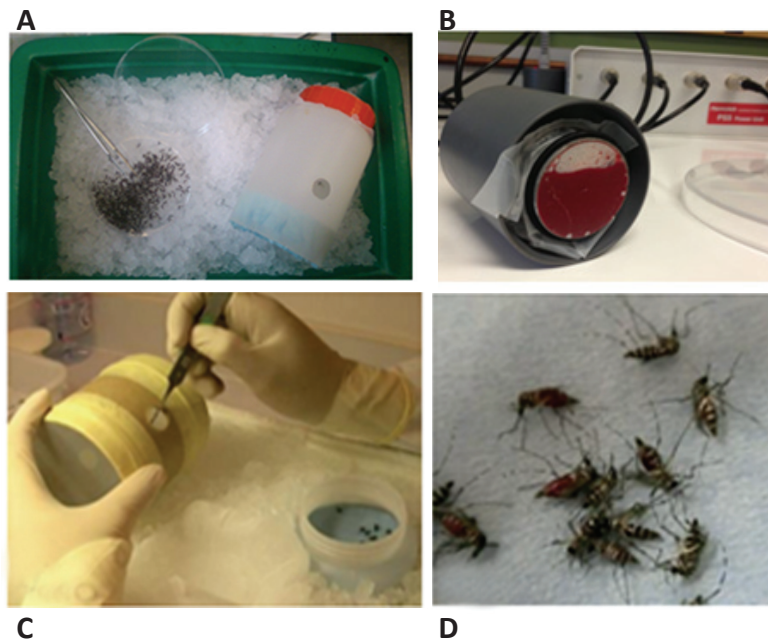
CHIKV 06.21 strain isolated from La Reunion Island in November 2005 from a newborn male presenting symptoms of meningo-encephalitis (Schuffenecker et al., 2006). The DENV-2 strain, provided by Leon Rosen (Institut Pasteur, Paris, France) was isolated in 1974 from human serum in Bangkok (Thailand) (D2BN32). Viral stocks have been amplified in *Aedes albopictus* C6/36 cell line. Cells are maintained in L-15 medium (Gibco, Invitrogen) supplemented with Amino acid non-essential (AANE), 10% SVF, and 1% PS. For chikungunya virus, the C6/36 cells were infected at a multiplicity of infection (MOI) of 0,1 and incubate at 28°C for 2 days. The DENV-2 is collected after 5 days post infection at a MOI of 0,1, and transferred onto newly non-infected cells to have a sufficient volume. After 5 days, supernatants are harvested and concentrated with PolyEthylenGlycol solution (PEG). 10ml of concentrated virus is obtained after an incubation at 4°C over night followed by a centrifugation at 3200rpm during 45min.

Virus titers are determined on VeroE6 cells cultivated with Dulbecco's Modified Eagle Medium (DMEM - Gibco) supplemented with 10% SVF and 1% PS. Serial dilution are performed and 200 µl of dilutions are used to infect cells. 72h post infection, cells are fixed in 4% paraformaldehyde for 15min. After permeabilization with 0,1% triton solution and a blocking step with 1% BSA and 0,1% Tween for 30min, cells are incubated with 4G2 monoclonal antibody diluted at 1:1000 for 1h. Following of 3 PBS

washes, the secondary antibody (anti-mouse-HRP, 1:500, BioRad) is used for 1h. Peroxidase activity is detected by using Vector Kit (Vector).

### Oral infection of mosquitoes with CHIKV and DENV-2

*Aedes aegypti* mosquitoes were infected sequentially with CHIKV and DENV-2 viruses. 7 days post emergence, and 24h prior the blood feeding, female mosquitoes are prepared and were conditioned as 60 individuals per plastic jar and deprived of sugar (Figure 34A). Blood rabbit is washed three times with PBS to prepare the infectious blood meal, deprived of plasma. The infectious blood meal was comprised of two-thirds washed rabbit erythrocytes, one third viral suspension, ATP (as a phagostimulant) at a final concentration of  $5 \times 10^{-3}$  M and viral titers of  $5.10^8$  PFU/ml (Figure 34B). Mosquitoes are in contact with infectious blood through cellulose membrane during 15min and bloods fed mosquitoes were separated using a cold table, transferred to clean cartons and maintained as noted above (Figure 34C-D).



**Figure 34. Preparation for oral infection of *Aedes aegypti* mosquito with cellulose membrane and selection of blood fed mosquitoes.**

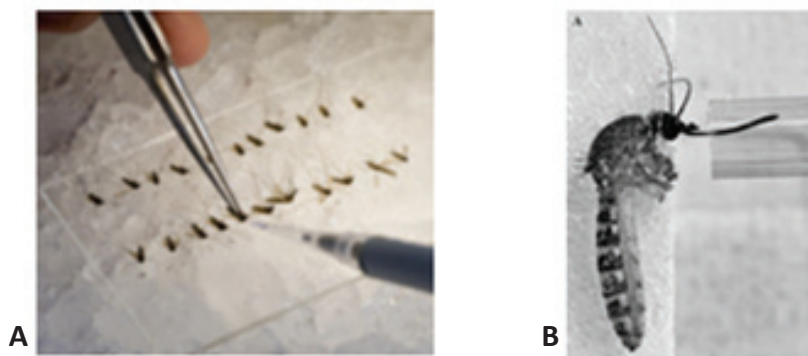
Females' mosquitoes were selected and transferred into clean plastic jar on ice (A). The infected blood meal is mediated on a cellulose membrane with hemotek system heating at 37°C (B). The blood meal takes not more than 15 minutes per plastic jar. After the blood meal, mosquitoes were put at 4°C to sleep and engorged females with a red belly (D) are selected and transferred into clean cartons (C and D).

## Dissection

Midgut and salivary glands were dissected at different time point after the blood meal, 3, 7, 10 and 14 days post feeding. Placed in tubes containing 350  $\mu$ l RA1 buffer and 3,5  $\mu$ l of Bmercaptoethanol (BME). After grinding, the RNA extraction was conducted using Nucleospin RNAII Kit advices (Macherey Nagel).

## Salivation

10 days after the blood meal, mosquitoes were anaesthetized in cold table and the saliva is collected (Figure 35A). The proboscis was introduced into tips with 10  $\mu$ l of L-15 medium for 25 min (Figure 35B). Among this 10  $\mu$ l, 2  $\mu$ l were adding to 2  $\mu$ l of lysis buffer RIPA (RadioImmunoPrecipitation Assay, Tris pH8 20mM, NaCl 150 mM, EDTA 2mM, NP40) to detect the saliva by Dot Blot. The 8  $\mu$ l reminders will be analyze by RT-qPCR after RNA extraction. Mosquito body was also isolated and grinded in 350  $\mu$ l RA1 buffer and 3,5  $\mu$ l of BME.



**Figure 35. Salivation method of *Aedes aegypti* mosquitoes.**

Females mosquito are disposed on glass slide (A) and proboscis was delicately introduced into tips containing 10  $\mu$ l of culture medium (B). Legs and wings were previously removed to facilitate the procedure. After 30 minutes, volume in the tip potentially containing the saliva is disposed in tubes with RIPA buffer to analyze.

## Dot Blot

1  $\mu$ l of saliva mixed with RIPA buffer is put on a PVDF membrane. Salivary glands extracts were used and L-15 medium as positive and negative samples, respectively. After 1h with a blocking solution composed of PBS-5% milk, an antibody against saliva of *Aedes* mosquitoes (rabbit) were added over night at 4°C. The secondary antibody diluted in PBS-5% milk (anti-rabbit HRP) was then incubated 1h, after 3 washes in PBS – 0,01% Tween 20. The peroxidase activity was detected by using ECL Pierce substrate (Invitrogen).

### **DENV-2 and CHIKV quantitative real time RT-PCR (Q-PCR)**

Total RNA from mosquitoes or MGs was extracted using the Nucleospin® RNA II kit (Macherey-Nagel) according to the manufacturer's instructions. RNA was eluted in 40 µl RNase-free H<sub>2</sub>O by centrifugation at 11,000 g for 1 min.

One-step reverse transcription quantitative PCR RT-q PCR (RT-qPCR) was performed using the Power Sybr Green RNA-to-Ct one step kit (Applied Biosystems). The CHIKV primers were selected in the E2 structural protein regions: sense Chik/E2/9018/+ (CACCGCCGCAACTACCG); anti-sense Chik/E2/9235/- (GATTGGTGACCGCGGCA). The DENV-2 primers were selected as described in Lanciotti et al., 1992: sense CGCCACAAGGGCCATGAACAG, antisense (TCAATATGCTGAAACGCGCGAGAAACCG). RT-qPCR was performed using a Fast-Real-Time PCR Systems 7500 with 7500 v.2.0.1 software (Applied Biosystems). Briefly, 0,08 µl of enzyme, 5 µl of RT-PCR mix, 0,1 µl of each primer composed the premix. 2 µl of samples had been used in duplicate. Reactions were performed in a microseal 384 microplates covered with optically clear caps, and placed in a Applied Biosystems 6 Flex cycler. Settings were: 48°C for 10 min, followed by the inactivation of DNA polymerase a 95°C for 10 min. 40 cycles of 95°C for 15 sec, 60°C for 1 min, and 95°C for 20 sec for fluorescence measurement. A time of 20 min was added to ascertain the melting curve, in order to confirm product specificity.

Synthetic RNA transcripts for CHIKV and DENV-2 were generated to construct a standard curve. Signals were normalized to the standard curve using serial dilutions of synthetic RNA transcripts.

### III. Results

#### 1. *Aedes aegypti* mosquito susceptibility to sequential blood meal

The first challenge of this study was to realize a sequential blood meal. The efficiency of the first blood meal had been optimized, by controlling the composition of the medium of the viral suspension. It had been observed that a medium without serum and without additives increase the percentage of engorged females, 16,6 to 37,5% between the two trials. However, the efficiency of the second blood meal for females that had already received the first one was still weak, around 17% (Table 6). This result indicates that a second blood meal performed after four days following the first one is limited. Moreover, during the second blood meal, it has been observed that the amount of blood ingested is lower in condition of females pre-infected with CHIKV than in non-infected condition (data not shown).

	1 <sup>st</sup> blood meal	2 <sup>nd</sup> blood meal	
	CHIKV (%)	DENV-2 (%)	CHIKV + DENV-2 (%)
<b>Trial 1</b>	240 (16,6)	80 (26,6)	20 (16,6)
<b>Trial 2</b>	540 (37,5)	180 (50)	65 (17)

**Table 6. Engorged female portions and rates (%) during the first and the second blood meal.**

1440 females are exposed with the first blood meal for both trials. For the second blood meal, 80 and 180 mosquitoes are exposed to DENV-2 (engorged females). The percentage of engorged females has been increased in the second trial, from 16,6% to 37,5% (CHIKV) and from 26,6% to 50% (DENV-2). The percentage of secondary engorged mosquitoes pre-exposed with CHIKV is still low, close to 17% for both trials.

In addition, for both blood meals, three engorged females have been collected and the viral loads have been analyzed to control the presence of virus in mosquitoes after ingestion. For CHIKV, both trials used a blood meal at a titer of  $3.3 \times 10^8$  FFU/ml. We are able to detect  $5.7 \times 10^4$  and  $5.6 \times 10^4$  copies RNA/mosquito. All of the three mosquitoes were positive for CHIKV detection (Table 7). In contrast, for DENV-2, only one mosquito present DENV-2 positive detection for the first trial, with a titer of  $2.3 \times 10^4$  copies RNA/mosquito. The titer of the blood meal was  $3.3 \times 10^7$  FFU/ml. For the second trial, the increase of the viral load in blood meal ( $1.7 \times 10^8$  FFU/ml) allows to increase the percentage of infected mosquito and the viral load detected in abdomen after ingestion ( $2.1 \times 10^5$  copies RNA/mosquito).

