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Renée Zakhia

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Ecole doctorale des Sciences et Technologie

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Laboratoire d'Immunologie et de Maladies à Transmission Vectorielle, Faculté de Santé Publique

Epidemiology of West Nile Virus in Lebanon

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Thèse de doctorat de Virologie et Entomologie Médicale

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Summary

West Nile virus (WNV) and Rift Valley Fever virus (RVFV) are two emerging arboviruses that have never been reported in Lebanon. They can be transmitted by *Culex pipiens* mosquito species including two biotypes: *pipiens* and *molestus*. During this project, we assessed the circulation of WNV among mosquitoes, human, horse and chicken populations in Lebanon. Moreover, we evaluated, under experimental conditions, the capacity of local *Cx. pipiens* biotypes to transmit both viruses.

Adult mosquitoes were collected, identified and tested to detect WNV RNA. Besides, human, horse and chicken blood samples were collected and screened for WNV antibodies using an in-house ELISA and then confirmed by neutralization assay. Moreover, local *Cx. pipiens* specimens were experimentally infected with WNV lineage 1 or RVFV Clone 13 strain. The viral infection, dissemination and transmission were then estimated at different days post infection. The vector competence was compared between *Cx. pipiens* biotypes.

Entomological results revealed that 87.2% of collected adult mosquitoes were *Cx. pipiens*. Screened mosquitoes were negative for WNV. Seroprevalence rates were 1.01% and 1.98% among humans and horses respectively. Besides, local *Cx. pipiens* were highly competent for WNV transmission and to a lesser extent to RVFV. The *molestus* biotype was able to transmit WNV earlier than *pipiens* biotype.

The present study provides new evidence of a low circulation of WNV among human and horses in Lebanon. *Cx. pipiens* is the suspected vector and is experimentally competent to ensure transmission. Therefore, there is a need to establish surveillance program to predict and prevent potential outbreaks.

Keywords: West Nile Virus, Rift Valley Fever Virus, *Culex pipiens*, Lebanon, Vector competence, Seroprevalence.

Résumé

Le Virus du Nil Occidental (VNO) et le Virus de la Fièvre de la Vallée du Rift (VFVR) sont deux arbovirus transmis par le moustique *Culex pipiens* comprenant deux biotypes: *pipiens* et *molestus*. Au cours de ce projet, nous avons évalué la circulation du VNO au Liban dans des populations de moustiques, des humains, des chevaux et des poulets. Nous avons aussi évalué la compétence vectorielle des populations locales de *Cx. pipiens* à transmettre le VNO et le VFVR.

Des moustiques ont été récoltés et testés pour la présence d'un gène spécifique du VNO. En plus, des sérums humains, de chevaux et de poulets ont été analysés pour rechercher des anticorps spécifiques par ELISA puis confirmés par neutralisation. En outre, des spécimens de *Cx. pipiens* ont été infectés avec la lignée 1 du VNO ou la souche de VFVR Clone 13. Ensuite, les taux d'infection, de dissémination et de transmission ont été déterminés à différents jours après infection des moustiques. La compétence vectorielle a été comparée entre les différents biotypes.

Les résultats entomologiques ont révélé que *Cx. pipiens* est dominant (87.2%). Tous les moustiques analysés étaient négatifs pour le VNO. Les taux de séroprévalence étaient de 1.01% et 1.98% parmi les humains et les chevaux respectivement. De plus, *Cx. pipiens* s'est révélé bien plus compétent pour transmettre le VNO que le VFVR. Le biotype *molestus* est capable de transmettre le VNO plus tôt que celui de *pipiens*.

Cette étude présente des preuves sur une faible circulation du VNO au Liban. *Cx. pipiens* s'est révélé compétent pour assurer cette transmission. Ainsi, il est essentiel d'établir des programmes de surveillance pour prévenir les éventuelles épidémies.

Mots Clés: Virus du Nil Occidental, Virus de la fièvre de la Vallée du Rift, *Culex pipiens*, Liban, Compétence Vectorielle, Séroprévalence.

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List of Abbreviations

%	Percentage
°C	Degree Celsius
<i>Ae.</i>	<i>Aedes</i>
BAGV	Bagaza Virus
BHK	Baby Hamster Kidney
bp	base pairs
BSA	Bovine Serum Albumin
Bti	Bacillus thuringiensis toxin
CDC	Centers for Disease Control and Prevention
cDNA	Copy Deoxyribonucleic acid
CEC	Cecropin
CHIKV	Chikungunya Virus
CSF	Cerebrospinal Fluid
<i>Cx.</i>	<i>Culex</i>
DDT	Dichlorodiphenyltrichloroethane
DENV	Dengue Virus
dpi	days post infection
eCDC	European Centre for Disease Prevention and Control
EIP	Extrinsic Incubation Period
ELISA	Enzyme Linked Immunosorbent Assay
EMR	East Mediterranean Region
FAO	Food and Agriculture Organization of the United Nations
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
HRP	Horseradish Peroxidase
IFA	Immunofluorescence Assay
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IGR	Insect Growth Regulator

IgY	Immunoglobulin Y
IRS	Indoor Residual Spraying
ITN	Insecticide treated nets
JEV	Japanese Encephalitis Virus
MENA	Middle East and North Africa
MR	Magnetic Resonance
NBC	Nahr El Bared Camp
NCR	Non Coding Region
NS	Nonstructural
NY	New York
OD	Optical Density
ODN	Optical Density on Negative antigen
ODP	Optical Density on Positive antigen
OIE	World Organization for Animal Health
OP	Organophosphate
ORF	Open Reading Frame
PBS	Phosphate Buffered Saline
PBS-T	Phosphate Buffered Saline Tween
PBS-T-BA	Phosphate Buffered Saline Tween Bovine Albumin
PCR	Polymerase Chain Reaction
PFU	Plaque Forming Units
pi	post infection
PO	Phenoloxidase
PRNT	Plaque Reduction Neutralization Test
RNA	Ribonucleic Acid
RNAi	Ribonucleic Acid interference
RNP	Ribonucleoprotein
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RVF	Rift Valley Fever
RVFV	Rift Valley Fever Virus
<i>s.l.</i>	<i>sensu lato</i>

<i>s.s.</i>	sensu stricto
SIT	Sterile Insect Technique
SLEV	St. Louis Encephalitis Virus
SPNL	Society for the Protection of Nature in Lebanon
SPSS	Statistical Package for the Social Sciences
TBE	Tris Borate EDTA
TMB	3,3',5,5'-Tetramethylbenzidine
UNRWA	United Nations Relief and Works Agency
USA	United States of America
USUV	Usutu Virus
VC	Vector Capacity
WHO	World Health Organization
WHOPES	World Health Organization Pesticide Evaluation Scheme
WNE	West Nile Encephalitis
WNF	West Nile Fever
WNND	West Nile Neurological Disease
WNV	West Nile Virus
YFV	Yellow Fever Virus
ZIKV	Zika Virus

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General Introduction

West Nile virus (WNV) is a mosquito-borne zoonotic agent member of the Flavivirus genus (Flaviviridae family). It was first isolated in Uganda in 1937 (Smithburn et al., 1940). Usually, only 20% of infected individuals develop symptoms and less than 1% develops serious and potentially fatal neurological illnesses such as meningitis and encephalitis. Birds are considered the main reservoir and migratory birds are involved in the introduction of the virus into new regions (Andreadis, 2012). WNV infections have been reported in many tropical and temperate countries in Africa, Europe, Asia and America (CDC, 2017; Failloux et al., 2017). The East Mediterranean region (EMR) has long been considered endemic for WNV. Locally acquired cases have been recently reported in Israel (Paz and Semenza, 2013), Greece (Papa, 2017), and Turkey (Kalaycioglu et al., 2012). In addition, evidence of WNV circulation has been reported from Jordan (Batieha et al., 2000) and Egypt (Soliman et al., 2010). It is worth noting that the introduction of WNV into the United States in 1999, which constitutes a turning point in WNV epidemiology, is thought to have originated from the EMR (Israel) following introduction from Africa (Giladi et al., 2001; Lanciotti et al., 1999).

WNV is mainly transmitted by mosquitoes of the *Culex* genus especially those belonging to the *Culex pipiens* complex (Turell, 2012). In the Mediterranean basin, this complex is mainly represented by *Cx. pipiens s.s.* under its two biotypes: *Cx. pipiens pipiens* and *Cx. pipiens molestus*.

To date, no WNV cases have been reported from Lebanon. However, the country is considered at risk for WNV outbreak. Not only it is geographically part of an endemic region, but also Lebanon is located on the flyways of migratory birds. Besides, entomological studies conducted in 1999-2001 showed a widespread of *Culex pipiens* (Knio et al., 2005). Additional entomological surveys conducted by our laboratory (Laboratory of Immunology and Vector-

Borne Diseases, Lebanese University) during the last decade confirmed the spread of this mosquito that colonizes urban and rural habitats (unpublished data).

The abundance of *Culex pipens* is considered a risk factor for local transmission, not only of WNV, but of also other arboviruses. Indeed, this species is a possible vector of Rift Valley Fever Virus (RVFV). This virus is of particular interest for Lebanon and the Mediterranean countries. It is mainly spread in the Sub-Saharan region in Africa (Himeidan et al., 2014). Recorded substantial expansion to areas close to the Mediterranean basin, such as Egypt (Meegan, 1979), Mauritania (El Mamy et al., 2011), Senegal and even the Arabian Peninsula (Saudi Arabia and Yemen (Ahmad, 2000)) should be considered as a health threat for Mediterranean countries, including Lebanon. Intensive livestock trade between Lebanon and these countries increases the risk of RVFV introduction.

During this PhD research project, we aimed to assess the circulation of WNV in Lebanon and the capacity of local strains of *Cx. pipiens* to transmit WNV and RVFV under experimental conditions. Therefore, our project is divided into two sections:

The first section covers the epidemiology of WNV in Lebanon and attempts to provide evidence of WNV circulation by screening mosquito populations for WNV RNA and by detecting WNV specific antibodies among human, horses and chicken populations.

The second section of this study includes an assessment of the vector competence of local populations of *Cx. pipiens* against both WNV and RVFV. For this, experimental transmission assays were conducted in Biosafety Level 3 (BSL3) facilities at the Unit of Arboviruses and Insect Vectors (Institut Pasteur, Paris, France). Moreover, the difference of vector competence between both *Cx. pipiens* biotypes was investigated.