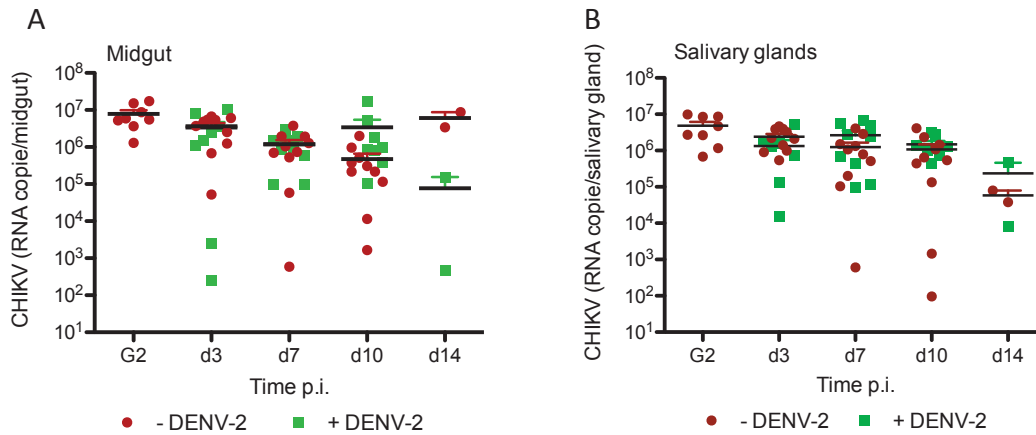
	CHIKV		DENV-2	
	copies RNA/mosquito	N and %	copies RNA/mosquito	N and %
<b>Trial 1</b>	$5.7 \times 10^4$	3 - 100	$2.3 \times 10^4$	3 - 33,3
<b>Trial 2</b>	$5.6 \times 10^4$	3 - 100	$2.1 \times 10^5$	3 - 100

**Table 7. Infectious particles in abdomen of engorged females after blood meals.**

For both trials, the CHIKV titer was at  $3.3 \times 10^8$  PFU/ml, whereas the titer of DENV-2 was at  $3.3 \times 10^7$  FFU/ml for the first trial and at  $1.7 \times 10^8$  FFU/ml. For CHIKV and DENV-2 blood meal, 3 females had been collected and analyzed after blood meal. N refers to the total number of females tested.

## **2. Chikungunya detection in midgut and salivary glands in *Aedes aegypti***

Dissections start at the moment of the second blood meal, and have performed until 14 days after the second blood meal. At the moment of the second blood meal with DENV-2, CHIKV has already colonized the midgut, and the replication reaches the salivary glands (Figure **36A and 36B – G2**). This is in accordance with to previous study which explain the potential transmission of virus only two days after infection (Dubrulle et al., 2009). At 3 days post infection, the second blood meal seems to impact the viral load of CHIKV in midgut and in salivary glands. In some mosquitoes, the RNA load of CHIKV decreases in compare to a mono-infection (Figure **36A and 36B – d3**). The titer decreases from  $10^7$  to  $10^3$  RNA copies in midgut and from  $10^6$  to  $10^4$  RNA copies in salivary glands. This effect is not observed for the following days. The viral load decreases naturally few days after infection. However, 14 dpi, the decrease is more important for the co-infection condition in midgut only (Figure **36A-d14**), rising from  $10^7$  to  $10^5$  or  $10^3$  RNA copies. These results suggest that DENV-2 infected blood meal moderately impact the CHIKV replication before 14 days in these target organs.

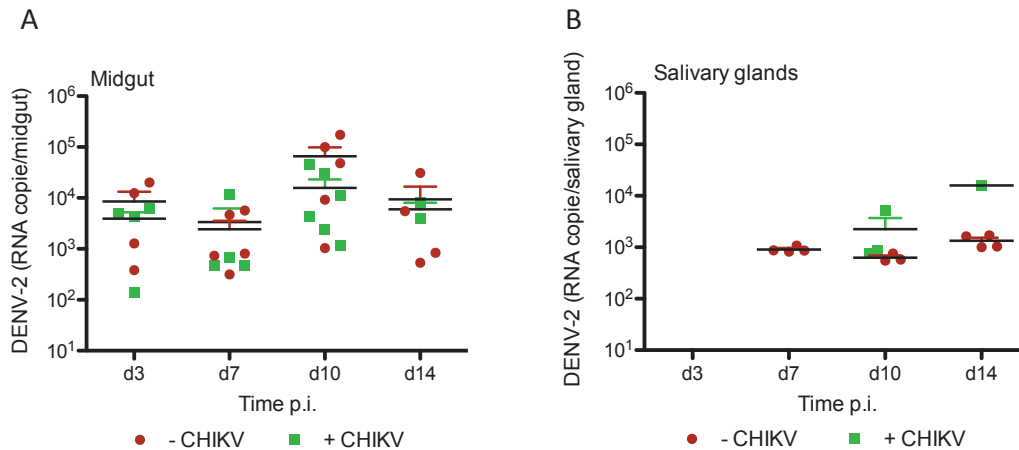


**Figure 36. CHIKV infection in *Aedes aegypti* midgut and salivary glands in co-infection or not with DENV-2.**

During the second blood meal, and every three days, 5 mosquitoes for each condition are collected, to dissect midgut and salivary glands. At day 14, only 2 surviving mosquitoes were analyzed. Collected into buffer, viral RNAs were extracted and RT-qPCR was performed to detect CHIKV RNA in midgut (A) and in Salivary glands (B). Results are reported as RNA copies per organ, and correspond of two independent trials.

### 3. DENV-2 detection in midgut and salivary glands in *Aedes aegypti*

DENV-2 viral load detection has been performed in same samples. Virus is only detected in midgut in day 3-post infection (Figure 36A). Naturally, DENV replication in mosquito is slower with a low titer in comparison to CHIKV replication. The pre-infection with CHIKV does not affect the viral replication of DENV-2 in midgut for all days analyzed (Figure 37A). The number of RNA copies reaches 10<sup>5</sup> at day 10 (d10), and then decreases. However, the effect is different in salivary glands. The DENV-2 virus is detected from 7 dpi, only for the mono infection condition. The period between the infectious blood meal and the moment where the mosquito became infectious, called extrinsic period, may vary according to environmental conditions, viral load, mosquito or virus strains (Kramer et al., 1983). At 10 and 14 dpi, the pre-infection seems to increase the DENV-2 viral load, in compare to mono infection condition (Figure 37B – d10 and d14). The number of RNA copies increase from 10<sup>3</sup> to 5 x 10<sup>3</sup> at 10 dpi, and from 10<sup>3</sup> to 10<sup>4</sup> at 14 dpi. Unfortunately, at late time, few mosquitoes are still alive, only one mosquito at 14 dpi for the co-infection condition. This high mortality limits the accumulation of data. At this stage of the study, we can only highlight an upward trend of DENV-2 RNA in co-infection condition in salivary glands.

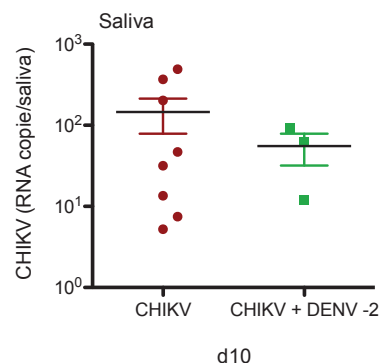


**Figure 37. DENV-2 infection in *Aedes aegypti* mosquito midgut and salivary glands following a pre-infection with CHIKV.**

On the same samples of dissected organs than used for the detection of CHIKV, DENV-2 RNA was detected in midgut (A) and in Salivary glands (B) after the pre-infection with CHIKV. Results correspond of two independent trials and are reported as RNA copies per organ.

#### 4. Saliva analyze for DENV-2 and CHIKV transmission

At 10 dpi, 10 mosquitoes in each condition were used for saliva extraction. Then, positive saliva samples have been analyzed to detect viral load for DENV-2 and CHIKV. Surprisingly, none of saliva was positive for DENV-2 in mono and co-infection conditions (data not shown). Although DENV-2 was not present in saliva, virus was in salivary glands as shown previously (Figure 36B). For CHIKV, 8 of 10 saliva possessed CHIKV particles. DENV-2 presence does not impact the viral load of CHIKV in saliva (Figure 37). However, it's important to notice the weak viral load, around 100 RNA copies/saliva.



**Figure 38. Detection of CHIKV in saliva of *Aedes aegypti* mosquito after 10 days post infection.**

10 days post infection, 10 mosquitoes per condition are disposed on glass slide and their proboscis are introduced into a tip containing 10 µl of media L-15. After testing of saliva in tube by Dot blot, RNA of positive saliva were extracted and viral RNA were detected by RT-qPCR using set of primer specific for both virus. DENV-2 was not detected in mono and co-infection conditions. CHIKV is detected in both conditions, in every positive saliva samples.

## IV. Discussion

*Aedes* mosquitoes are able to transmit a large part of arboviruses. Among them, the chikungunya virus (*alphavirus*) and the dengue virus (*flavivirus*), each one responsible of numerous outbreaks in tropical and temperate regions. Because of the enhancement of endemic areas, and the overlapping distribution of both viruses, the detection of co-infections in humans continues to increase since several decades. Observed during the recent outbreaks in Caribbean region and in Africa, investigations performed in the *Aedes albopictus* mosquito reveal that mosquito can carry both viruses and, in theory, can transmit simultaneously both viruses to a novel host (Caron et al., 2012; Vazeille et al., 2010). The *Aedes aegypti* mosquito, which is also known to transmit viruses, has been identified as the principal vector during the simultaneous outbreaks in Central America since 2013. Recently, *Ae. aegypti* were exposed simultaneously to CHIKV, ZIKV and DENV-2 and results show the possible co-infection of mosquitoes and the transmission of all combinations of viruses (Ruckert et al., 2017). However, the infection, dissemination and transmission of each virus are not impacted by co-infections. Another study demonstrates also the potential co-transmission of CHIKV and ZIKV via a unique bite after a simultaneous inoculation (Göertz et al., 2017). The originality of our study consists to investigate the sequential infection, leading to co-infection. The potential transmission in the field is largely random and all possible models of co-infection need to be investigated. Although there are no significant variations between all combinations of co-infection in previous studies performed with simultaneous exposure, the sequential infection could lead to differences in term of the viral dissemination or the transmission. The vector competence could be modified during simultaneous outbreak in favor of one virus, or could induce important severe cases due to the infection enhancement. At the cellular scale, our results indicated that pre-infection with CHIKV, promote DENV infection. All serotypes have the same increase in the presence of CHIKV. Another alphavirus member (SFV) increase also the DENV-2 infection. However, the infection with another arbovirus (VSV) did not impact by the chronic CHIKV infection. These different setting should be tested in vivo to confirm of not the generalization of the phenotype to all DENV serotypes and to SFV. Similarly, a

VSV infection may be carried in CHIKV infected mosquito to test specificity of *flavivirus* increase.

However, successive infections on *Aedes* mosquitoes are rarely performed due to the complexity of the experimentations. Firstly, time between the two blood meals was determined in function of gonotrophic cycle of mosquitoes. Blood meal triggers the egg deposition after 3-4 days. A second blood meal before this step is not efficient. We observed that the composition of infectious blood meal is crucial for an efficient sequential blood feeding and no additives should be added in virus medium. In addition, the viral load needs to be high, around  $10^8$  FFU/ml to ensure the infection by both viruses. Finally, in our setting, we observed a high mortality of mosquito, especially co-infected mosquitos. We need to evaluate more in details if the co-infection results in increased mortality due to explosive non-controlled infection in different organs or if it is a coincidence.

Two distinct organs were investigated as they are playing a key role for the vector competence in mosquito. The midgut is the first organ infected after the infectious blood meal. Viral load has to be sufficient to infect epithelium cells of midgut and to go through the epithelium barrier. The viral dissemination in secondary organs is then possible via the hemolymph.

The transmission to a novel host via the saliva is mediated by the infection of salivary glands. This organ is also crucial for the viral infection. Saliva containing viruses plays a key role for the transmission (Schneider and Higgs, 2008). With vasoconstrictants, and anti-inflammatory proprieties, injection of saliva proteins during the blood meal inhibits the vessels constriction (Ribeiro, 1987).

Our present study shows the absence of impact of the pre-infection with CHIKV on the DENV-2 replication in the midgut. This first result may be explained by the fact that the localization of replication in midgut for each virus is quite different. Indeed, DENV-2 localization in midgut is preferentially in the inferior part and rarely the superior part (Salazar et al., 2007), whereas the CHIKV infects the superior part of midgut. In this study, the tropism in midgut being different, there is no co-infection of the same cell, no interaction between CHIKV and DENV and therefore no increase in DENV infection. In order to validate this hypothesis, we need to dissect and isolate midgut of co-infected mosquito at different time points to make an immunostaining of midgut and localize CHIKV and DENV infections.