This manuscript is divided in two parts Literature Review and Empirical Studies. The first part is composed of four chapters. Chapter 1 is an overview of the mosquito vector *Culex pipiens*, its biology and medical importance. In this chapter, we present classical and advanced methods to control mosquito populations and discuss the related challenges. Chapters 2 and 3 describe West Nile Virus and Rift Valley Fever Virus, their epidemiology, diagnosis and pathogenesis. Chapter 4 discusses the knowledge related to the vector-virus interactions. Chapter 5 specifies the study objectives of the present research project. The second part presents the work performed during this thesis project. It is composed of three chapters. In chapter 6, we assess the circulation of WNV in Lebanon by conducting entomological and seroprevalence surveys. In chapter 7, we assess the vector competence of Lebanese strains of *Culex pipiens* against West Nile Virus and Rift Valley Fever Virus. Chapter 8 presents the general discussion and conclusion related to the epidemiology of WNV in the country.

Part 1
Literature Review

Chapter 1

Overview on *Culex pipiens* mosquito vector

Culex pipiens complex mosquitoes belong to the Culicidae family, Culicinae subfamily. They have a global distribution and are considered vectors of several medically important arboviruses such as West Nile Virus (WNV) and Rift Valley Fever Virus (RVFV).

1. Classification

There are more than 3500 species of mosquitoes, all belonging to the Culicidae family (Service, 2007) that is divided into three subfamilies: Toxorhynchitinae, Anophelinae (anopheline) and Culicinae (culicine). The subfamily Culicinae comprises more than 40 genera (Service, 2007) among which *Culex* and *Aedes* include an important number of medically important species. *Culex* mosquitoes are widespread in almost all the continents with more than a thousand species (Becker et al., 2010). Within this genus, we recognize *Culex pipiens* which is considered a complex or assemblage of medically important species (Harbach, 2012).

Culex pipiens mosquitoes are found worldwide, but they are absent from the extreme northern parts of the temperate zones. The taxonomy of the *Cx. pipiens* complex is controversial and has constantly changed. Based on Harbach's last revision (Harbach, 2012), the *Cx. pipiens* complex includes *Cx. pipiens s.s.*, *Cx. quinquefasciatus* and the form *pallens* and possibly, *Cx. australicus* (Harbach, 2012; Harbach et al., 1985; Turell, 2012). The females of the complex members cannot be distinguished morphologically. *Cx. pipiens s.s.* includes two biotypes or subspecies: *Cx. pipiens pipiens* and *Cx. pipiens molestus* (Fonseca et al., 2004; Harbach, 2012). Harbach (2012) considered that the nominal forms *pipiens* and *quinquefasciatus* are

phylogenetically distinct species; they do not interbreed in the region where they co-exist. They are distinct outside the areas of introgression where the form *pallens* is described. According to this author, the *pallens* form does not meet the criteria for subspecies status. Harbach did not conclude on the status of *Culex australicus* and considered that this taxon “may or may not be a member of the *pipiens* assemblage” (Harbach, 2012).

2. Geographic distribution

A worldwide general distribution map of the *Culex pipiens* complex species was elaborated by Farajollahi and collaborators (2011) (Figure 1). *Culex pipiens* s.s. (including both biotypes and their hybrids) is present in European and Asian countries in addition to all Mediterranean countries including Lebanon (Farajollahi et al., 2011; Knio et al., 2005). This species has been accidentally introduced into temperate zones in North and South America, while only *Cx. pipiens* biotype *molestus* has been introduced to cities in Japan, Republic of South Korea, and Australia (Farajollahi et al., 2011; Fonseca et al., 2004). *Cx. quinquefasciatus* thrives in tropical and subtropical regions including the African lowlands, Americas, Asia, and Australia as shown in the distribution map (Figure 1). A recent study conducted by Shaikevich and collaborators (2016) provided the first molecular evidence of the presence of hybrids between *Cx. quinquefasciatus* and *Cx. pipiens* in coastal regions of Southern Europe and Mediterranean region (Morocco) (Shaikevich et al., 2016). In addition, they confirmed the presence of hybrids between *pipiens* and *molestus* biotypes in the study areas in Europe and around the Mediterranean Sea (Shaikevich et al., 2016).

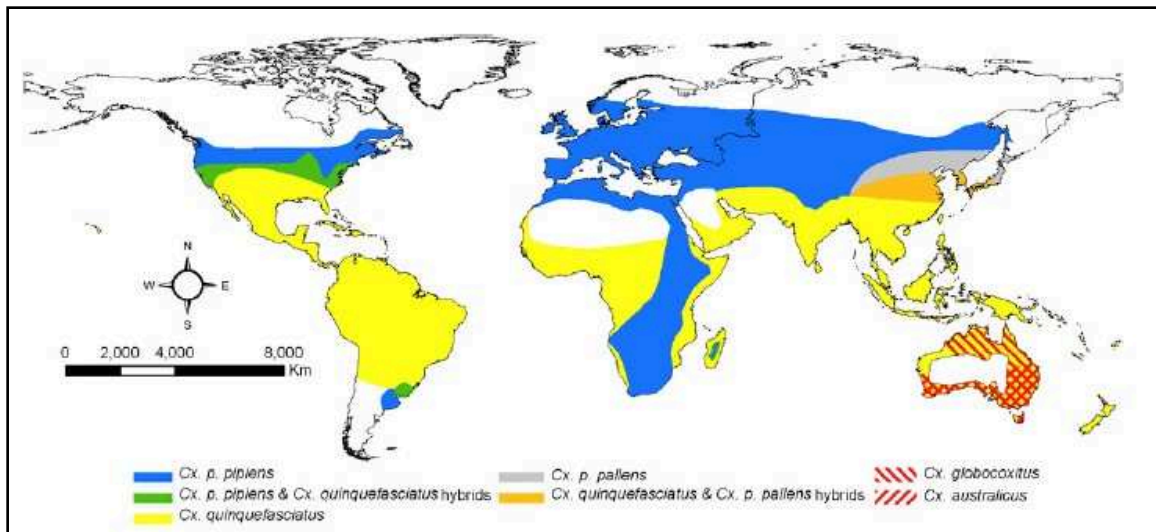


Figure 1. Global distribution of *Culex pipiens* complex mosquitoes (Farajollahi et al., 2011).

3. External morphology of adults

Adults of *Culex pipiens*, as any other mosquito, have a body that is divided into three parts: a head, thorax and abdomen (Figure 2). The head has a conspicuous forward-projecting proboscis and carries a pair of segmented antennae. Mosquitoes can be sexed by examining their antennae: individuals with feathery antennae are males while those with only short and rather inconspicuous antennal hairs are females. In addition, the palps (below the antennae) of female mosquitoes (as female Culicines) are very short while in males they are long. Arising between the palps is the single long proboscis which in females contains the piercing mouthparts, thus only female mosquitoes can bite.

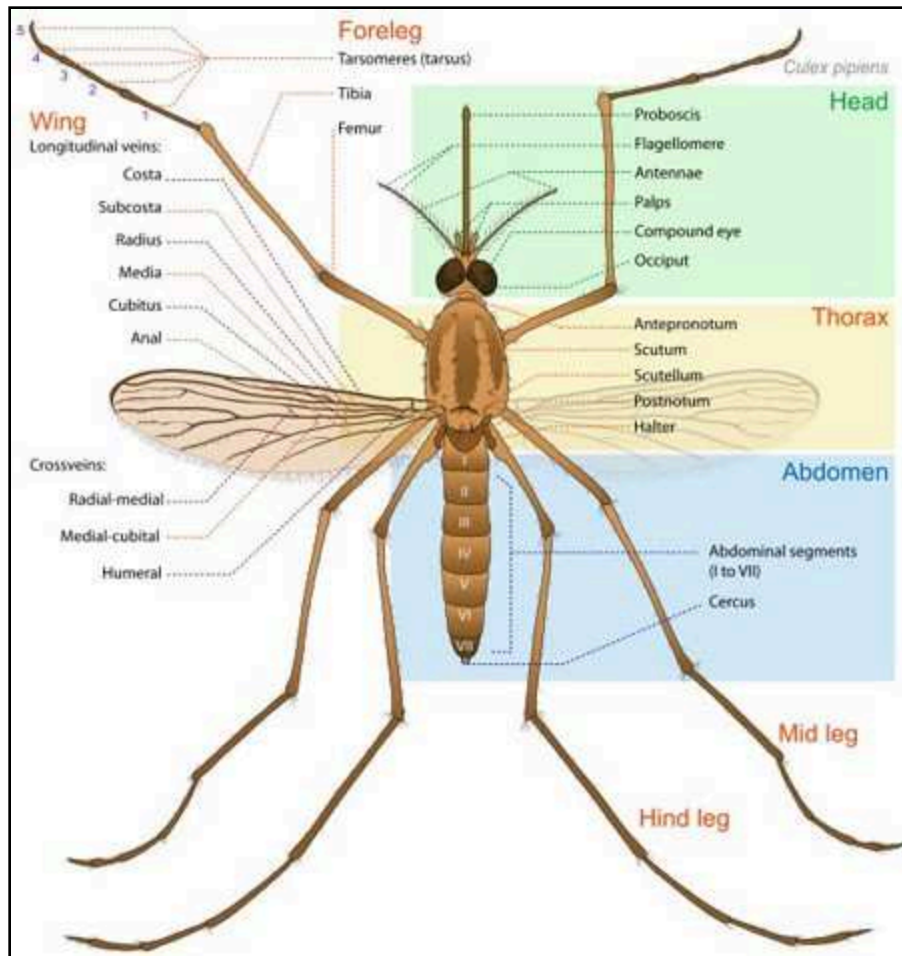


Figure 2. *Culex pipiens* anatomy diagram (Credit: Callista Images).

The thorax is ornamented with numerous scales that allow the discrimination between species. Moreover, the thorax provides an insertion site of three pairs of legs and one pair of functional wings. The abdomen of mosquitoes is composed of 10 segments, but only the eight are visible. The last two segments are reduced and form the genitalia (Service, 2007). The abdomen is usually covered dorsally and ventrally with brown and white scales. The dorsal parts of the abdomen are called tergites, they are important criteria for *Culex* species identification (Service, 2007).