In the salivary glands, the quantity of RNA DENV-2 is higher in co-infection condition than mono-infection condition, at day 10 and 14 post infection. These results are consistent with our in vitro results previously obtained. In salivary glands, the DENV-2 replication is not detected before 10 days, as previously observed (publication in preparation) and the presence of CHIKV do not accelerate the dissemination up to salivary glands. In saliva, none contained DENV-2. However, even if the saliva did not contain DENV-2, salivary glands were infected with DENV, and in theory, could have impacted the transmission of CHIKV in saliva. However, the level of RNA CHIKV in mono and co-infection conditions is equivalent. Altogether, the CHIKV is not impacted by the second infection with DENV-2, for its dissemination into mosquito, and for its transmission via the saliva. For DENV, the conclusion is not yet clear. The complexity of experiment limits the number of mosquito per conditions. Statistics are not possible at this moment. However, a novel experiment is programmed (July 2017) in order to confirm preliminary results. In this future experiment, we will focus on key time points of co-infection: a short time (Day 3) and late time for the DENV-2 replication in salivary glands (Days 10 and 14). By doing this, the number of mosquitoes per time points will be increased and it will allow a more precise analysis of the transmission of viruses in salivary glands and in saliva.

In the long term, this study will provide the proof of concept that sequential infections with two different viruses lead also to co-infected *Aedes aegypti* mosquitoes. In addition, with this setting, the preliminary CHIKV infection modifies the outcome of DENV. In a context of enhancement of co-circulation areas of various arboviruses in the world, the outcome of this model of co-infection must be further studied in the field to design new objectives during simultaneous outbreaks, and to understand pathogens interactions in the same host.

# Experimental Report

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## Chapter 3. Identification of viral proteins implied in the promotion of *flaviviruses* infection during co-infection with *alphaviruses*

## I. Introduction

Dengue (*flavivirus*, DENV) and Chikungunya (*alphavirus*, CHIKV) viruses are two mosquito-borne arboviruses that cause human epidemics of several million serious clinical cases over the last decade (Caron et al., 2012). Because of the transmission by same mosquito vectors, *Aedes albopictus* and *Aedes aegypti*, these two arboviruses are present in the same geographical areas and co-circulations are not uncommon. Recently, with the re-emergence of the CHIKV, the number of reported cases of patients infected by both DENV and CHIKV is increasing since the first case in 1967 (Furuya-Kanamori et al., 2016; Myers and Carey, 1967). In theory, mosquitoes may simultaneously carry out both viruses, and transmit them to a novel host. In the field, mosquitoes has been identified as co-infected by CHIKV and DENV-2, and experiments in laboratory reveal the possibility of co-transmission of both viruses through the infected saliva (Vazeille et al., 2010). These co-transmission and co-infection may impact the outcome of one virus during the infection. To characterize any interference or synergy between DENV and CHIKV during replication, sequential co-infection of the *Aedes albopictus* C6/36 cell line have been performed and results have shown that the permissiveness and the production of DENV is enhanced in presence of CHIKV. On the contrary, the DENV pre-infection had no effect on subsequent CHIKV co-infection. At the cellular level, DENV and CHIKV share interactions with common cellular pathways. To date, no investigations have shown interactions between viral proteins of DENV and CHIKV during their replication in common target cells.

The CHIKV RNA genome encodes two open reading frames (ORF), with the nonstructural proteins (nsPs) 1 to 4 in 5' ORF, which are involved in the viral replication and interacted with the immune cellular pathways. The 3' ORF regroup the five structural proteins, the capsid and the envelope glycoproteins (E3, E2, 6K and E1). On the contrary, DENV RNA genome constitutes one open reading frame, which is translated as a single polyprotein. The cleavages induced by host and viral proteases lead to the formation of three structural proteins (C, prM and E) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). While the virion is formed from structural proteins, the non-structural proteins are required for synthesis

of viral RNA by forming a replication complex. CHIKV and DENV mature virions are released from infected cells by exocytosis, which depends on the host secretory pathway.

When studied independently, both viruses are able to replicate in insects and mammals. In mammals, the infection is often associated with disease and infected cells develop cytopathic effect (CPE). In insect and mammalian cells, these viruses are interacting with different cellular factors, especially with proteins of the innate immune system (Fusco et al. 2013; Yasunaga et al. 2014). The majority of studies performed in mammal cells indicates that CHIKV and DENV viruses bypassed cell antiviral strategies into cells to successfully and efficiently replicate their genome.

In comparison, the infection in the vector became persistent and nonpathogenic infection, which results in a continuous transmission of these viruses in nature. Since several decades, it is known that arboviruses persist in insect cells through the suppression and/or by evading immune defense (RNA interference, Toll, Jak/STAT and Imd/Jnk) (Lin et al. 2004). However, little is known about the interactions between CHIKV or DENV with the immune cellular pathways in mosquito vectors. The inhibition of the mosquito innate immune pathways seems to be implying in this persistence, as demonstrated for the Semliki Forest Virus (SFV) in 2007 (Fragkoudis et al., 2007). Recent RNAi screen performed in *Drosophila* cells show the key role of immune pathways activation against *alphaviruses* infection (Yasunaga et al. 2014). Moreover, the Toll and the JAK STAT pathways are certainly involved in the control of DENV infection in mosquito as well as the siRNA system (Jupatanakul et al., 2017; Xi et al., 2008).

All these antiviral strategies are activated following infection, and probably by the interaction with viral proteins with cell factors. So far, the precise molecular mechanism is still unclear.

The CHIKV nsP2 protein seems to play a crucial role in the interaction with immune response. Involving in the inhibition of the phosphorylation of the STAT factors in the Interferon response pathway, in the induction of the autophagy or apoptosis pathways, the modulation of these pathways lead to an efficient replication and dissemination of virus (Fros et al., 2010; Joubert et al., 2012b; Krejbich-Trotot et al., 2011b). The CHIKV nsP2 has been shown to induce both extrinsic and intrinsic apoptosis pathways. Found

in apoptotic blebs, CHIKV can propagate into professional phagocytosis cells as macrophages. For the dengue viruses, studies highlighted the implication of several viral proteins, NS5 has been identified to interplay in the Interferon response pathway (Green 2014) whereas NS3 and NS4A induces the autophagy process (Lee et al., 2008; McLean et al., 2011).

Our preliminary results, indicated that CHIKV infection of *Aedes* cells can increase the secondary infection by DENV. In the co-infection context, it is possible that the modulation of immune system in insect cells during the first infection could play a key role on the pattern of the second viral infection.

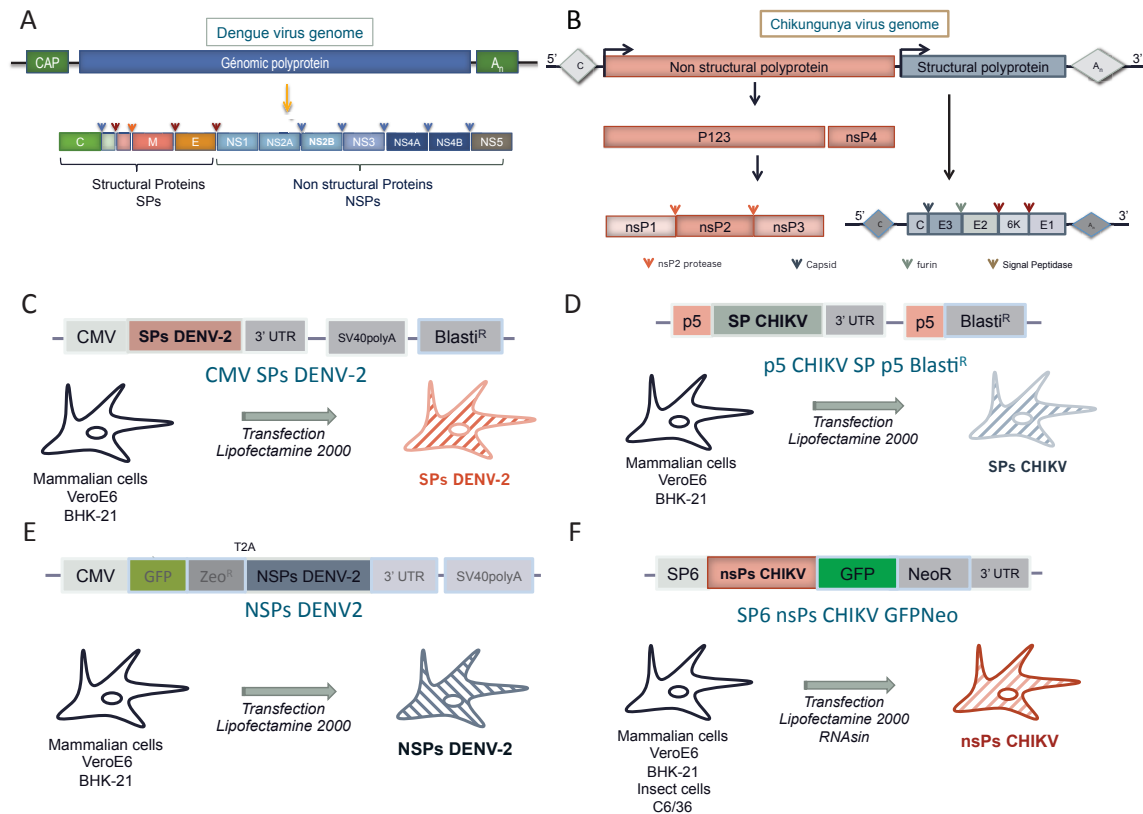
In this work, we want to explain the molecular mechanism induced during co-infection and that will explain DENV increase infection. For this aim, stable line cells were established by expressing viral proteins in insect cells. In parallel, we also made stable expression in mammalian cells. Indeed, due to CPE, sequential infection by CHIKV or DENV is not possible. Therefore, protein expression followed by virus infection is a way to generalize or not the phenotype observed in insect cells. Independently, structural and non-structural proteins (SPs and NSPs) of each virus were transfected into cells and selected during several days. Then we performed infection with the opposite virus in order to identify the proteins responsible of the previously observed effects during co-infection. In insect cells, the stable expression of CHIKV non-structural proteins allows an increase of DENV-2 infection. This preliminary result suggests the implication of one of non-structural proteins on facilitation for DENV replication, observed in previous works. Further investigations are needed to determine the role of each protein independently. In parallel, preliminary results indicated that DENV-2 non-structural proteins inhibit the initial stages of replication of CHIKV in mammalian BHK-21 cells. Altogether, a better understanding of the interaction between CHIKV and DENV, in mosquitoes and in mammals, may provide clue for the epidemiology of simultaneous outbreaks.

## II. Methods

### **Viral protein expression in mammalian and insect cells.**

African Green Monkey kidney epithelial cells (VeroE6) and Baby Hamster Kidney 21 (BHK-21) cells were grown in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal bovine serum (FBS). *Aedes albopictus* larval C6/36 cells were grown in Schneider's (Gibco, Invitrogen) and Mitsuhashi-Maramorosh medium (Bioconcept, Allschwil, Switzerland) (50%-50%) supplemented with 10% FBS. All culture media were supplemented with 10U/ml of Penicillin and 10µg/ml of Streptomycin (Gibco®, Invitrogen).

Target cells were seeded at  $1 \cdot 10^6$  cells per well in a 12-well plate and transfected with 1µg of plasmid coding DENV-2 Structural proteins (SPs DENV-2), DENV-2 nonstructural Proteins (NSPs DENV-2), CHIKV Structural proteins (SPs CHIKV) are transfected with Lipofectamine 2000 (Invitrogen) for mammalian cells and Effectene (Qiagen) for mosquito cells, according to manufacturer's protocols. For the transfection of CHIKV Nonstructural proteins (nsPs CHIKV), mammalian cells are electroporated with 10 µg of viral RNA and insect cells are transfected with 1 µg using lipofectamine 2000 supplemented with RNasin. 24h hours post transfection or electroporation, media was removed, and fresh media was supplemented with drugs for the selection of transfected cells. Zeocin (300 µg/mL) for Dengue Nonstructural proteins, blasticidin (5 µg/mL) for Dengue Structural proteins and Neomycin (500 µg/mL) for Chikungunya Non-structural proteins. Media and drugs were changed every two days until selection was achieved. Plasmid constructions are represented in Figure 39.