Culex pipiens s.l. are characterized by the following morphological criteria:

At the *Culex* genus level:

- a scutellum clearly trilobate in thorax (Figure 3.A)
- absence of both pre-spiracular and post-spiracular setae in thorax
- sensory organs at the apex of the tarsomere 5 with an empodium and 2 pulvilli
- a wing characterized by: a fringe of scales in the alula and the apex of the vein 1-A localized beyond the mcu/cuA fork (Figure 3.B)

At the species level:

- a wing with an entirely dark costal vein (Figure 3.B)
- entirely dark legs (femur, tibia and tarsomeres of the 3 pairs of legs)
- basal pale band on their tergites (Figure 3.C)

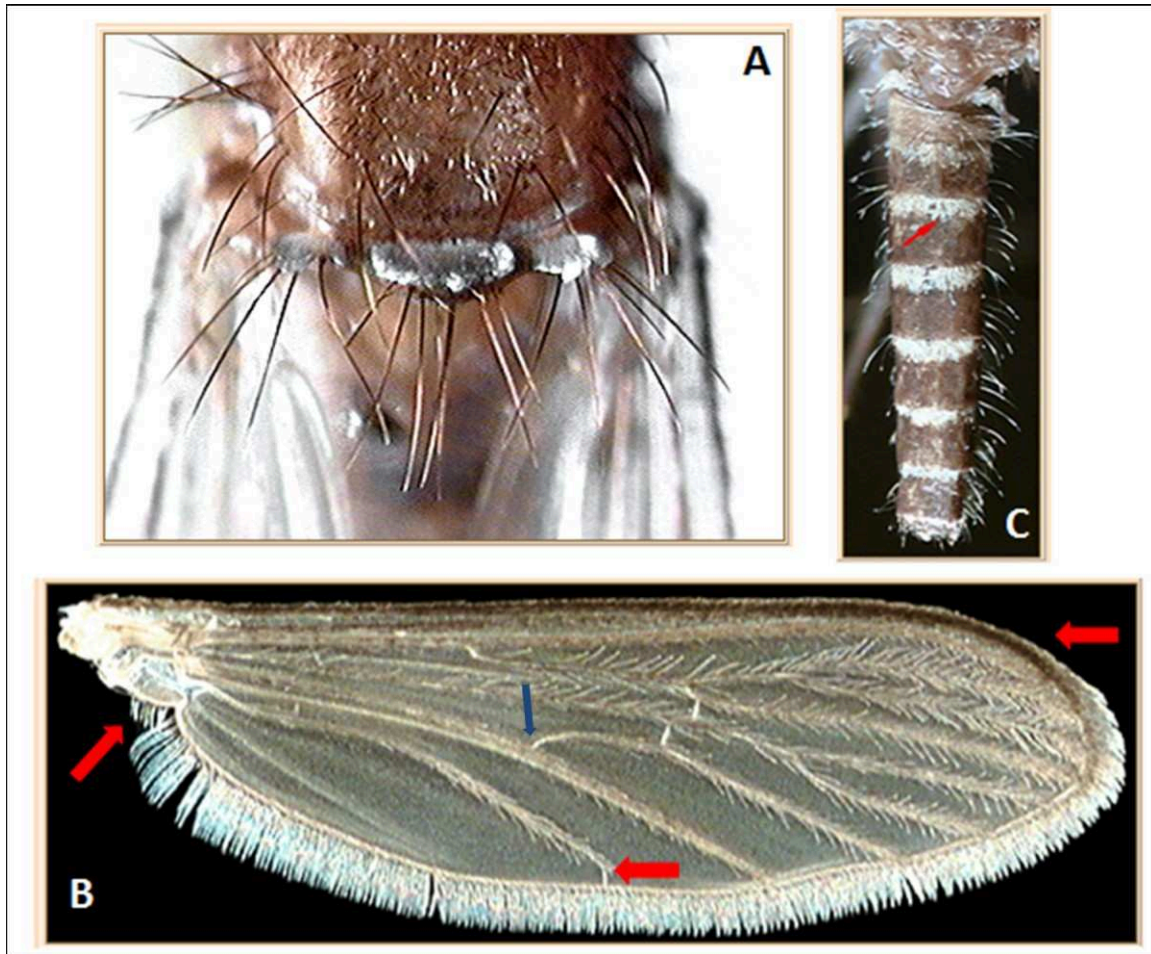


Figure 3. *Culex pipiens* morphological characteristics: A. Trilobate scutellum. B. Wing characteristics: fringe of scales in the alula (left arrow), dark costal vein (right arrow), apex of the vein 1-A (middle red arrow) beyond the mcu/cuA fork (blue arrow). C. Pale basal band on tergites (The mosquitoes of the Mediterranean Africa, IRD, France, CD-ROM, 1999).

Morphological discrimination between members of the *Cx. pipiens* complex is very difficult and sometimes not possible. *Cx. pipiens pipiens* and *Cx. pipiens molestus* can only be distinguished using molecular techniques such as Polymerase Chain Reaction (PCR) (Bahnck and Fonseca, 2006; Fonseca et al., 2004; Price and Fonseca, 2015).

4. Life cycle and biology of different stages

Culex pipiens mosquitoes, as all *Culex* species, go through four separate and distinct stages during their life cycle: egg, larva, pupa, and adult (Figure 4).

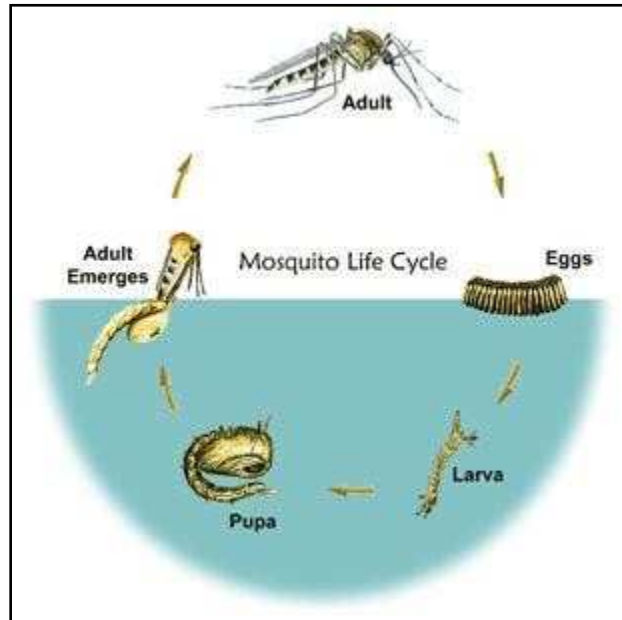


Figure 4. *Culex pipiens* life cycle (AMCA, 2017).

a. Oviposition and biology of eggs

Female *Culex pipiens* mosquitoes lay their eggs on the surface of fresh water or in temporarily wet areas like flood plains (Figure 5). They lay up to 300 eggs in one oviposition directly on the water surface (Service, 2007). Eggs are usually brown, long and cylindrical of 1mm or less in length. They are laid upright in several rows on the water surface and held together by surface tension to form an egg raft that floats on the water. These eggs cannot survive to desiccation and die when dry. Time from egg hatching to larvae stage varies from 2 to 3 days in the tropical regions, 7 to 14 days in cooler temperate areas (Becker et al., 2010).



Figure 5. Female *Culex pipiens* laying eggs. Photograph by Sean McCann (University of Florida).

b. Larval biology

Larvae have a well-developed head bearing a pair of antennae and a pair of compound eyes. The thorax is roundish and usually has long and conspicuous hairs. The last segment of the abdomen differs in shape from the other segments. It ends with two pairs of transparent sausage-shaped anal papillae, which are concerned with osmoregulation (Becker et al., 2010). *Cx. pipiens* larvae must come to the water surface to breath. Atmospheric air is taken in through a pair of spiracles situated at the end of a single tube termed the respiratory siphon. *Cx. pipiens* larval siphon is often long and narrow, possessing always more than one pair of subventral tufts of hairs.

There are four active larval instars. They feed on yeasts, bacteria, protozoans, decaying organic matter found in the water. Larval development (time from egg hatching to pupation) depends on areas, temperatures and species, it can be 5 to 7 days, but may also last about 7 to 14 days (Becker et al., 2010).

Larval habitats of *Culex pipiens* mosquitoes, as most *Culex* species, are ground collections of water such as pools, puddles, ditches, borrow pits and rice fields. In urban areas, this species lays eggs in man-made container-habitats such as tin cans, water receptacles, bottles and water-storage tanks with variable amount of organic matter (Service, 2007).

c. Pupae biology

Cx. pipiens mosquito pupae are aquatic and comma-shaped (Service, 2007). The head and thorax are combined to form the cephalothorax, which dorsally possesses a pair of respiratory trumpets. Pupae do not feed but spend most of their time resting at the water surface to take air (Service, 2007). The pupal development may take 2-3 days in tropical areas, but in cooler temperate regions, pupal period can last 9-12 days. At the end of pupal life the skin on the dorsal surface of the cephalothorax splits, and the adult mosquito struggles out (Becker et al., 2010).

d. Adult biology and behavior

Most *Cx. pipiens* mosquitoes mate shortly after emergence. Sperm from a male enter the spermatheca of a female, and this usually serves to fertilize all eggs laid during her lifetime (Becker et al., 2010). Female mosquitoes must bite a host and take a blood-meal to obtain the necessary nutrients for the development of eggs. This is referred as anautogenous development (Service, 2007). However, some *Cx. pipiens* forms can develop the first batch of eggs without a blood-meal, and more rarely, subsequent batches. This process is called autogenous development (Service, 2007). The blood-meal digestion depends on temperature and varies from 2 to 3 days in hot climates, to 7–14 days in colder areas. After full digestion, the *Cx. pipiens* female is gravid and ready to lay eggs in suitable larval habitats (Service, 2007). Male *Cx. pipiens* mosquitoes,

along with other species, cannot bite but feed on the nectar of flowers and other natural sugar secretions, along with females in order to obtain energy for flying.

Adult *Cx. pipiens* mosquitoes mainly disperse only a few hundred meters from their emergence sites. In Japan, a mark–release–recapture of *Culex pipiens pallens* showed that the maximum flight distance was estimated as 1217m with a mean distance flight that did not exceed 517m (Tsuda et al., 2008). In some cases, this distance is longer when the mosquitoes are wind-assisted. In tropical areas, adult female mosquitoes live 1–2 weeks in average whereas in temperate countries, adult life may be extended to 3–4 weeks (Becker et al., 2010). Species that hibernate live much longer: for instance in Europe, some fertilized female *Culex pipiens* survive in hibernation from season to another (Becker et al., 2010). Adult male mosquitoes have a shorter life span than females.

Cx. pipiens subspecies differ significantly in physiology, behavior and ecology (Table 1). *Cx. pipiens* biotype *pipiens* is mainly present in temperate areas, ornithophilic (bird biting host preference), anautogenous and eurygamous (mating in outdoor swarms only) whereas the biotype *molestus*, thrives in sewers in sub-tropical regions, is mammophilic (prefers biting mammals including humans), autogenous and stenogamous (ability to mate in a confined space) (Fonseca et al., 2004; Harbach, 2012). The hybrids of these two biotypes show opportunistic and different feeding behaviors. Another member of the complex, *Culex quinquefasciatus* is a noticeably domestic species and common in urbanized areas. Especially in towns with poor and inadequate drainage and sanitation, this mosquito breeds mainly in water polluted with organic matter (Harbach, 2012).

The adult females of the *Cx. pipiens* complex bite humans and animals throughout the night, indoors and outdoors (Harbach, 2012). During the day, they are inactive and are often found

resting in dark corners of rooms and shelters. They may also rest outdoors on vegetation and in tree holes in forested areas (Harbach, 2012).

Table 1. Behavioral and physiological traits associated with three nominal forms of the *pipiens* assemblage (Harbach, 2012).

Trait	<i>pipiens</i>	<i>molestus</i>	<i>Cx. quinquefasciatus</i>
Host feeding	ornithophilic, zoophilic	anthropophilic, mammophilic, ornithophilic	anthropophilic, mammophilic, ornithophilic
Feeding location	exophagic	endo- / exophagic	endo- / exophagic
Resting location	exophilic	endo- / exophilic	endo- / exophilic
Egg development	anautogenous	autogenous	anautogenous
Mating space	eurygamous	stenogamous	stenogamous
Diapause	heterodynamous	homodynamous	homodynamous
Larval habitat	clean water normally above ground	clean / polluted water below ground	clean / polluted / brackish water above ground

5. Medical importance

In several countries, members of *Culex pipiens* complex cause considerable nuisance, annoyance and irritation because of their troublesome bites. They are considered as main vectors, and sometimes reservoirs, of several medically important pathogens that infect humans including filarial worms, wild life pathogens such as avian malaria (*Plasmodium* species) and some arboviruses like Saint Louis Encephalitis Virus (SLEV), WNV, and RVFV.

Culex pipiens fatigans (now referred as *Culex quinquefasciatus*) was considered a vector of Bancroftian filariasis in East Africa (White, 1971). This species was found infected with microfilariae of *Wuchereria bancrofti* in North-Eastern Tanzania between 1968 and 1970 (White, 1971). *Culex pipiens* mosquitoes were implicated as natural vectors of Dirofilariasis in Central Italy (Cancrini et al., 2007). In 2002-2003, mosquito collections were performed using dog-baited traps and *Dirofilaria immitis* and *Dirofilaria repens* were found in three pools of *Cx.*

pipiens (Cancrini et al., 2007). In addition, three pools of field-collected *Cx. pipiens* were found naturally infected with *Dirofilaria immitis* in Central Turkey (Yildirim et al., 2011). In addition, avian malaria parasites can be transmitted by *Cx. pipiens* species complex. *Plasmodium* parasites were detected in *Cx. pipiens* collected by bird-baited traps in 2004 in New York (Kimura et al., 2010).

In the United States, from 1977 to 1986, nine outbreaks of SLEV occurred (Monath and Tsai, 1987) during which both *Cx. pipiens* and *Cx. quinquefasciatus* were found naturally infected with the virus (Monath and Tsai, 1987). Two strains of SLEV were isolated from overwintering *Cx. pipiens* mosquitoes collected in Maryland and Pennsylvania during January and February 1977 (Bailey et al., 1978). Moreover, a Saint Louis encephalitis epidemic in Grand Junction, Colorado, occurred in 1985. No human cases were reported, but mosquito surveillance disclosed SLEV in *Culex tarsalis* and *Culex pipiens pipiens* field collected mosquitoes (Tasi et al., 1988).

One of the predominant vectors of WNV is *Cx. pipiens*. Field-collected *Cx. pipiens* mosquitoes were found to be naturally infected with the virus in several countries in the world (Hubálek and Halouzka, 1999). WNV was isolated from *Cx. pipiens* mosquito pools in Romania (Tsai et al., 1998), Portugal (Almeida et al., 2008), Italy (Calzolari et al., 2010), Egypt (Soliman et al., 2010), Israel (Orshan et al., 2008), Greece (Papa et al., 2013; Patsoula et al., 2016), and Turkey (Ergunay et al., 2013). Hubalek and Halouzka (1999) mentioned in their review that WNV was also isolated from *Cx. pipiens* in South Africa, Czech Republic and Bulgaria. In addition, the same authors stated that WNV was as well isolated from *Cx. quinquefasciatus* in India, Pakistan, and Madagascar. Moreover, a study conducted by Andreadis (2012) showed that, WNV isolated from field collected *Cx. quinquefasciatus* mosquito pools were predominant in Southeast and Southwest United States, while *Cx. pipiens* was the predominant vector of WNV

in northeastern United States (Andreadis, 2012). Furthermore, a study showed that *Cx. pipiens* mosquitoes were able to vertically transmit WNV (Dohm et al., 2002). This type of transmission amplifies the virus and increases the transmission risk.

Cx. pipiens complex is also considered a secondary vector of RVFV. Some countries reported field-collected *Cx. pipiens* mosquitoes naturally infected with the virus (Tantely et al., 2015). RVFV was successfully isolated from *Cx. pipiens* mosquitoes in Egypt (Hoogstraal et al., 1979) and Sudan (Seufi and Galal, 2010). In addition, the virus was isolated from *Cx. quinquefasciatus* in Kenya (Sang et al., 2010).

Cx. pipiens was indicated as a suspected vector of Usutu virus (USUV). In Italy, USUV was detected in 54 *Cx. pipiens* pools (Calzolari et al., 2010). Moreover, sequences of this virus were found in one pool of this species in Spain in 2006 collections (Busquets et al., 2008).

6. Mosquito control methods and challenges

Mosquito control manages medically important mosquito populations including *Culex pipiens* in order to reduce their impact on human health, economies, and pathogen transmission. Several mosquito control measures are directed at either the immature aquatic stages of the mosquito or the adults using environmental management, physical, biological, chemical and genetic methods. In addition, personal protection measures that limit people contact with adult mosquitoes are also implemented. It is a key component in the prevention of mosquito nuisance and mosquito-borne diseases. Measures may include: simply staying indoors, wearing clothing that minimizes exposed skin, use of personal repellents on skin or clothing, and use of Insecticide treated nets (ITN) (Becker et al., 2010; Service, 2007).

Several environmental management concepts aiming to reduce mosquito breeding sites can be applied, but these measures differ between urban and rural areas and may sometimes be difficult to implement especially in urban areas (Becker et al., 2010). Habitats for many mosquito species can be diminished by monitoring and eliminating wide range of water bodies and also by, appropriately designing urban structures and sanitation to prevent formation of breeding sites.

Several physical control techniques exist for both immature aquatic mosquito stages and adults. For example, larvae and pupae can be suffocated and deprived from water tension using polystyrene beads and monomolecular surface films. Physical control techniques are attractive since they are effective against mosquitoes resistant to conventional insecticides.

Biological control is performed by the reduction of the target mosquito population, to an “acceptable” level, by the use of predators, parasites, pathogens, competitors, or toxins from microorganisms. These measures are mostly efficient against the immature aquatic stages of the mosquito, for which some vertebrate and invertebrate predators can be used successfully such as the mosquito fish (*Gambusia affinis*), amphibians, birds and several others (Becker et al., 2010). Pathogens such as fungi can also be adopted targeting larval stages of mosquitoes. These measures must be controlled in order to maintain biodiversity and seek for the minimal impact on the environment. The use of toxins such as *Bacillus thuringiensis* toxin (Bti), is largely adopted in many industrialized countries. This environmentally safe product remains expensive and therefore rarely used in low-income regions.

The development of chemical insecticides has led to many major advances in mosquito control. Several families of insecticides are used indoors or outdoors: chlorinated hydrocarbons (organochlorines), organophosphates (OP), carbamates, and pyrethroids, and a special class

referred to as insect growth regulators (IGRs) (Becker et al., 2010). According to the World Health Organization Pesticide Evaluation Scheme (WHOPES), the most used organochlorine in the African region is Dichlorodiphenyltrichloroethane (DDT). In other regions, the most used insecticides are the organophosphate Malathion and the pyrethroid Permethrin. The carbamate Propoxur is also used for indoor residual spraying (IRS). There are two major groups of IGRs that differ in their mode of action. The first is the chitin synthesis inhibitors which interfere with new cuticle formation, resulting in moult disruption (Becker et al., 2010). The second is the juvenile hormone analogs that interfere with the metamorphic processes affecting development to the adult stage (Becker et al., 2010).

The main constraint remains the difficulty to target the adult and larval stages in their resting or breeding sites. The auto-dissemination approach is a relatively new approach that aims to better target the adult stage. It is based on exposing wild females to artificial resting sites contaminated with pyriproxyfen. Then females can disseminate this juvenile hormone analogue to larval habitats, thus interfering with adult emergence. This approach was applied on *Aedes aegypti* and *Aedes albopictus* (Caputo et al., 2012; Devine et al., 2009). It was also experimentally tested on *Cx. quinquefasciatus* populations and provided very promising results (Mbare et al., 2014).

Over time, repeated uses of insecticides lead to the development of insecticide resistance, especially to organochlorines and organophosphates, becoming a problem in several mosquito populations and particularly in *Culex pipiens* in different countries including Lebanon (Osta et al., 2012), Iran (Salim-Abadi et al., 2016) and the USA (Paul et al., 2005). This resistance became a limiting factor for the efficacy of control efforts.

Today, genetic control of mosquitoes relies on two main fronts. The first consists of Sterile Insect Technique (SIT) that aims to eliminate an entire population of a particular mosquito species. One example is the sterile male technique or male sterilization that consists of releasing a mass of modified male mosquitoes that mate with indigenous females to produce no fertile offspring. The second is the population replacement consisting to replace wild populations by genetically-modified mosquitoes rendered refractory to a pathogen (Becker et al., 2010).

SIT is currently in operational use more than the population replacement technique. In Florida, in 1969, *Culex quinquefasciatus* populations were successfully suppressed and eliminated after the massive release of chemo-sterilized males (Patterson et al., 1970). In France, in 1970, a reduction in *Cx. pipiens* populations was obtained after the release of hundred thousand sterile specimens following chromosomal translocation (Laven et al., 1971). More recently, in La Reunion Island, a field test was implemented to control *Aedes albopictus* populations; a two-fold reduction of the wild population fertility was obtained after the release of males sterilized by irradiations with Cesium-137 (Oliva et al., 2012).

In addition, recent work has shown that the introduction of an over replicating strain (wMelPop) of the bacteria *Wolbachia* into *Ae. aegypti* can lead to a cytoplasmic incompatibility and inhibits the dissemination of Dengue Virus (DENV) as well as Chikungunya Virus (CHIKV) (Moreira et al., 2009). The release of *Aedes* specimens infected with appropriate strains of *Wolbachia* was proposed to control Zika Virus (ZIKV) in Brazil (Callaway, 2016). However, the use of *Wolbachia* to reduce transmission of pathogens by *Culex* species must be studied carefully. Indeed, a recent study demonstrated that the transmission of WNV could be improved by the presence of the *Wolbachia* strain *wAlbB* in the mosquito *Culex tarsalis* (Dodson et al., 2014).