**Figure 39. Plasmid constructions to the expression of viral proteins in mammalian cells.**

The genome of dengue virus is composed of one open reading frame, encoding structural (pink part) and non-structural proteins (blue part) (A). In contrast, two open reading frames constitute the chikungunya genome. One encodes non-structural proteins (pink part), and the other one encodes structural proteins (blue part) (B). To express DENV-2 structural proteins, the construction from Pierson et al., was used (C). The Blastidin resistance gene allows the selection of transfected cells. To express CHIKV structural proteins, capsid, and glycoproteins genes are cloned under control of p5 promoter, (D). Both constructions were introduced into mammalian cells by using lipofectamine 2000 reagent. The DENV-2 non-structural proteins were also derived from Pierson et al (E). All of these three constructions were transfected into cells using lipofectamine 2000 reagent according to manufacturer advices. The CHIKV non-structural proteins were cloned in fusion with GFP reporter gene and Neomycin gene resistance. Proteins were under control of SP6 promoter. After in vitro transcription, RNA was transfected with lipofectamine 2000 in addition with RNasin (F). For each construction, a 3'UTR and poly A from Simian Virus 40 regions were added following the interest genes.

Transfected or transduced cell were washed and detached with ice-cold phosphate-buffered saline (PBS) and lysed in cold cell lysate buffer (20 mM TRIS pH 7.5, 1% Triton, SDS 0.05%, Na Deoxycholate Acid 0.5% and 150 mM NaCl) for western blot. Nuclei and membranes were precipitated by centrifugation at 12000 rpm and supernatants were harvested. Cell lysates were then denatured at 95°C for 5 minutes in reducing loading buffer (5X Blue Loading Buffer: 200 mM TRIS HCl pH 6.8, 10% SDS, 500 mM  $\beta$ -mercaptoethanol and 50% glycerol) and electrophoresed on a 12% SDS-polyacrylamide gel at 100V. Proteins were transferred onto a nitrocellulose membrane by electroblotting (BioRad) for 10 min at 2,5 A constant up to 25V. After saturation in TBST

(20 mM Tris-HCl, pH 7.5 150 mM NaCl, Tween 0.05%) – milk 5% for 1h, membranes were incubated with primary antibody for the detection of DENV-2 proteins (NS1-DENV-2 1:1000; E-DENV-2 1:500) and for the detection of CHIKV proteins (IgG CHIKV 1:1000; C-CHIKV 1:100) in TBST– milk 5% over night at 4°C. After three washes of 15 minutes using TBST, membranes were then blotted with horseradish peroxidase (HRP) anti-human or anti-rabbit secondary antibody at 1:5000 dilution in TBS for 1 hour at room temperature. After three washes in TBST, membranes were incubated with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). Signal was revealed with Chemidoc MP system (Biorad).

### **Viral production and infection**

DENV-2 strain 16681 isolated in 1964 from a DHF/DSS patient in Bangkok, Thailand in 1970 by Halstead (Halstead et al., 1970), Chikungunya CHIKV-LRic strain LR2006 OPY1, (Tsetsarkin et al., 2006) and Chik-mCherry (generated in the laboratory) were produced by electroporation of BHK21 cells by *in vitro* transcribed RNA derived from molecular clones.

*Alphaviruses* pseudo-particles were produced in HEK-293T cells by transfection with an equal mass (8 µg) of retroviral core MLV and reporter gene (GFP), and 1-4 µg of *alphavirus* glycoprotein gene deleted of capsid gene, using a calcium phosphate transfection protocol (Bartosch et al., 2003). 16h post transfection, media and DNA-calcium phosphate precipitates DNA complexes were washed off and replaced with DMEM-FCS 10%. Supernatants were harvested 48 h post-transfection, filtered 0.45µm, and then used to infect target cells or frozen at -80°C.

Infections with replicative virus on mammalian and insect cells are performed at two different multiplicity of infection (MOI) of 0,1 and 1 in 96 well plates. After 2h of infection, supernatants were removed for add fresh media. Analysis was performed by TCID<sub>50</sub>. *Alphaviruses* pseudo-particles are used to infect mammalian cells in 24 well plates using 2 different volumes of infection. Media is removed after 6h post infection, and cells are harvested 3 days after, resuspended in 2% paraformaldehyde (PFA) prior to FACS analysis. FACS analyzes were performed with a MACSQuant® Analyzer MACS

Miltenyl biotec cytometer using MACSQuantify™ Software. The percentage of infected cells in GFP positive cells was measured to calculate the infectious titers (IU/mL).

### III. Results

#### 1. DENV-2 proteins expression

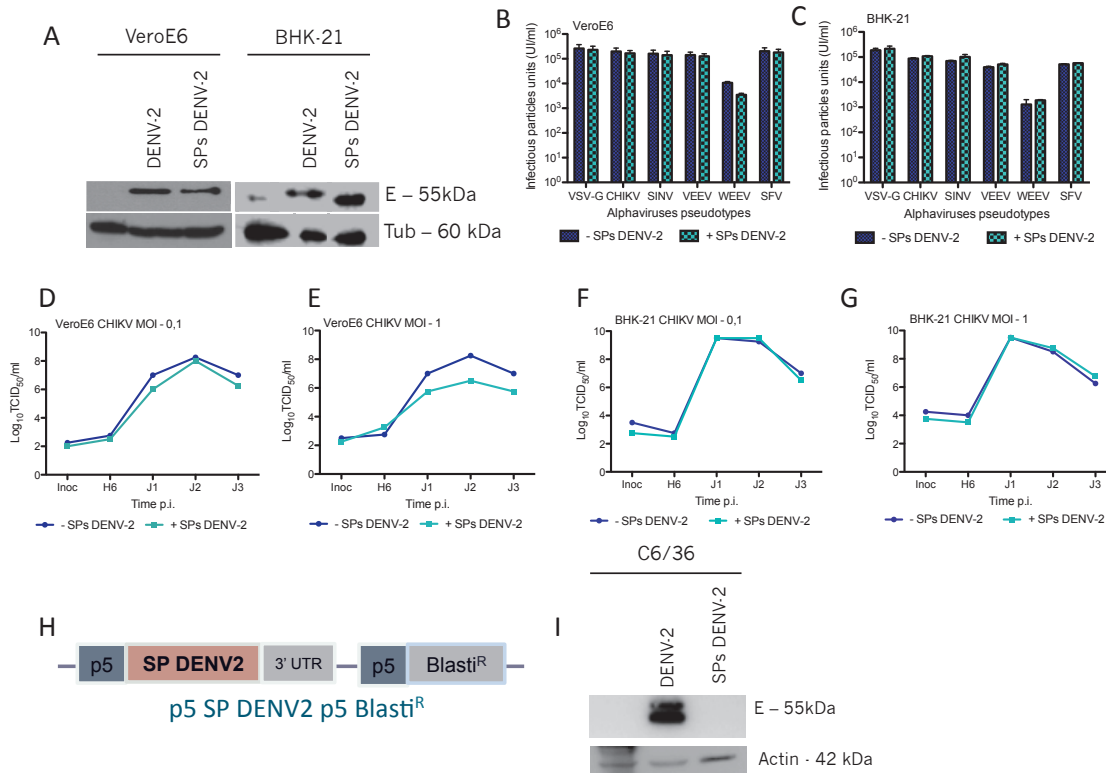
##### 1. 1. DENV-2 Structural proteins

After the transfection and selection of genes encoding structural proteins in VeroE6 and BHK-21 cells, proteins are detected by western blot. In both cell lines, E protein (55kDa) is visualized at the expected size (Figure 40A).

The entry process was firstly investigated in presence or not of DENV-2 structural proteins (SPs DENV-2). Pseudo-particles are composed of *alphaviruses* glycoprotein, anchored onto a retroviral core and they transduce a reporter gene that allows a precise quantification of the entry step. As shown in Figure 40B and 40C, the presence of DENV-2 structural proteins do not impact the *alphaviruses* pseudo-particles entry in VeroE6 and BHK-21 cells (Figure 40B and 40C). In order to test if DENV-2 SPs may have an impact of other steps of the *alphavirus* replication cycle, cells stably expressing DENV-2 SPs were infected by replicating CHIKV. Supernatants were harvested until 3 days following the infection. The expression of SPs DENV-2 in VeroE6 cells seems to limit the CHIKV replication at MOI 1 in comparison to replication in naïve VeroE6 cells (Figure 40E). At MOI 0,1, the inhibition is less significant (Figure 40D). However, this first result was not similar in BHK-21 cells (Figure 40F and 40G). No differences in CHIKV infection were observed in presence or not of SPs DENV-2 in this cell types. This discrepancy may be due to more rapid cell mortality in VeroE6 than in BHK21. More experiments will be needed to confirm the preliminary results.

The expressions in mammalian and in insect cells are very different. Many tests were performed in order to find the most efficient expression in insect cells, and the promoter p5 from parvovirus was selected. This promoter is efficient and it is smaller than the actin 5 promoter from *Drosophila* allowing cloning of bigger fragments. The CMV promoter was substituted by P5 promoter and the blasticidin resistance gene allowed the selection of transfected cells (Figure 40H). Expression of DENV-2 Structural proteins

in C6/36 cells lines had been analyzed after transfection and selection (Figure 40I). However, unfortunately, up to now, no expression was detected by western blot.



**Figure 40. Structural proteins of DENV-2 expression in mammalian cells and impact on CHIKV entry and replication.**

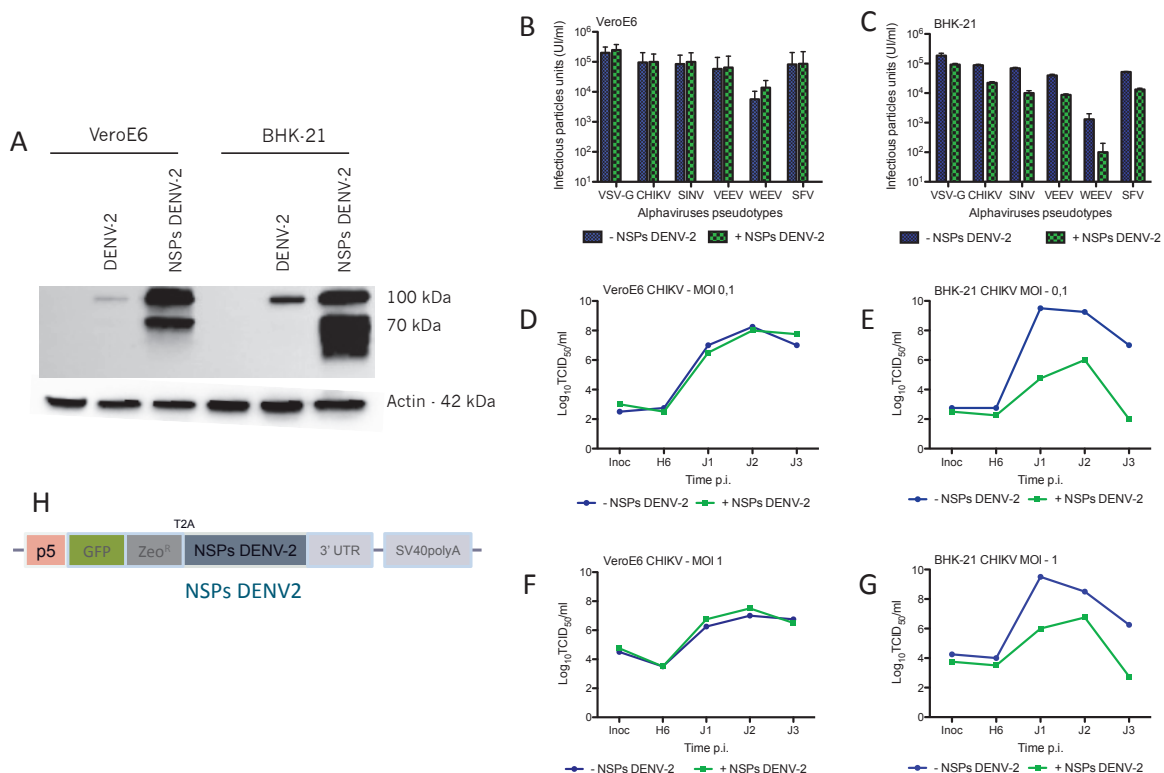
After transfection into mammalian cells (VeroE6 and BHK-21), the detection of viral proteins (E protein, 55kDa) were performed by western blot (A). DENV-2 infectious cell lysate was used as positive control. Cells lines expressing SP-DENV-2 are represented in lighter blue color, in compare of wild type cell lines in darker blue color. *Alphaviruses* pseudoparticles produced into 293T as previously described (Bartosch et al., 2003) were used to investigate the impact of expression of SP DENV-2 on the entry process in VeroE6 (B) and BHK-21 cells (C). Growth curve experiments in VeroE6 cells and in SP DENV-2 VeroE6 cells (D and E) and in BHK-21 cells and in SP DENV-2 BHK-21 (F and G) with CHIKV at the indicated MOI. Inoculum and supernatants were harvested from 6H to day 3 p.i., and the viral titers were obtained by limiting dilution assays in VeroE6. Results are reported as 1,5 log<sub>10</sub> (TCID<sub>50</sub>/ml). No differences were observed for the entry of the replication of CHIKV into cells expressing SP DENV-2 in comparison of wild type cell types. In order to express viral proteins in insect cells, the CMV promoter have to be replaced by parvovirus p5 promoter (H). The blasticidin resistance gene under the same promoter lead to the selection of cells expressing viral proteins. After transfection into insect cells (C6/36), the western blot does not allow the detection of the E DENV-2 protein (I), in contrast of DENV-2 infectious cell lysate used as positive control.

## 1. 2. DENV-2 Non Structural proteins

Following similar protocols than for DENV-2 SPs, non-structural proteins were expressed in VeroE6 and BHK-21 cells. After selection, the viral proteins expression was analyzed by western blot as shown in Figure 41A. The stable expression of DENV-2 NSPs is strongly suggested by the positive detection of NS1 (homodimer around 80 kDa). In order to test if the stable expression of DENV-2 NSPs may influence *alphavirus*

entry, cells were infected with different *alphavirus* pseudotypes. While the presence of DENV-2 NSPs in VeroE6 had no impact on *alphaviruses* entry (Figure 41B), the entry of CHIKV, SINV, VEEV, WEEV and SFV pseudo-particles is decreased in BHK-21 cells (Figure 41C). The effect of DENV-2 NSPs on the whole cell cycle of *alphavirus* was then tested with wild type viruses. The replicative CHIKV virus is also drastically decreased with two different MOI 0,1 and 1 (Figure 41E and 41G) in presence of NSPs DENV-2 in BHK-21 cells, but not in VeroE6 cells (Figure 41D and 41F). These results, to be confirmed, suggest that NSPs DENV-2 may have a cell type specific effect and they inhibit early step of the CHIKV entry/replication only in BHK-21 cells.

To express NSPs DENV-2 in insect cells, CMV promoter had to be replaced by parvovirus p5 promoter, efficient in insect cells, as shown in Figure 41H. The size of plasmid is consistent and the construction is still ongoing. Based on results in mammalian cells, the presence of NSPs DENV-2 in insect cells may influence the CHIKV replication steps.



**Figure 41. Non Structural proteins of DENV-2 expression in mammalian cells and impact on CHIKV entry and replication.**

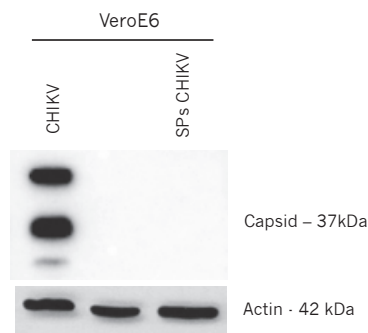
Transfected into VeroE6 and BHK-21, genes encode for non-structural proteins lead to their expression as reveal the staining of NS1 protein by western blot (A). DENV-2 infectious cell lysate was used as positive control. Cells lines expressing SP-DENV-2 are represented in blue color, in compare of wild type cell lines in green color. As described previously for the cells expressing SP DENV-2, *alphaviruses* pseudoparticles were used to analyze the impact on the entry process of NSP DENV-2 with VeroE6 (B) and BHK-21 (C), and growth curve experiments with CHIKV at the indicated MOI in VeroE6 and BHK-21 cells expressing NSP DENV-2 (D-E-F-G). Inocula and supernatants were

harvested from 6H to day 3 p.i., and the viral titers were obtained by limiting dilution assays in VeroE6. Results are reported as 1,5 log<sub>10</sub> (TCID<sub>50</sub>/ml). No differences were observed for the entry of the replication of CHIKV into VeroE6 cells expressing NSP DENV-2 in comparison of wild type cell types (D and F). In BHK-21 cells, expression of NSP DENV-2 leads to a reduction of CHIKV replication, with two MOI (E and G). Experiment was performed only once and need to be reproduced to confirm results. In order to express viral proteins in insect cells, the CMV promoter have to be replaced by parvovirus p5 promoter in the plasmid (H).

## 2. CHIKV proteins expression

### 2. 1. CHIKV Structural proteins

Structural proteins of CHIKV (SPs CHIKV) were firstly expressed in VeroE6 cells. Proteins were under control of a promoter p5 from parvovirus, efficient in both mammalian and insect cells. However, no detection of viral protein was observed by western blot, as shown in Figure 42.



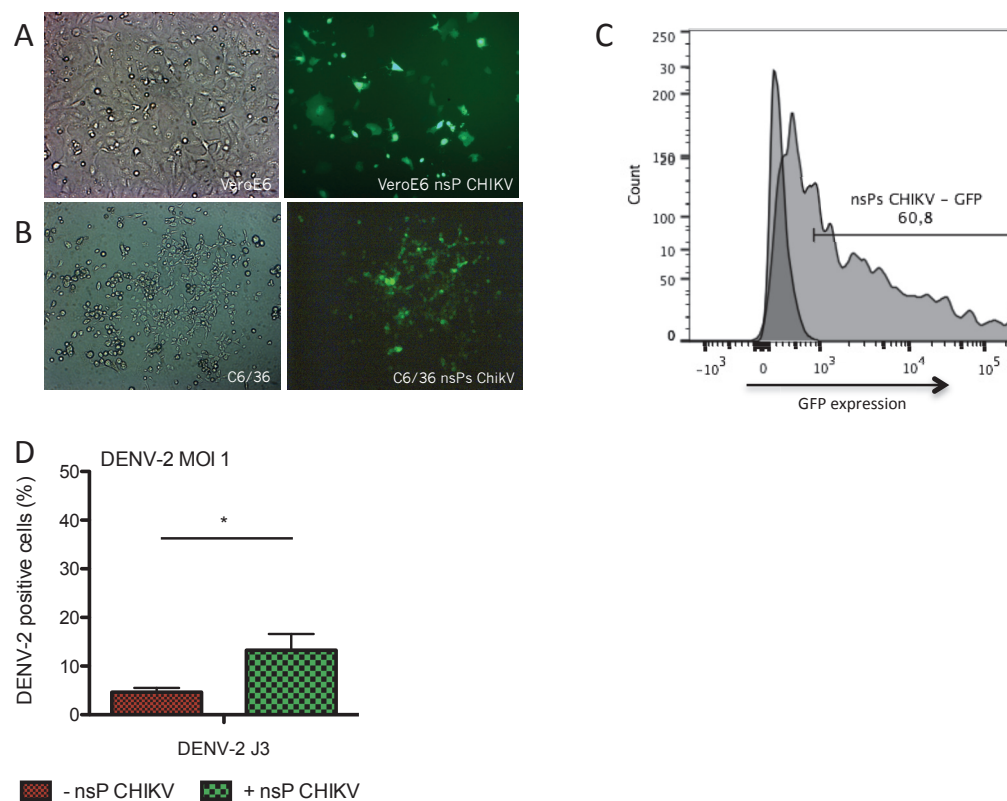
**Figure 42. Structural proteins of CHIKV expression in mammalian cells.**

Plasmid containing SP CHIKV under the control of parvovirus p5 promoter was transfected into mammalian cells (VeroE6), and cells were lysed to detect viral proteins by western blot. Using an antibody against Capsid protein (37 kDa), none protein is detected, in compare of CHIKV infectious cell lysate used as positive control.

### 2. 2. CHIKV Non-structural proteins

In order to express CHIKV non-structural proteins (CHIKV NSPs) we generated a replicon with a SP6 transcription promoter. RNA has been transcribed *in vitro* and transfect in mammalian (VeroE6) and insect (C6/36) cells. The visualization of reporter gene GFP by microscopy confirmed the good expression of the GFP proteins and indirectly of the viral proteins (Figure 43A). The selection of transfected mammalian cells was not possible. It was not surprising, as, in mammalian cells, nsP2 gene is known to induce cytopathic effect, impeded the stable expression of viral proteins. The super-infection with DENV-2 on these cells was not possible, considering the state of cells.

Contrary to mammalian cells, cytopathic effect was not observed in insect cells. After few weeks, the selection of transfected C6/36 cells allows to detect 50% of cells which express GFP protein as shown by microscopy (Figure 43B) and by flow cytometry (Figure 43C). It is possible that after a few cycles, the replicon excludes the GFP protein, which prevent to reach 100% of cells expressing CHIKV NSPs. Western blot will be performed in the future to detect non-structural proteins when antibody will be available in the laboratory. Super-infection with DENV-2 was performed and infected cells were stained 3 dpi. As shown in Figure 43D, the presence of nsPs CHIKV in insect cells lead to the increase of DENV-2 infection. DENV-2 infects 5% of C6/36 cells and the presence of nsPs CHIKV in C6/36 cells enhances the infectivity to 20%. This preliminary result suggests that nsPs CHIKV are implied in the previously observed effect during co-infection in insect cells. To go further, independent proteins should be expressed in cells in order to investigate the impact of each protein.



**Figure 43. Non-structural proteins of CHIKV in mammalian and insect cells and super-infection with DENV-2.**

RNA encoding CHIKV Non-structural proteins were transfected into VeroE6 and C6/36 cells (A and B). Because of the induction of cytopathic effect of nsP2 protein in mammalian cells (A), the selection of transfected cells leads to the death. The efficiency of transfection in VeroE6 was observed 48h post transfection. The transfected insect cells were observed by microscopy (B) and by flow cytometer (C). After selection with Neomycin drug, C6/36 cells expressing CHIKV nsPs were infected with DENV-2. 3 dpi, cells were harvested are stained with E DENV-2 antibody. The percentage of infected cells was determined by flow cytometry, and reported as percentage of positive cells (D).

## IV. Discussion

Transmitted by the same vector, the *Aedes* mosquito, chikungunya and dengue viruses (*alphavirus* and *flavivirus* families respectively) are considered as two pathogens causing important outbreaks in the world since several decades. During simultaneous outbreaks, viruses induce similar symptoms and, specific diagnoses were for a long time-limited. Reported co-infection cases are increasing but the correlation between co-infection and severe cases is still not clear. However, human and mosquito co-infection can in theory induce a modification of virulence, as well as of vector competence. Previously, *in vitro* works highlighted the enhancement of infection of *flaviviruses* in mosquito cells pre-infected with *alphaviruses* (CHIKV and SFV). The *flaviviral* production was also increased in presence of these *alphaviruses*. *In vivo* experiments on *Aedes aegypti* mosquito confirmed these *in vitro* results. In salivary glands, the sequential infection with CHIKV and DENV-2 lead to an enhancement of DENV-2 replication. This observation suggests the potential promotion of DENV-2 transmission during simultaneous outbreaks. However, all these results do not explain the molecular mechanism under the process observed. Some *alphaviral* proteins in collaborative work with cellular pathways probably induce the enhancement of DENV-2 replication and production. To try to identify any possible mechanism, the CHIKV genome had been separated in two parts, to produce separately structural and non-structural proteins. Although the expression of structural proteins is not yet established, the expression non-structural proteins of CHIKV in C6/36 cells lead to an enhancement of DENV-2 infected cells. In this setting, all non-structural proteins are expressed and the genome is also replicating. Both can induce the DENV increase. However, in the literature the nsP2 is known to be multifunctional. It is involved in the viral RNA replication, the host cell shutoff and the inhibition of antiviral response in insect and mammalian cells. A large proportion of viral protein is localized in the nucleus, and the conserved C terminal domain of nsP2 is involved in the suppression of interferon signaling (Breakwell et al., 2007; Fros et al., 2013). The nsP2 is also involved in the shutoff of the unfolded protein response (UPR). Identified as the cellular response against high concentration of misfolded proteins in the ER, this process regulate the production of several proteins involved in the degradation and the apoptosis pathway (Kohn, 2010). The individual expression of nsP2 is sufficient to inhibit the UPR pathway, and another study reveals

the suppression of this pathway by the CHIKV nsP4 protein (Rathore et al., 2013). Finally, nsP2 is also known to be important for autophagy that is necessary for both CHIKV and DENV replication. Therefore, different functions of nsP2 (mainly counteracting of interferon and induction of autophagy) may explain the increase of DENV replication. In addition, employed by insect against *flaviviruses* and *alphaviruses*, the RNA interference pathway is a major antiviral defense mechanism resulting in the degradation of viral RNA. Viruses develop strategies, as the encoding of viral suppressor of RNAi (VSRs) to counteract the pathway. In 2016, Mathur et al., identified the importance of nsP2 and nsP3 in the exhibition of RNAi suppressor activity in insect cells, and in mosquitoes. Altogether, the expression of viral proteins after CHIKV infection leads to a suppression of antiviral responses with a balance to maintain cells alive. All these modifications following CHIKV infection are appropriated for an efficiency and rapid replication of DENV and *flaviviruses*. However, nsP2 is toxic alone and it is difficult to test this hypothesis. We may generate different mutants that lost some cell functions to test nsP2 influence.