Chapter 2

West Nile Virus

West Nile Virus (WNV), an arthropod-borne virus, is the causative agent of West Nile Fever (WNF). This virus is transmitted from infected birds to humans by the bite of a broad range of mosquito species, but mainly members of the *Culex* genus, most importantly *Culex pipiens* complex species.

1. Transmission cycle

In an enzootic cycle, mosquitoes get infected by feeding on a bird having a sufficiently high viremia. The virus then replicates inside the mosquito before it infects another host (Figure 6). Vertical transmission by mosquitoes provides the mechanism of virus overwintering (Chancey et al., 2015). Only females having high natural infection titers succeeded to pass WNV to their overwintering adult progeny (Nelms et al., 2013). Migratory birds ensure the seasonal spread of the virus between countries. Thus, mosquito species feeding on these infected birds during their stopovers transmit the virus to local bird species. Transmission to people and horses is accidental; it occurs when an infected mosquito feeds on a person or a horse thereby transmitting the virus via its salivary gland secretions. People and horses are dead end hosts because they do not develop viremia high enough to infect a mosquito. Experimental infections of horses with the NY99 strain of WNV revealed low viremia levels (a maximum viral titer of 10^3 plaque forming units (PFU)/mL) of short duration and all mosquitoes that fed upon viremic horses were not able to transmit the virus (Bunning et al., 2002). Therefore, infected horses are unlikely to serve as important amplifying hosts for WNV in nature. In fact, infectious doses required to infect *Cx.*

pipiens should be greater than 10^5 PFU/mL (Turell et al., 2000). Horizontal transmission between birds is possible in cases of fecal or oral fluids contact (Van der Meulen et al., 2005). WNV can also be transmitted between humans by blood transfusion, organ transplantation, transplacental transmission, and via breast milk.

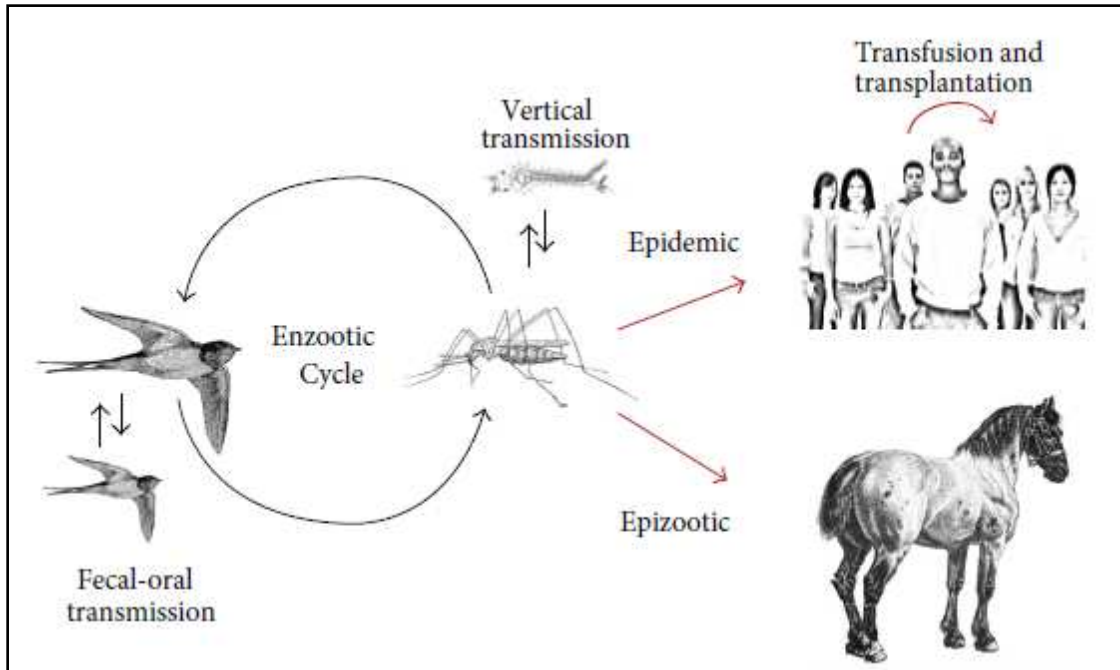


Figure 6. WNV transmission cycle (Chancey et al., 2015).

Birds are presumed to be the most important amplifying hosts of WNV. In laboratory studies, bird species of Passeriformes, Charadriiformes, Strigiformes (owls), and Falconiformes developed viremia levels sufficiently high to infect most feeding mosquitoes (Komar et al., 2003). Birds like house sparrows (*Passer domesticus*) which are common in many countries including Lebanon, develop high viremia and therefore contribute to mosquito infection without developing clinical symptoms (Komar et al., 2003).

2. Geographic distribution

WNV was first isolated from a febrile woman in Uganda in 1937 (Smithburn et al., 1940). WNV infections have been reported in many tropical and temperate countries in Africa, Europe, Asia and America. The circulation of this virus is being continuously traced by eCDC (Figure 7). The Middle East and North Africa (MENA) region has been long considered as a WNV-endemic area in which hundreds to thousands of human and equine cases have occurred. A series of outbreaks from the 1990s brought WNV into the spotlight. Epidemics in Algeria, Morocco, Tunisia, Italy, France, Romania, Israel, and Russia were associated with uncharacteristically severe human disease, including neurologic complications and death (Marka et al., 2013; Murgue et al., 2001a; Platonov et al., 2001; Tsai et al., 1998). Recent outbreaks have affected Israel in 2000 (Weinberger et al., 2001), Greece in 2010 (Danis et al., 2011) and 2011 (Vrioni et al., 2014), and Turkey in 2010 (Kalaycioglu et al., 2012). In addition, evidence of recent human WNV circulation has been reported in Libya (Shaibi et al., 2017), Jordan (Batieha et al., 2000), Egypt (Soliman et al., 2010) and Israel (Kopel et al., 2011).

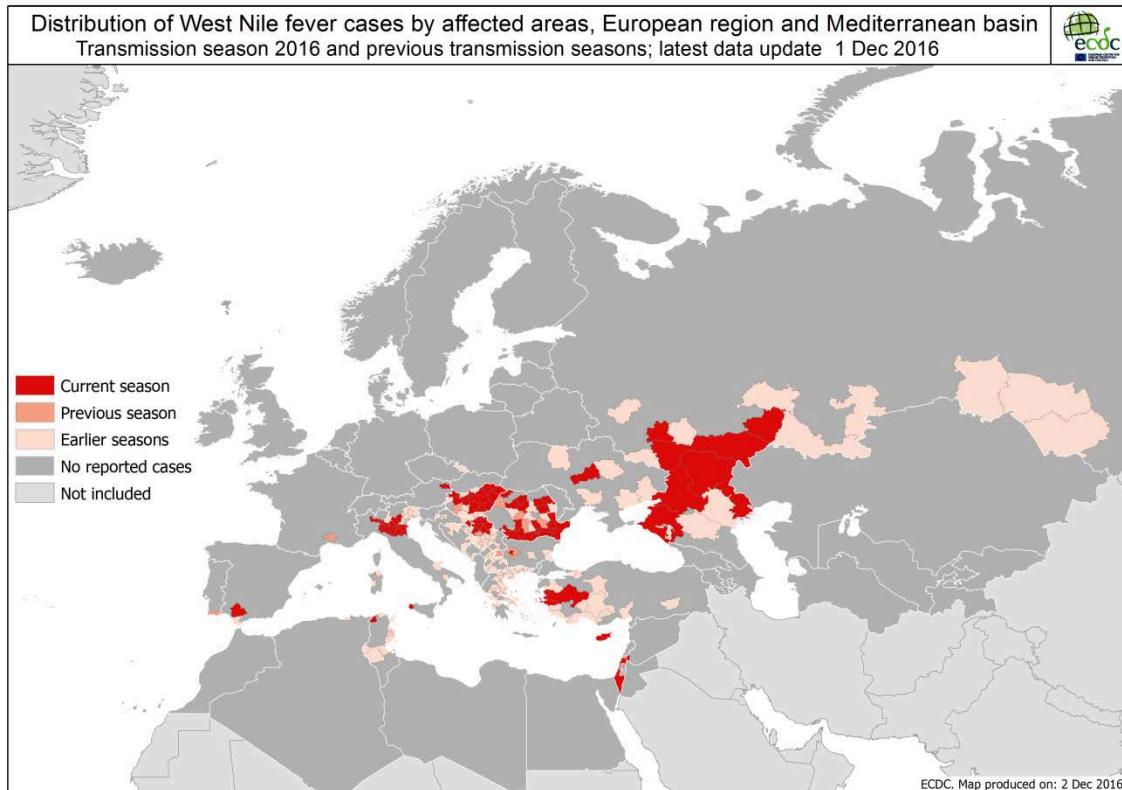


Figure 7. Past and present WNV transmission activities in the Euro-Mediterranean region (eCDC, 2017).

The introduction of WNV into the United States of America (USA) in 1999 which constitutes a turning point in WNV epidemiology, is thought to have originated from Israel following introduction from Africa (Giladi et al., 2001; Lanciotti et al., 1999). In the summer 2001, the virus was found in 10 states and WNV continued to spread until a large outbreak that occurred in 2006, with 177 deaths and 1495 cases of West Nile neurological disease (WNND) out of a total of 4260 cases (WHO, 2017). Through 2010, it was estimated that 1.1% of the United States population had been infected by WNV (WHO, 2017).

The presence of WNV was first discovered in Europe in 1958 in Albania with detection of neutralizing antibodies in human sera (Hubálek and Halouzka, 1999). The first documented outbreak of WNV occurred in southern France in 1962-1963, causing WNND in both humans and horses (Murgue et al., 2001a). Notable outbreaks with human cases include the reemergence

of WNV in France in 2000-2003, Italy in 2008-2009, and Hungary in 2008 (Bakonyi et al., 2013; Calistri et al., 2010; Di Sabatino et al., 2014; Zeller and Schuffenecker, 2004). Recently, human WNV cases were reported in several European countries.