The hypothesis of direct interaction with CHIKV and DENV proteins may also be investigated. Some viral proteins of CHIKV and DENV co-localized at the endoplasmic reticulum during the life cycle of viruses. Both viruses use intracellular organelles, and potential interactions between proteins are still undetermined. However, the budding process is different for each virus, the assembly occurs on the surface of the ER for DENV particles, and at the plasma membrane for CHIKV virion (Martín et al., 2009). In order to test this hypothesis, we will express individual proteins or part of them to test any heterologous incorporation or formation of hybrid particles.

Even if we did not found mammal cells where we can achieve CHIKV or DENV chronic infection, we initiate some study to evaluate any interaction between CHIKV and DENV in mammal cells. In our study, expression of DENV proteins in mammalian cells revealed that the non-structural proteins inhibit the early step of CHIKV replication, and the replication in BHK-21 cells. However, previous co-infections work in insect cells reveal that the pre-infection with DENV-2 does not affect the CHIKV replication. Competitive effects between structural and non-structural proteins, resulting in a neutral effect during co-infection experiments, could explain these contradictory results. Another explanation can be that the effect is species dependent. Therefore, expression of nonstructural DENV proteins in insect cells need to be achieved. Also activated after the

DENV infection, the RNA interference pathway is counteracted by the action of DENV NS4, acting as an RNAi suppressor (Kakumani et al., 2013). A common pathway could be an enhancer for DEN viruses. Mutations on NS4 to affect the RNA interference regulation could reduce differences of permissiveness and viral production observed in mono and dual-infection.

In conclusion, the CHIKV infection, with expression of viral proteins, mobilized complex mechanisms for its own efficient replication, and this mobilization is beneficial for a secondary infection with *flaviviruses* (DENV serotypes and YFV). The implication of non-structural proteins in this co-infection process needs still to be determined.

## General Discussion

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Nowadays, with the improvement of modern genetic technics, it is important to consider the pathosystem in host as the resultant of the partnership between various pathogens in a given host. Acting in synergy or in interference, pathogens interactions may conduce to the modification of parameters for pathogens and for hosts. The infection competence, the tropism or the capacity of dissemination of pathogen is potentially influenced in multiple infection condition. In evidence, the host pre-infected with a first pathogen, will not respond in the same way compare to a non-infected host. Clinical manifestations may be attenuated or on the contrary, accentuated, causing severe symptoms, which could be lethal for individuals. In the context of arboviruses transmitted by *Aedes* mosquitoes, the potential of co-infection both in human and in vector is non-negligible. Mosquitoes, which have the capacity of transmitting several viruses independently, in the case of overlapping geographic distribution of pathogens and in the case of simultaneous outbreaks, can co-transmit many pathogens. Independent studies reveal simultaneous outbreaks in endemic regions notably for *flaviviruses* (DENV, YFV) and *alphaviruses* (CHIKV) (Caron et al., 2012; Leroy et al., 2009; Manore et al., 2014). Frequently, co-circulation leads to co-infections in vectors, *Aedes albopictus* and *Aedes aegypti*, and in humans. Certainly link to the improvement of diagnosis methods, co-infections in human are in a constant augmentation during latest 20 decades (Furuya-Kanamori et al., 2016). However, still very few studies reveal the co-infection in mosquito.

During this PhD work, sequential infections with two arboviruses causing recent outbreaks have been performed firstly in mosquito cells, and secondly in insect using the *Aedes aegypti* mosquito. The results of in vitro experiments are represented schematically in Figure 44. We performed sequential co-infection in *Aedes albopictus* but also *Aedes aegypti* cell lines. After a chronic infection with a first virus, cells are infected with the second virus. The permissiveness and the viral production are analyzed during several days post infection. We found that the permissiveness of DENV-2 is enhanced in presence of CHIKV in cells (Figure 44, co-infection CHIKV-DENV). Three different cell types were used, C6/36 and U4.4 cells from *Aedes albopictus*, and Aag2 cells from *Aedes aegypti*. The titers of DENV virus are increased in the presence of CHIKV. In parallel, the viral RNA in C6/36 cells was extracted and the DENV-2 RNA is also increased in co-infection condition. The CHIKV titers or RNA is not affected by a secondary infection with DENV-2.

The replication of CHIKV is very rapid and the antiviral responses of cells are mobilized to control infection. The enhancement of DENV may be induced by the decreased antiviral defense, or by the activation of cellular pathways beneficial for an active DENV replication. We know that CHIKV and DENV both interplay with RNA interference pathway, but also with Imd pathway (Avadhanula et al., 2009; Kakumani et al., 2013; Mathur et al., 2016) or autophagy.

Viral proteins have a key role in the activation of antiviral pathways during infection. The CHIKV non-structural protein 2 (nsP2) is involved in the viral RNA replication, but also in the host shutoff and the inhibition of antiviral response. In mammalian cells, this protein localizes in a large proportion in the nucleus, and affects the Interferon response (Breakwell et al., 2007). The C-terminal domain seems to be crucial for this repression (Fros et al., 2013). The unfolded protein response (UPR) is also regulated by nsP2 protein. This cellular defense mechanism is developed to degrade high concentration of misfolded proteins in the endoplasmic reticulum (ER), and to limit viral protein expression. Experiments on nsP2 have shown that this protein was sufficient to inhibit the UPR (Fros et al., 2015). However, the host shutoff is well described in mammalian cells, the process could differ in insect cells.

The most important antiviral pathway known in insect is the RNA interference response, resulting in the degradation of viral RNA in cytoplasm. With evolution, viruses develop strategies to counteract RNAi pathway, by encoding viral suppressor of RNAi (VSRs). Again, nsP2, with nsP3 were found to enhance the RNAi suppressor pathway (Mathur et al., 2016). Altogether, the non-structural proteins, with nsP2, seem to play a crucial role in the regulation of anti-viral responses in mammalian and insect cells, in addition to promote the RNA replication.

Also activated after the DENV infection, the RNA interference pathway is counteract by the action of DENV NS4, acting as a RNAi suppressor (Kakumani et al., 2013). A common pathway could be an accelerant for DEN viruses.

To go further in the characterization of co-infection in mosquito cells, the third part of the project was to determine the molecular process involved in the enhancement of *flaviviruses* by the pre-infection with *alphaviruses*.

Plasmid constructions were generated to express CHIKV structural and non-structural proteins independently in insect cells. This experiment needed many developments as very few tools are available to study proteins in mosquitos. Only CHIKV nsP were expressed but interestingly, they promoted DENV infection. This result suggests that one non-structural protein or complex of proteins or the replication of CHIKV genome, influence one or more cellular pathways in favor of DENV replication, as described previously.

The acceleration of DEN replication in insect cells result in the enhancement of DEN particles in supernatants detected by titration and by RT-qPCR. If the number of release particles is increased, and more infectious, the number of infected cells will be also increase.

During the second CHIKV infection in chronic DENV-2 infected C6/36 and U4.4 cells, the numbers of CHIKV and DENV-2 RNA levels in cells and numbers of infected cells are stable in both mono and co-infection conditions (Figure 44, co-infection DENV-CHIKV). In contrast, while the CHIKV RNA level is also stable in supernatant, the RT-qPCR specific to DENV-2 reveals that lower quantity of RNA is detected in co-infection condition, these results indicate a retention of DENV-2 particles, probably due to a competition for components of the exocytosis pathway by CHIKV, such as the protein beta-1 tubulin. Exocytosis requires the proteins of the cytoskeleton and investigations in *Aedes aegypti* mosquitoes reveal that the protein beta 1 tubulin involved in the cytoskeleton is down regulated by CHIKV infection. Also used by DENV to ensure its entry into mosquito cells (Paingankar et al., 2010).

Although the number of particles is reduced by the presence of CHIKV in cells, the titration of supernatant to measure the infectivity of particles reveal an enhancement in presence of CHIKV. This enhancement of infectivity could be due to a better maturation of virion by the furin protease. Indeed, both viruses used the cellular protease for the maturation of their virion. The furin has been identified to cleave the pE2 precursor leading to the formation of the CHIKV E2 and E3 proteins (Klimstra et al., 1999). DEN viruses exist in variants depending on the degree of precursor membrane prM protein cleavage. Immature particles containing prM proteins are non-infectious, while the

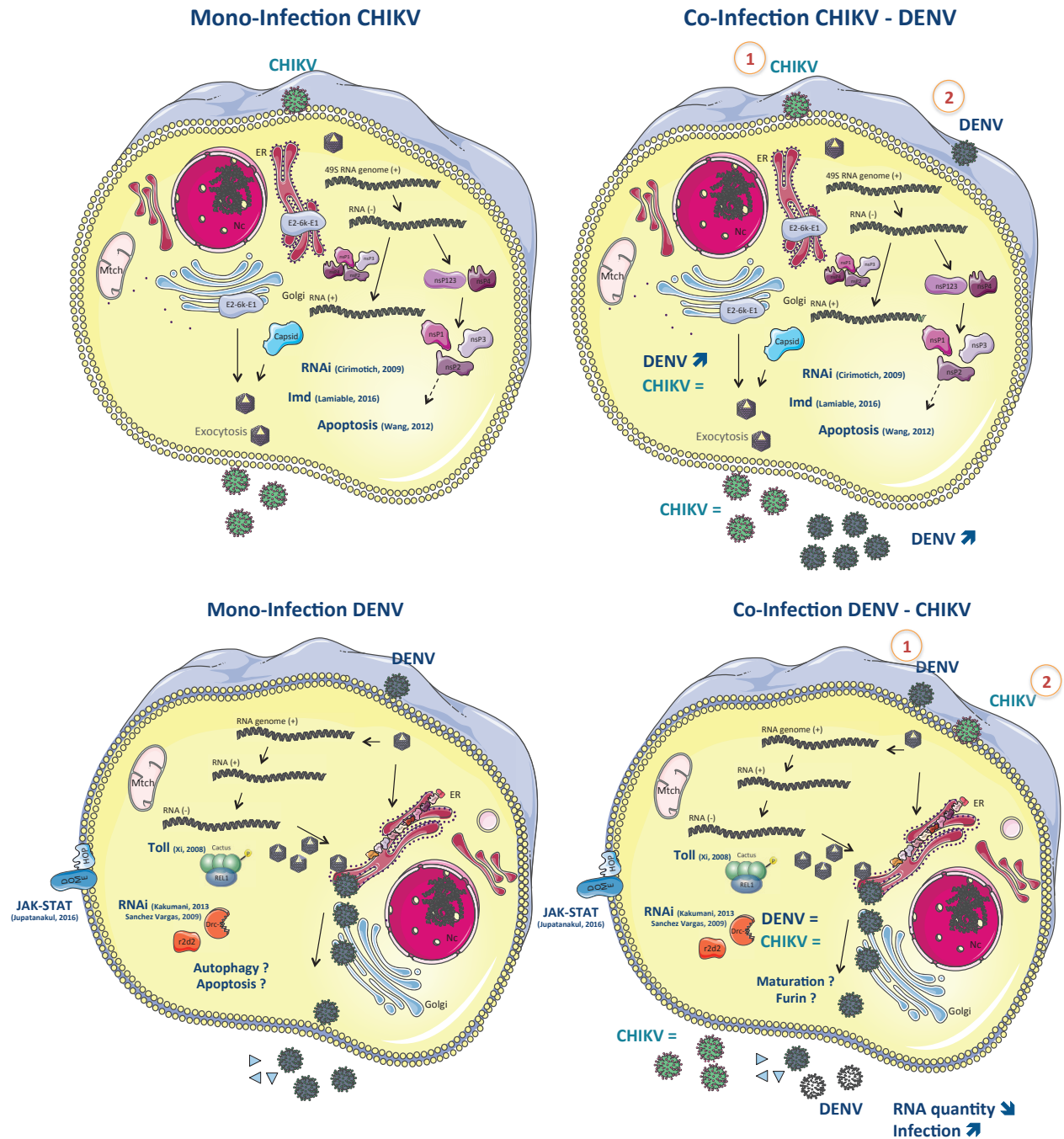
cleavage of prM proteins lead to maturation of infectious particles (Junjhon et al., 2008). The maturation occurs in the trans-Golgi network with an acidification inducing conformation changes and exposition of furin cleavage sites, between pr and M proteins, at the surface of the virion (Pierson and Diamond, 2012).

During co-infection model, the fast replication of CHIKV could induce the biosynthesis of proteins such as furin, which is favorable for the enhancement of DENV maturation.

Moreover, it is well established that assembly of membrane-containing virus requires both host and virus chaperones, in intracellular compartment or in the plasma membrane. Potential competition for the release of virion and interactions for maturation is still remained. The role of defective particles in the different populations during co-infection in insect cells has to be investigated.

The direct interactions between CHIKV and DENV proteins are not excluded in our investigations. Even if the replicative cycles occur in different ways, both viral proteins interact with the cellular compartments to generate proteins, and direct interactions between viral proteins are not yet described.

In C6/36 cells, the enhancement of permissiveness and the viral production by CHIKV was also expanded to other *flaviviruses* members, DENV serotypes (DENV-1, -3, -4) and the Yellow Fever Virus (YFV). The viral production of Zika virus (ZIKV) is also promoted by the pre-infection with CHIKV in C6/36 cells. Moreover, another *Alphavirus*, the Semliki Forest Virus (SFV) is able to induce the enhancement of DENV-2 permissiveness and production. Altogether, these complementary results suggest that the process is conserved between *alphaviruses* and *flaviviruses*. The absence of impact of CHIKV infection on the Vesicular Stomatitis Virus (VSV) infection in C6/36 cells confirms this hypothesis. It will be interested to detect all these viruses in mosquitos in the field to identify any “favorable” association, which may reflect synergy between some viruses *in vivo*.



**Figure 44.** Schematic representation of *in vitro* results during co-infection in *Aedes* cells.

The descriptions of CHIKV and DENV replication cycles have already been described. During CHIKV infection in insect cells, balance between antiviral pathways and viral replication lead to the survey of cell with an efficient replication of virus. It has been notified that RNA interference, Imd and apoptosis pathway can reduce CHIKV infection, and CHIKV counteract pathways for its survey (Cirimotich et al., 2009; Lamiabie et al., 2016; Wang et al., 2012). During a second infection with DEN viruses, investigations performed in C6/36, U4.4 (*Aedes albopictus*) and Aag2 (*Aedes aegypti*) cells, in order to study the impact of a first infection on the outcome of the second one. The permissiveness and the viral production of both viruses were analyzed. In cells, the chronic infection is stable, both in the number of infected cells (detected by flow cytometry), and the RNA quantity (detected by RT-qPCR). The DENV-2 RNA quantity is detected in higher concentration in co-infection condition, and the number of infected cells is also enhanced, suggesting that CHIKV promote the DENV replication in insect cells. Agrees with cellular results, the chronic production of CHIKV is similar to the co-infection condition, and supernatants of DENV in presence of CHIKV (co-infection condition) contained more DENV particles with an increased infectious titer.

The DENV replication cycle takes generally more time than CHIKV cycle. The infection activated many antiviral pathways, some independently to CHIKV infection. The JAK-STAT, and Toll pathways are involved in the control of DENV infection, on the contrast of CHIKV (Jupatanakul et al., 2014; Xi et al., 2008). Similarly to mammalian pathways, the homolog of autophagy and apoptosis may play a crucial role in the limitation of replication in insect cells. Following the DENV-2 infection, the CHIKV infection enter into C6/36 and U4.4 cells without any differences in cells, both in mono and co-infection conditions. The production of CHIKV particles in the supernatant is also stable with any conditions. The number of DENV-2 infected cells, and the DENV-2 RNA quantity is stable after the addition of CHIKV. However, the quantity of DENV-2 particles released in the supernatant is decreased by the addition of CHIKV, but the infectivity of these released virions is higher than mono-infection. The involvement of efficient maturation of particles by cleavage with protease (furin) is possible.

To complete our *in vitro* results, we designed an *in vivo* model miming a sequential oral co-infection in *Aedes aegypti* mosquito. Contrary to *Aedes albopictus*, *Aedes aegypti* was choose for the flexibility and the adaptability during blood meal. Simultaneous infection with CHIKV and DENV-2 have demonstrated for the first time in laboratory the possibility of co-infect *Aedes albopictus* mosquitoes, and the potential co-transmission, with the presence of both viruses in saliva (Vazeille et al., 2010). Recently, two publications reproduced these results in *Aedes aegypti* mosquitoes, using CHIKV, DENV-2 and ZIKV. These co-infected mosquitoes can transmit all combination of viruses via a single bite. Furthermore, in both study, the co-infection does not affect drastically the viral dissemination and the transmission in mosquitoes, suggesting that the vector competence of *Aedes aegypti* is not influence by the presence of various viruses simultaneously (Göertz et al., 2017; Ruckert et al., 2017). However, in our condition, we observed different outcomes. It is not unexpected that the infection in the long term with a first virus can modulate the antiviral response, and by this way modulate the second infection differently. In the field, the proportion of simultaneous and sequential co-infection is not yet known, and we can suppose that it occurs randomly during simultaneous outbreaks. All possibilities have to be exploited.

Even if challenging, we succeeded to set up the condition of two sequential blood meals in laboratory. In our study, 4 days after a first blood meal containing CHIKV with a titer surrounding  $10^8$  FFU/ml, female mosquitoes were exposed to a second blood meal containing DENV-2 virus at  $10^9$  FFU/ml. We were able analyzing the infection in the midgut, the dissemination through the salivary glands and the potential transmission in the saliva. RNA levels of CHIKV in midgut and in salivary glands were not impacted by the second infection with DENV-2. However, the pre-infection with CHIKV impacted the RNA level of DENV-2 in salivary glands. *In vivo* results are represented schematically in Figure 44. *In vivo* experiments are often more complex than *in vitro* models. *In vivo*, a lot of parameters come into play in results, such as the life story of the mosquito (age,

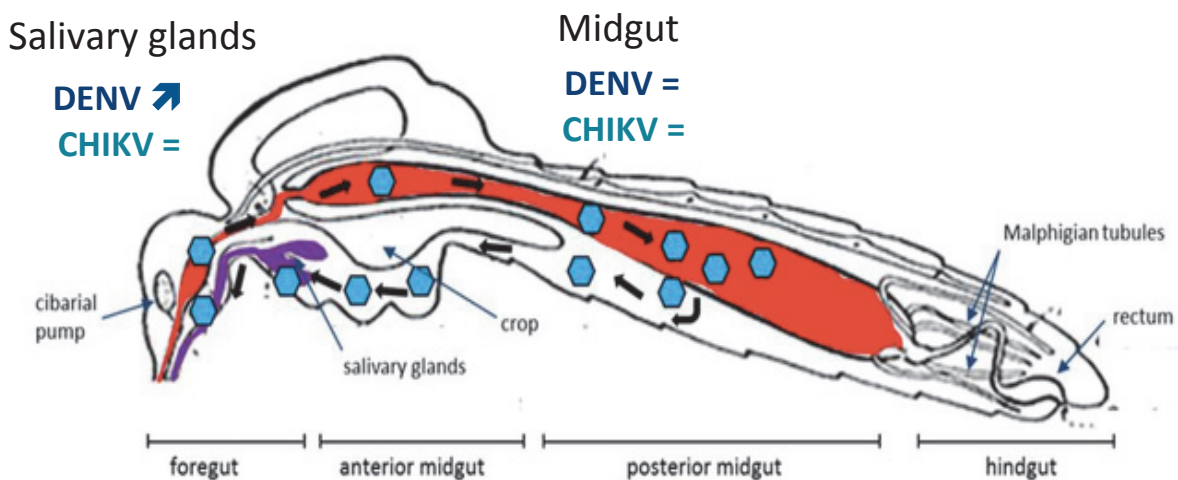
temperature, environment), the natural immune system activated after a viral infection, the multiplicity of tissues and cell types, or experimental variations. The midgut is the major barrier to pathogen transmission. In this organ, each virus modulates antiviral pathways differentially to facilitate the viral replication, and the dissemination through other organs. As described in a previous study, the DENV-2 replicated in the posterior part of the midgut, whereas the CHIKV replicates in the anterior part (Salazar et al., 2007; Tchankouo-Nguetcheu et al., 2010). The differential of replication site in midgut for both viruses may explain the absence of influence on the replication on each other in this organ.

Some proteomic analysis has been conducted on infection of mosquito (Tchankouo-Nguetcheu et al., 2010) and a list of modulated proteins in midgut by CHIKV or DENV infection has been established. Results indicate common regulation of some pathways, such as the endocytosis and the exocytosis pathways, but also some proteins involved in the metabolism, as the transferrin protein. In contrast, antagonist actions induced by CHIKV and DENV-2 have been noticed. CHIKV down regulate involved in the stress responses, while DENV-2 up-regulate it proteins. Pathways involved in the energy production of cells, as the glycolysis is also modulated differentially, with an up regulation by CHIKV and a down-regulation for DENV-2 (Tchankouo-Nguetcheu et al., 2010). Both viruses used same pathways and same cellular proteins for their replication in the midgut, and for the dissemination and the transmission. However, the infection may differentially affect pathways. Antagonist modulation induced by CHIKV and DENV-2 during the co-infection experiments may lead to cancelling of observable effect.

Altogether, RNA Sequencing and proteomic analysis in mono and co-infection conditions in *Aedes aegypti* mosquito could give tracks onto the modulation of common or antisens immune pathways.

In salivary glands, the DENV-2 RNA quantity is higher in co-infection than mono-infection condition, at day 10 and day 14pi. It is known that the duration of extrinsic period for DENV-2 in *Aedes aegypti* mosquito may take until 10 days. In comparison, at 7 dpi, DENV-2 has just started to escape midgut, while CHIKV reached the salivary glands between 2 and 4 days. The mechanism of the enhancement of DENV-2 in CHIKV infected mosquitoes is still to be determined. The modulation of salivary glands proteins with CHIKV infection may be involved in this process. Among them, CHIKV infection lead to

the up-regulation of proteins involved in virus transport (beta-1 tubulin) or in the intracellular cell signaling processes (Tchankouo-Nguetchou et al., 2012). The tubulin, major component of microtubule constitutes the cell cytoskeleton and play a key role in the trafficking. Viruses used this cellular trafficking during the entry by endocytosis and the exit by exocytosis. CHIKV and DENV have been identified among these viruses. Secreted protease inhibitor and Immunity related protein are considerably increased following the CHIKV infection in salivary glands, and may contribute to the enhancement of DENV-2 replication in this organ.



**Figure 45. Schematic representation of in vivo results during sequential infection with CHIKV and DENV-2 in *Aedes aegypti* mosquitoes.**

The RNA level of CHIKV in mono and co-infection conditions is stable in midgut and salivary glands. However, the RNA level of DENV-2 is not affected by the presence of CHIKV in midgut, in contrast of findings in salivary glands. The pre-infection with CHIKV enhances the DENV-2 replication in salivary glands.

The transmission cycle mosquito-human occurring during outbreak could be investigated using the transmission mosquito-mouse model. *In vivo* studies in mouse model allow to increase the understanding of arboviruses infection and pathogenesis (Couderc et al., 2008; Gardner et al., 2010; Metz et al., 2013). Following the transmission, target cells and organs could be analyzed, according to symptoms in patients. The persistence of virus in several tissues is still unclear and could influence dual-infections, with variation of the viral kinetic and the pathogenesis.

In human, the persistent infection with *alphaviruses* and *flaviviruses* can be found in specific compartments. Viral persistence of CHIKV is detected in muscle and joint several weeks following the infection (Gardner et al., 2010). Macrophages and dendritic cells also play a key role in the persistence of infection (Jessie et al., 2004; Labadie et al., 2010).