Epizootics in horse populations occurred in Morocco in 1996 in which 94 cases in equines (including 42 deaths) and 1 case in a human were reported (Schuffenecker et al., 2005). During the late summer of 1998, veterinary authorities in Tuscany, Italy, received reports of neurologic disease cases among horses (Autorino et al., 2002). In 2000 in USA, 63 horses with neurologic and other illnesses due to WNV infection were reported from 26 counties in seven states (Marfin and Gubler, 2001). Cases were reported in France in 2000, where WNV cases were confirmed in 76 horses, and 21 of them died (Murgue et al., 2001b). Recent equine outbreaks occurred in Italy in 2008 (14 horse cases), Hungary in 2008 (12 equine neuroinvasive cases) (Angenvoort et al., 2013). Since 2010, WNV infections among horses were repeatedly reported in the Mediterranean basin and other European countries, namely Algeria (Lafri et al., 2017), Jordan (Abutarbush and Al-Majali, 2014), Bulgaria, Czech and Slovak Republics (Hubálek et al., 2013), Croatia, Greece, Italy, Spain (Abad-Cobo et al., 2016; Jiménez-Clavero et al., 2010), Macedonia, Morocco, Portugal (Barros et al., 2011), and Romania (Angenvoort et al., 2013).

WNV has been detected in more than 150 species of wild and domestic birds worldwide. The susceptibilities of birds to WNV infection differ among bird species. Severe disease and deaths were not reported in natural WNV infections of birds until 1998, when an outbreak occurred in Israel, during which 160 of 400 domestic goslings developed fatal encephalitis (Di Sabatino et al., 2014). In 1999, during the outbreak of WNV in USA, thousands of deaths in more than 150 species of native and exotic birds were detected (Nash et al., 2001; Steele et al., 2000). In the following years, bird infection with WNV and sometimes death were observed in several

countries around the world. During the summers of 2004-2005 in Southeast Hungary, the first clinical cases were described and WNV infections in birds were detected. It corresponds to the first evidence of a neuroinvasive WNV lineage 2 circulation in continental Europe (Erdélyi et al., 2007). Subsequently, a similar WNV lineage 2 strain was molecularly identified and characterized from an Eurasian magpie hunted in Greece in 2010, during a WNV outbreak in humans (Valiakos et al., 2011).

In a serosurvey study conducted in Camargue France, a low WNV seroprevalence was observed among birds in a 3-year period (2005-2007). It revealed the existence of an enzootic activity of WNV without any detectable emergence of an epizootic (Balança et al., 2009). Moreover, in Germany during 2005-2009, a study discovered WNV-neutralizing antibodies in 10 species of migratory birds, but not in resident birds (Seidowski et al., 2010). For the first time in Serbia, WNV lineage 2 RNA were detected by RT-qPCR in pooled tissue samples of investigated birds in 2012 (Petrović et al., 2013).

3. Classification and lineages distribution

West Nile Virus (WNV) belongs to the *Flavivirus* genus that includes the largest number of viral species compared to other genera (*Pestivirus* and *Hepacivirus*) composing the Flaviviridae family. The *Flavivirus* genus includes at least 53 species divided into 12 serogroups. WNV belongs to the Japanese Encephalitis Virus (JEV) group that comprises eight viral species, among which we cite, in addition to WNV, JEV, SLEV, Murray Valley Encephalitis that are frequently associated with widespread human and animal disease outbreaks (Schweitzer et al., 2009).

WNV is a genetically and geographically diverse virus. Up to nine distinct WNV genetic lineages with inconsistent numbering, have been proposed in the literature based on phylogenetic analyses of the complete genome or of the NS5 gene. Their genomes differ from each other by more than 20-25% and correlate well with the geographical point of isolation. Rizzoli and collaborators (2015) suggested a harmonized lineage numbering into seven lineages (Figure 8). Lineages 1 and 2 of WNV have been associated with significant outbreaks in humans (Chancey et al., 2015).

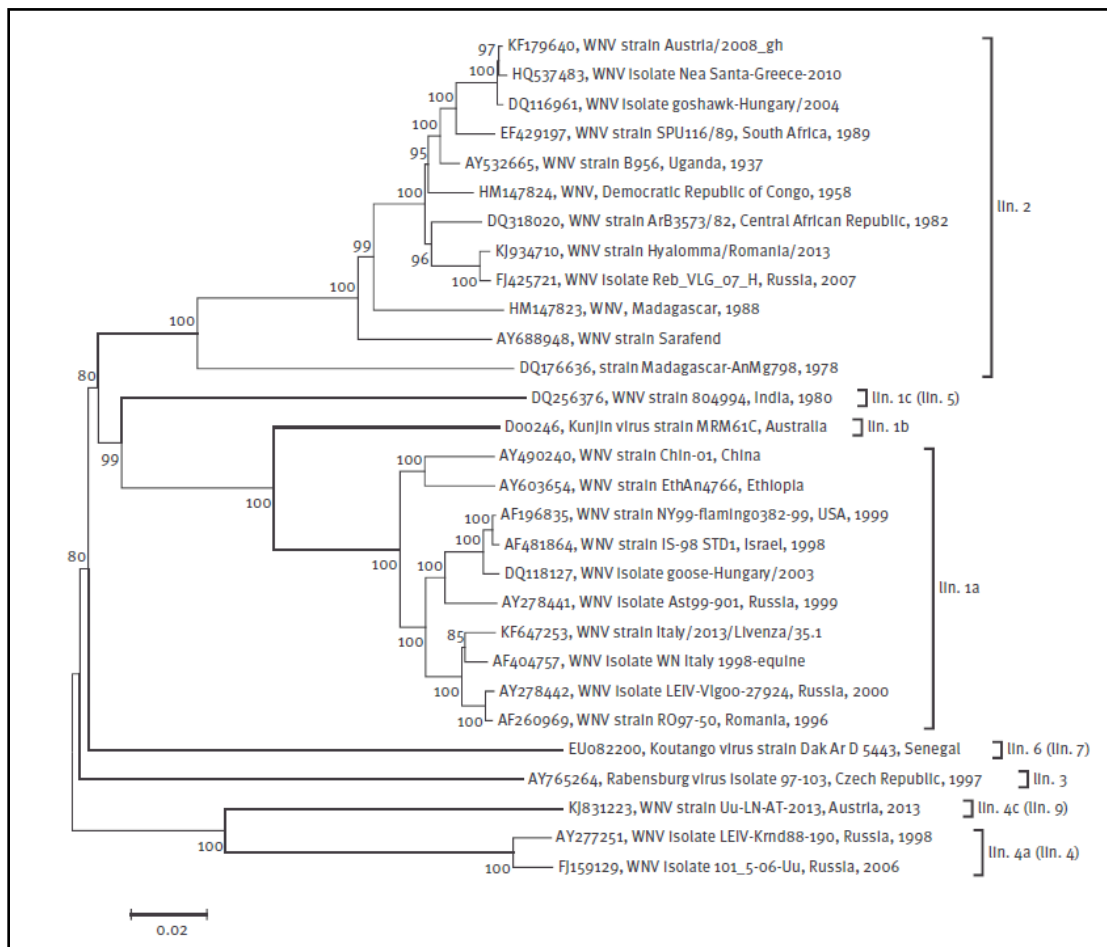


Figure 8. Phylogenetic tree of WNV lineages based on complete genome sequences (Rizzoli et al., 2015).

Lineage 1 is the most widespread, containing isolates found in Europe, North America, Asia, Africa and the Middle East (Chancey et al., 2015; Rizzoli et al., 2015). This lineage is further divided into different clades: WNV-1a is found mainly in Africa, Europe, North America and Asia (May et al., 2011). WNV 1-b contains the Australian Kunjin virus (May et al., 2011). Clade 1c of lineage 1, formerly considered lineage 5, includes isolates from India (Bondre et al., 2007; Rizzoli et al., 2015).

Lineage 2 strains are mainly distributed in Sub-Saharan Africa and Madagascar, and detected in humans and horses (Venter and Swanepoel, 2010). But in the last decade, Lineage 2 WNV has been introduced in Europe. Ciccozzi and collaborators (2013) suggest, based on E and NS5 gene analysis, that there were at least two introductions of this lineage to Central Europe in 1999 and to Russia in 2000 from the African continent. Since 2004, strains have been observed in Hungary from birds of prey (Bakonyi et al., 2006). In 2010, it caused outbreaks in Romania and Greece (Valiakos et al., 2011) and in 2011, it was detected for the first time in Italy (Ciccozzi et al., 2013).

Lineage 3 contains strains circulating in certain *Culex* and *Aedes* mosquito species in southern Moravia, Czech Republic; and it is not known to be pathogenic to mammals (Bakonyi et al., 2006). Lineage 4 comprises three clades of viruses as well: clade 4a was detected in a tick specimen from the Caucasus (May et al., 2011). Clade 4b, previously noted as lineages 6 and 7, contains a number of isolates from *Culex pipiens* mosquitoes captured in Spain in 2006 (Vazquez et al., 2010). And, Clade 4c, previously classified as lineage 9, comprises viral strains isolated from *Uranotaenia* mosquitoes in Austria (Rizzoli et al., 2015). A re-classification of Sarawak Kunjin virus as lineage 6 has been proposed as this strain is different from other Kunjin viruses

(Lineage 1-b). Lineages 6 (called Koutango virus) and 7 comprise isolates of the virus from Senegal (Chancey et al., 2015; Rizzoli et al., 2015).

4. Virus description and genome structure

WNV virion is a 50 nm icosahedral particle, enveloped by a lipid bilayer (Figure 9). It has a single stranded positive-sense RNA genome of approximately 11 kb (Knipe and Howley, 2013). The virion presents a host-derived lipid membrane surrounding a nucleocapsid core (Knipe and Howley, 2013). The envelope, derived from the host cell membrane, is composed of two virally derived major proteins: protein E (association with the outer viral envelope) and protein M. The protein E is a viral hemagglutinin and the primary target of the host immune response while the protein M acts as a stabilizing factor to protect the protein E as it is transported from the infected cell during replication (Schweitzer et al., 2009).