Moreover, an important factor for the sexual transmission of Zika virus is the long-term persistence of virus in the genital tract (Hamer et al., 2017; Tang et al., 2016). In the field, reported cases of co-infection with CHIKV and ZIKV have been reported since the recent outbreak Central America (Sardi et al., 2016). Although infection with *alphaviruses* has not been reported for such tropism, co-infections could modulate the tropism and genital tract could be a potential site of sequential infection.

The neurotropism of *alphaviruses* and *flaviviruses* could also be influenced during co-infections. Since the recent outbreak, reported cases of neurological symptoms have been attributed to CHIKV infection (Robin et al., 2008). ZIKV infection was characterized by causing common symptoms and an astonishing number of microcephaly in fetus and new-borns in Brazil (de Oliveira et al., 2017). Without epidemiology reports during the latest simultaneous outbreak, it is difficult to linked the severe cases with co-infection. However, the brain and neuronal cells could be an interesting part in the future investigations, to improve the understanding of pathogenesis.

All these investigations could be explored in collaboration with Dr Valerie Choumet in Pasteur Institut of Paris. They developed mouse model to study persistence infection in *alphavirus* infection context (unpublished data). The expertise in live imagery and the infection by CHIKV and DENV with luciferase gene reporters, could be used to visualize the localization of both viruses in mouse model after the viral transmission.

With recent improvement of single cell experiments, the analysis of the spacio-temporal dynamics of viral RNA with complex cell population during infection is possible. Investigations on YFV vaccine strain (YFV-17D) demonstrated interactions with murine and human hematopoietic cells (Douam et al., 2017). Authors have track the (+) and (–) sens viral RNAs at single cell resolution in mouse model, and they highlighted potential virus-host interactions important in the regulation of virulence and attenuation.

In conclusion, we have shown that sequential *alphaviruses* and *flaviviruses* infections are possible both in insect cells and in mosquito. The co-infection in insect cells modulates the permissiveness and the viral production. The sequential infection with CHIKV facilitates DENV-2 viral replication in salivary glands of *Ae. aegypti* mosquito. These findings are essential as areas of co-circulation of various arboviruses are expanding throughout the world. An increase in such co-infections may alter the infectivity or pathogenicity of both viruses. The outcome of these mixed infections must be further studied to increase our understanding of pathogen-pathogen interaction in the host cell. These results also pave the way to understanding the antiviral response when more than one arbovirus is present in the mosquito.

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## Appendix

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*Appendix 1. Publication: Co-infection of mosquitoes with chikungunya and dengue viruses reveals modulation of the replication of both viruses in midgut and salivary glands of Aedes aegypti mosquitoes*

This work was submitted the 21th June 2017 and accepted the 28<sup>th</sup> July 2017 in International Journal of Molecular Sciences

## Article

# Co-infection of mosquitoes with chikungunya and dengue viruses reveals modulation of the replication of both viruses in midguts and salivary glands of *Aedes aegypti* mosquitoes

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**Abstract:** Arthropod-borne virus (arbovirus) infections cause several emerging and resurgent infectious diseases in humans and animals. Chikungunya-affected areas often overlap with dengue-endemic areas. Concurrent dengue virus (DENV) and chikungunya virus (CHIKV) infections have been detected in travelers returning from regions of endemicity. CHIKV and DENV-co-infected *Aedes albopictus* have also been collected in the vicinity of co-infected human cases emphasizing the need to study co-infections in mosquitoes. We thus aimed to study the pathogen-pathogen interaction involved in these co-infections in DENV/CHIKV co-infected *Aedes aegypti* mosquitoes. In mono-infections, we detected CHIKV antigens as early as 4 days post-virus exposure in both midgut (MG) and salivary gland (SG) whereas we detected DENV-2 antigens from day 5 post-virus exposure in MGs and day 10 post-virus exposure in SGs. Identical infection rates were observed for singly and co-infected mosquitoes, and facilitation of the replication of both viruses at various times post-viral exposure. We observed a higher replication for DENV-2 in SG of co-infected mosquitoes. We showed that mixed CHIKV and DENV infection facilitated viral replication in *Ae. aegypti*. The outcome of these mixed infections must be further studied to increase our understanding of pathogen-pathogen interactions in host cells.

**Keywords:** *Aedes aegypti*; arbovirus, chikungunya, dengue, co-infection

*Appendix 2. Publication: A protein coevolution method uncovers critical features of the fusion mechanism during Hepatitis C Virus entry into host cells*

This collaborative work was submitted in Nature Communication.

## A protein coevolution method uncovers critical features of the fusion mechanism during Hepatitis C Virus entry into host cells

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## SUMMARY

Amino-acid coevolution can be referred to mutational compensatory patterns preserving the function of a protein. In viral envelope glycoproteins, such coevolution confers to viruses the plasticity to evade neutralizing antibodies without altering entry mechanisms into host cells. Here, by characterizing the functional coevolution of the two Hepatitis C Virus (HCV) envelope glycoproteins E1 and E2, we shed light on the HCV fusion mechanism during virus entry. Computational coevolution analyses predicted that E1 and E2 refold interdependently during fusion through rearrangements of the E2 Back Layer domain (BLd). Consistently, a soluble BLd-derived polypeptide was able to inhibit HCV fusogenic rearrangements in vitro, as well as infection of humanized mice by patient-derived HCV particles. Beyond demonstrating HCV fusion as a unique mechanism, our work provides a proof of concept for a wider exploration of viral protein mediated-processes and highlights coevolution as a valuable tool for guiding the design of viral inhibitors.

### *Appendix 3 The evolution and consequences of Zika virus post translational modifications*

#### **The evolution and consequences of Zika virus post translational modifications.**

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The current explosive epidemic of Zika virus (ZIKV) poses a global public health emergency. Before outbreak, ZIKV infection was characterized by causing a mild disease presented with fever, headache, rash, arthralgia, and conjunctivitis. However, since the end of 2015, there is an increase in the number of Guillain–Barre syndrome associated cases and an astonishing number of microcephaly in fetus and new-borns in Brazil. According to genome sequence analyses, ZIKV has diverged into African and Asian/American lineages, of which the Asian lineage is responsible for the outbreaks occurring in the Pacific Islands and South America. The reason of the increase pathogenesis in human of the Asian lineage is not understood. It is crucial to clarify the potential viral determinants of human virulence. Using sequence alignments and in vitro experiments, we identified differential post translational modifications between African and Asian strains. We will discuss the analysis of the consequences of some modifications on pathogenesis.

*Appendix 4. List of presentations and abstracts*

**Highlighted Poster.** 5th Pan-American Dengue research network meeting Panama.  
Panama – Panama City – 20-23 Avril 2016.

***Dengue and Chikungunya viruses: interactions during co-infection in mosquito cells***

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Dengue (DENV) and Chikungunya (CHIKV) viruses are two mosquito-borne arboviruses that cause human epidemics of several million serious clinical cases over the last decade. These two viruses share the same mosquito vector, *Aedes albopictus*, and they share the same geographical area of distribution, particularly in sub-Saharan Africa and Southern Asia. Recently, with the re-emergence of the CHIKV, the number of reported cases of patients infected by both DENV and CHIKV is increasing. This co-infection is also possible in mosquitoes that can in theory transmit both diseases at the same time. At the cellular level, DENV and CHIKV share interactions with common cellular pathways. These similar host-virus interactions probably impact the outcome of one virus during co-infection. To characterize any interference or synergy between DENV and CHIKV during replication, we performed sequential co-infection of the *Aedes albopictus* C6/36 cell line. Cells are infected with a first virus for 2 weeks, leading to the chronic infection. Then, C6/36 cells are infected with the second virus, and the permissiveness is analysed for 6 days post infection. The viral production of both viruses is also analyzed. We found that the permissiveness and production of DENV-2 is enhanced in presence of CHIKV. On the contrary, there was no effect of DENV-2 pre-infection on subsequent CHIKV co-infection. Datas indicated that the CHIKV also increased the infection by DENV-1, DENV-3 and DENV-4, but also by another flavivirus, the Yellow Fever Virus (YFV). The interaction between CHIKV and DENV-2 viruses was similar in U4.4 (*Ae. albopictus*) and Aag2 cell lines (*Ae. aegypti*). Finally, we showed that a chronic infection by Semliki Forest Virus also increased a subsequent DENV-2 infection. Altogether, our results pave the way for the characterization of molecular interaction between DENV and CHIKV in mosquito cells, which may provide clue the epidemiology of simultaneous outbreaks.

## Abstract

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登革病毒、寨卡病毒(ZIKV) 和基孔肯亚 (CHIKV) 病毒的爆发和传播范围的扩大, 增加了共感染的风险。到目前为止, 尚无疫苗或治疗方法来对抗这些对人类健康和全球经济造成重大影响的病原。在最近的暴发中, 12% 的患者确诊为 CHIKV 和登革病毒的共感染。此外, 研究表明白纹伊蚊可以同时携带 CHIKV 和登革两种病毒。同时, 自然情况下的病理、流行病学不是简单的一个病毒感染一个宿主, 而是多种病原在一个有机体和微环境中的相互作用。因此, 研究必须要考虑第一个微生物感染宿主是否会改变宿主对第二个微生物感染的反应。目前, 针对甲病毒属和黄病毒属以及黄病毒属之间的虫媒病毒共感染研究尚属空白。我们的研究目标就是在细胞和分子水平研究这些病毒在蚊子和人类中的共同感染现象。为此, 我们在白纹伊蚊和埃及伊蚊的细胞中进行了共感染的比较研究, 结果发现, CHIKV 的感染可以加强登革病毒的易感性和复制。与此相反, 先感染登革病毒对 CHIKV 的感染没有影响。这种协同现象在黄病毒属虫媒病毒中广泛存在, CHIKV 的感染不仅可以增强 DENV-1, DENV-3 和 DENV-4 的病毒感染, 还可以增强黄热病毒和 ZIKV 的感染。重要的是, 我们成功地建立了共感染的埃及伊蚊模型, 验证了预感染有 CHIKV 的蚊子唾液腺中比单独感染含有更多的登革病毒 RNA。这一表型与我们在细胞水平观察到的现象一致。总之, 我们的研究为黄病毒属与甲病毒属虫媒病毒共感染提供了体内体外实验证据, 有助于更好地了解多个病毒同时爆发时期的疾病表征和流行病学特点。

Emergence and geographical extension of dengue (DENV), Zika (ZIKV) and chikungunya (CHIKV) viruses increase simultaneous outbreak in an increasing number of countries. To date, no vaccine or cure have yet been developed against these diseases those cause a tremendous impact on human health and in the economy worldwide. During recent simultaneous outbreaks, up to 12% of patients have been diagnosed to be co-infected by CHIKV and DENV. In addition, it was shown that the mosquitoes *Aedes albopictus* could carry and transmit simultaneously CHIKV and DENV. However, the pathology, as well as the epidemiology of a pathogen, relies on the interactions between several infectious agents present within an organism or a community in the environment. It is crucial to consider to which extent a host infected by a first microorganism is modified and whether its reaction to the infection by a second microorganism is consequently altered. However, there is no extensive report of *Alphavirus-Flavivirus* or *flavivirus-flavivirus* interactions. Our global objective is to characterize these co-infections in both mosquitoes and humans, at the cell and molecular level. To this aim, we started this project by performing sequential co-infection in different cell lines from *Aedes albopictus* and *Aedes aegypti*. We found that the permissiveness and production of DENV is enhanced in presence of CHIKV. On the contrary, there is no effect of DENV pre-infection on subsequent CHIKV co-infection. We generalized the synergistic phenomena and we showed that CHIKV pre-infection also increased the infection by DENV-1, DENV-3 and DENV-4, but also by two others re-emerging *flaviviruses*, the Yellow Fever Virus (YFV), and the Zika Virus (ZIKV). Remarkably, we succeeded to establish a mosquito model of co-infection of *Aedes aegypti* mosquito after by different two feedings at 4 days interval. Using this sequential co-infection, we were able to show that a pre-infection of *Aedes aegypti* by CHIKV increase the level of DENV-2 RNA in salivary glands compare to mono-infected mosquitos. This phenotype is reminiscent of the phenotype we observed *in vitro* during sequential infections. Altogether, our study paves the way to the characterization of molecular interaction between *flaviviruses* and *Alphaviruses* in mosquito *in vitro* and *in vivo*. This study can be crucial for a better understanding of disease and epidemiology during simultaneous outbreaks.

## Key Words

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Co-infection, Mosquito, Chikungunya, Dengue, Interactions

**共感染,蚊媒,基孔肯亚病毒,登革病毒,相互作用**