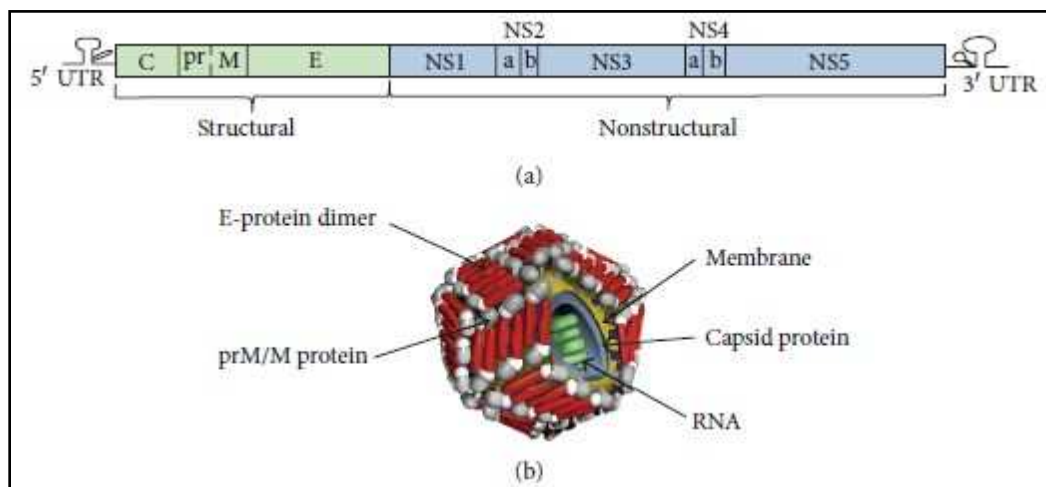


Figure 9. WNV genome organization and virion composition: (a) the viral genome is represented with one Open Reading Frame that includes 10 genes: 3 encode for structural proteins and 7 for nonstructural proteins. (b) Structure of WNV virion showing membrane and capsid proteins enveloping the RNA (Chancey et al., 2015).

The genome contains 10 genes flanked by 5' and 3' non coding regions (NCR) without a polyadenylation tail at the 3' end (Knipe and Howley, 2013). WNV genome has a single open reading frame (ORF) and encodes one large polyprotein that is co- and post-translationally processed and cleaved into 3 structural proteins that are encoded in the 5' end of the genome, and 7 non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) proteins that are encoded in the 3' end (Knipe and Howley, 2013).

Structural proteins of WNV include: the nucleocapsid protein (C); a precursor glycoprotein (prM) that is cleaved during virus maturation to yield the transmembrane protein (M); and the major spike glycoprotein of the envelope (E) (Knipe and Howley, 2013). The nucleocapsid is composed of C protein, which associates with the RNA genome and mediates viral assembly (Knipe and Howley, 2013). Heterodimers of prM and E protein become embedded in the lipid bilayer of the virus during assembly and are exposed on the virion surface until cleavage during the viral maturation process (Knipe and Howley, 2013). The E protein mediates binding of the receptor on the cell surface for viral entry. It also helps fusion with the membrane of the host cell and is considered the major target for neutralizing antibodies (Knipe and Howley, 2013).

All seven non-structural proteins appear to be directly or indirectly involved in viral RNA synthesis. NS1 plays an early role in regulation of viral RNA replication (Knipe and Howley, 2013). NS2A, NS2B, NS4A, and NS4B are small, hydrophobic proteins that do not have conserved motifs characteristic of known enzymes. NS3 has multiple enzymatic functions, serving as a viral serine protease which cleaves the other non-structural proteins from the viral polyprotein, in association with NS2B; and an RNA helicase in association with NS4A (Knipe and Howley, 2013). NS5 is the C-terminal protein of the viral polyprotein and is the largest and most conserved of the mature flavivirus proteins. It is necessary for viral replication, containing

RNA-dependent RNA polymerase activity in the C-terminal region and methyltransferase activity in the N-terminal region (Knipe and Howley, 2013).

The presence of the envelope makes WNV, like other Flaviviruses, susceptible to organic solvents. Moreover, these viruses are easily inactivated by heat and by common disinfectants (Knipe and Howley, 2013; Maclachlan and Dubovi, 2010).

5. Replication

WNV replicates well and cause cytopathic changes in many cell cultures such as Vero cell line (African green monkey kidney), and mosquito cell line (C6/36). Cellular attachment appears to be mediated by ligands on the E glycoprotein(s), although cellular receptors have not been unambiguously identified (Figure 10). After attachment, the viruses enter cells via receptor-mediated endocytosis. In the endosome, E proteins fuse the viral membrane with the endosomal vesicle membrane then release the nucleocapsid into the cytoplasm (Figure 10A). The genomic RNA is released from the capsid by an unknown mechanism and is translated (Figure 10B). The polyprotein is co- and post-translationally cleaved at multiple sites to generate the mature viral proteins (Figure 10C). The viral RNA dependent RNA polymerase (from the NS5 protein) copies complementary negative polarity strands from the positive polarity genomic RNA template (Figure 10D). The newly formed negative strand RNAs serve as templates for the synthesis of new genomic RNAs (Figure 10E). Virion assembly occurs in association with rough endoplasmic reticulum membranes (Figure 10F). Intracellular immature virions containing heterodimers of E and prM accumulate in vesicles and are then transported through the host secretory pathway where E and prM glycans are modified (Figure 10G). A portion of prM on

immature virions is cleaved in the *trans*-Golgi network and at the end, virions are transported to the plasma membrane in vesicles and released by exocytosis (Figure 10H) (Diamond, 2009).

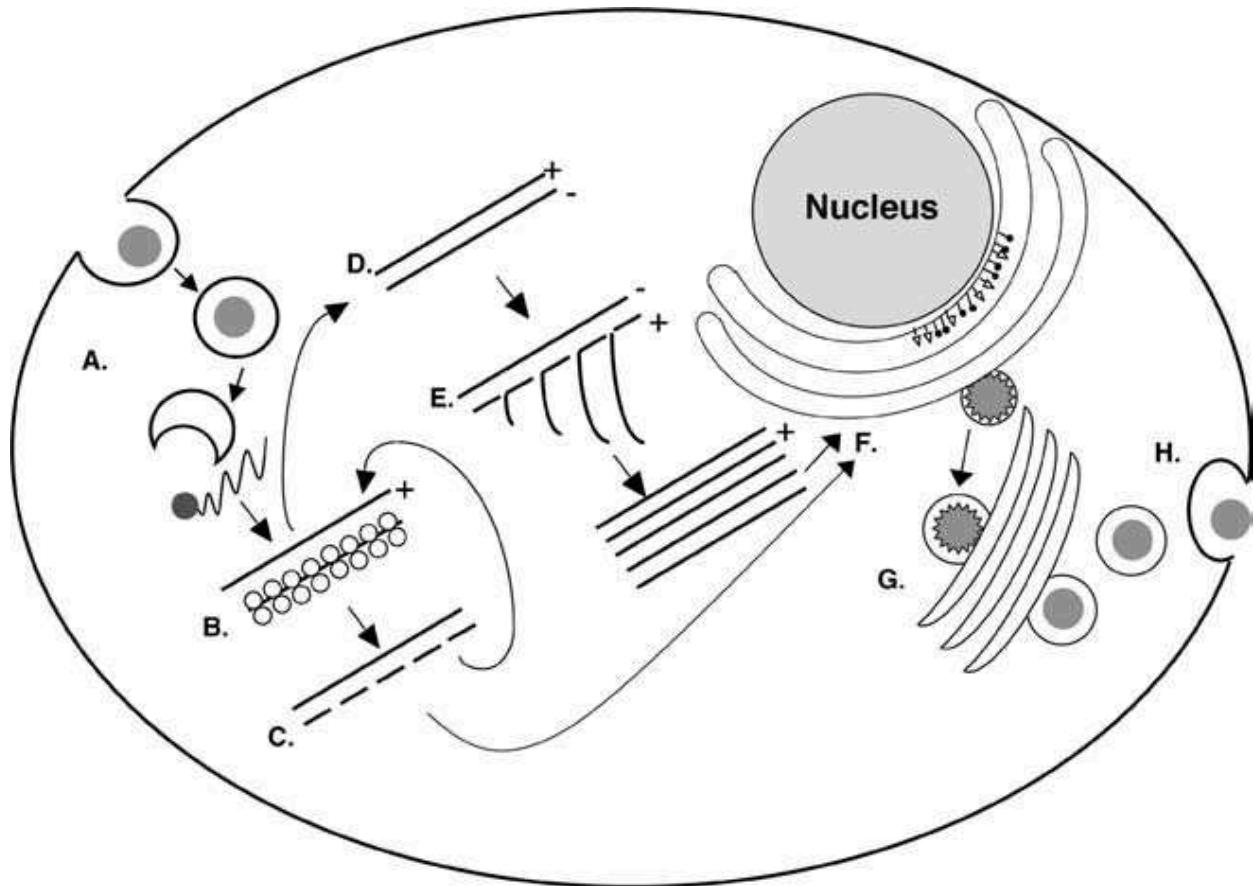


Figure 10. The replication cycle of WNV. The virus attaches to cell membrane, enters the host cell and releases the RNA that is translated and copied (A-C). The viral RNA negative and positive polarity are copied using viral RNA polymerase (D-E). The newly formed virions assemble with the endoplasmic reticulum membrane (F) then they are cleaved by the Golgi network (G). Finally virions are transported by vesicles and secreted by exocytosis (H) (Diamond, 2009).

6. Pathogenesis

The majority of WNV infections in humans are asymptomatic. Approximately, 20 to 30% of infected individuals develop flu-like clinical manifestations as an acute febrile illness accompanied by headache, polyarthropathy, rash and lymphadenopathy (Knipe and Howley, 2013). But WNV is also able to cause a neuroinvasive disease (WNND) in a limited

subpopulation of individuals (approximately 1 in 150). The clinical features of severe WNV infection vary and include muscle weakness, cognitive impairment, tremors, and a poliomyelitis-like flaccid paralysis. In some cases, infections can lead to encephalitis, causing long-term disability and rarely leading to death (Knipe and Howley, 2013).

Horses, just like humans, are dead-end hosts, meaning that while they become infected, they do not spread the infection. Horses infected with WNV mostly show no symptoms or rarely acquire mild febrile illness. However, neuroinvasive (encephalomyelitis) forms are also reported. In these cases, the disease shows reserved prognosis and 30-40% of horses would die (Maclachlan and Dubovi, 2010). Horses affected by West Nile Encephalitis (WNE) are usually euthanized. Many horses that recover from WNE have been reported to have some residual neurological deficits, such as gait abnormalities and a change in their usual temperament or behavior. The signs and course of the disease are highly variable since not all infected horses react similarly. Horses may become abnormally sensitive to sights, sounds, or touch. They may present signs of discomfort or anxiety, low-grade fever, or malaise. Neurologic signs can include muzzle twitching, impaired vision, trembling, lack of coordination, and muscle contractions. The most severe cases evolve to paralysis of the hind legs, terminal convulsion and death (Maclachlan and Dubovi, 2010).

In Europe, Africa, Middle East and Asia, mortality in birds associated with WNV infection is rare. In contrast, the virus is highly pathogenic for birds in USA (Maclachlan and Dubovi, 2010). Infection may result in a very high viremia titer and widespread necrosis, hemorrhage, and inflammation in several organs including heart, lungs, spleen, liver, kidney, and nervous system. These severe infections might lead to death (Maclachlan and Dubovi, 2010). In Serbia, evidence of WNV circulation (neutralizing antibodies or viral RNA) was reported in several bird species

such as Northern Goshawks (*Accipiter gentilis*), White-tailed Eagles (*Haliaeetus albicilla*), Legged Gull (*Larus michahelis*), Hooded Crow (*Corvus cornix*), Bearded Parrot-bill (*Panarus biramicus*), and Common Pheasant (*Phasianus colchicus*) (Petrović et al., 2013). In Southern France and Southern Spain, House sparrow (*Passer domesticus*) and Magpie (*Corvidae* family) birds were found infected with WNV (Balança et al., 2009; Ferraguti et al., 2016). In USA, Corvid species appear to be susceptible for WNV infection. Following the large outbreak in 1999, crows in addition to flamingos were found highly infected with this virus (Steele et al., 2000).

7. Diagnosis

The clinical signs of WNV infection among humans and horses are characteristic but not pathognomonic. Routine clinical laboratory analysis do not distinguish WNV infection from many other viral infections. Thus the diagnosis must be confirmed by further laboratory tests based on the detection of WNV RNA in blood and cerebrospinal fluid (CSF) or virus isolation in cell culture from serum samples (direct diagnosis) or the detection of a specific immune response against the virus (indirect diagnosis) (Rizzoli et al., 2015).

To date, the most consistent manner to verify WNV infection is serology (Rossi et al., 2010). Serological tests are performed on serum or cerebrospinal fluid (CSF) of acute or convalescent cases to determine the WNV specific antibody profile by ELISA. In order to detect recent infections, the test involves immunoglobulin M antibodies IgM-specific ELISA in which serum is collected within 8 to 21 days after the appearance of clinical symptoms. This test provides strong evidence of recent WNV infection. For seroprevalence studies, immunoglobulin G (IgG) antibodies are sought out by ELISA. One disadvantage of ELISA test is cross-reactivity with

infections caused by other Flaviviruses. Thus, Plaque reduction neutralization test (PRNT) is the gold standard method used for further confirmation of WNV infection (Rossi et al., 2010).

Moreover, virology tests can directly confirm the presence of the virus in patient serum or CSF. The virus is first amplified within permissive cells (such as Vero cells) and then the viral identity is confirmed by molecular methods. Nevertheless, molecular tools can be directly employed to confirm the presence of virus (or its RNA). Different quantitative reverse transcription polymerase chain reaction (RTqPCR) protocols have been developed to amplify minimal amounts of WNV RNA. But, viraemia is short-lived in dead-end hosts, so detection of viral genomes becomes increasingly challenging over time. Recent studies demonstrated that WNV RNA can be detected in urine much longer (up to 20 days after the onset of symptoms) and at higher concentrations than in serum or CSF in individuals infected with WNNND (Rizzoli et al., 2015). But the problem of PCR remains in certain protocols to miss some circulating WNV lineages, therefore a multiplex PCR is advised in order to avoid such issues (Rizzoli et al., 2015).

The presence of reactive lymphocytes or monocytes in CSF samples is indicative of WNV neurologic infection. In patients with WNV neuroinvasion, more than 40% of cells in the CSF are neutrophils. Furthermore, Magnetic Resonance (MR) imaging suggests abnormalities in the brain and meninges of WNV infected patients presenting a central nervous system disease (Rossi et al., 2010).

8. Treatment and vaccine development

Treatment for WNV infections is mainly supportive. No specific antiviral therapy is available. Management is focused on pain control and inflammation. There are no licensed

human WNV vaccines, but there are multiple equine vaccines. The development of safe and effective vaccines against WNV remains as a high priority. The only preventive methods to limit the spread of WNV are based on controlling the vector population and on vaccinating horse populations in endemic countries. Vector control is performed using several techniques such as personal prevention, the use of insecticides, impregnated bed nets and the implementation of some challenging genetic control protocols.

Three vaccine formulations are currently available for prevention of WNV in equids: two are inactivated vaccines and one is a non-replicating live canary pox recombinant vector vaccine (De Filette et al., 2012). The first licensed vaccine contains a formalin-inactivated whole WNV. This vaccine is currently commercialized in USA under the trade name West Nile-Innovator®. Another killed virus vaccine (Vetera® WNV vaccine) was developed and licensed. A third commercialized WNV vaccine in USA for horses is Recombitek® Equine West Nile Virus Vaccine which is a chimeric recombinant canary poxvirus vaccine. It expresses the prM and E genes derived from a 1999 New York isolate of WNV (De Filette et al., 2012). Administration, dosage and vaccination schedule differ between these three vaccines.

Although no approval from FDA (Food and Drug Administration) was obtained, vaccines for human use exist and currently, there are a number of ongoing clinical trials. There are several strategies being pursued for WNV vaccine development for humans including inactivated, recombinant live virus, chimeric virus (ChimeriVax-WN), DNA, and recombinant subunit vaccines (De Filette et al., 2012). ChimeriVax-West Nile vaccine (Acambis, Sanofi-Pasteur) utilizes the attenuated Yellow Fever Virus (YFV) vaccine strain to build a live chimeric virus that consists of the prM and E proteins of WNV in the context of the YFV capsid and non-structural proteins. It is the most advanced vaccine in development, and currently, it has

progressed beyond the second part of clinical phase II trial (De Filette et al., 2012). Although the live vaccine candidates induced high humoral and cell-mediated immune responses similar to that obtained with wild-type WNV, vaccination has raised some safety concerns in susceptible individuals (De Filette et al., 2012). Long-term immunogenicity, safety and cost effectiveness are the most important problems in current development of vaccines against WNV and potential candidates for use in humans.

Chapter 3

Rift Valley Fever Virus

Rift Valley Fever Virus (RVFV) is a mosquito-borne virus that affects humans and livestock. The main enzootic vectors belong to the *Aedes* genus. However, several *Culex* species, including *Cx. pipiens* are considered secondary vectors and contribute to the transmission of the virus.

1. Transmission cycle

RVFV circulates following two types of cycles: an enzootic cycle that occurs during the average rainfall season and an epizootic-epidemic cycle that occurs during the heavy rainfall season (Figure 11). During the enzootic cycle, the virus is maintained through transovarial transmission between the female mosquito belonging to the *Aedes* genus and her eggs and occasionally, through amplification in a vertebrate host such as African buffaloes. During the epizootic-epidemic cycle, the virus is over-amplified with massive hatching of *Aedes* eggs (including those RVFV-infected) following an abnormally high rainfall or floods. Obtained infected adults will infect susceptible livestock triggering an epizootic phase. A very high level of viremia is maintained for 3 to 5 days in infected sheep and cattle, allowing many more mosquitoes to become infected when feeding on infected animals. These mosquitoes may be transported for long distances in low level wind or air currents, which may lead to the rapid spread of the virus from region to region. Humans can become infected following the bites of *Aedes* (as a primary vector) or *Culex* (as a secondary vector) mosquitoes but the majority of human cases are thought to result from handling infected fluids and tissues: blood, secretions or

excretions of infected animals, notably after abortion via contaminated placentae (Maclachlan and Dubovi, 2010).

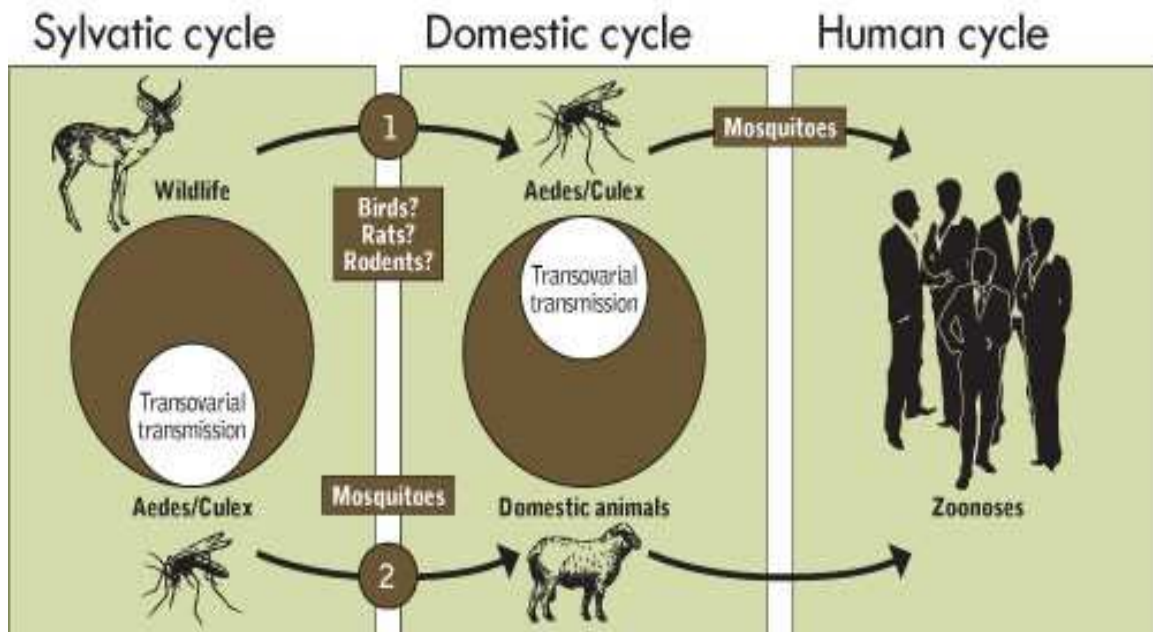


Figure 11. RVFV transmission cycle. An enzootic (sylvatic) cycle occurs during the average rainfall season in which the virus is maintained through transovarial transmission and through amplification in a vertebrate host. An epizootic-epidemic (domestic) cycle occurs during the heavy rainfall season in which the virus is over-amplified with massive hatching of mosquito eggs (FAO, 2017).

2. Geographic distribution

RVFV was first identified in an outbreak of abortions and deaths in wool sheep and illness in humans that occurred in the Rift Valley of Kenya after heavy rainfalls in 1930-1931 (Daubney et al., 1931).

Since then, outbreaks have been reported in the African region extending from sub-Saharan (Himeidan et al., 2014) up to Mauritania (El Mamy et al., 2011) (Figure 12). In Egypt in 1977, RVF was introduced via infected livestock trade along the Nile irrigation system and caused a large outbreak among both animals and humans resulting in over 600 human deaths (Meegan, 1979). Another example of RVF spillover into human populations occurred in West Africa in

1987, and was linked to construction of a dam on the Senegal river (Guillaud et al., 1988). The project caused flooding in the lower Senegal River area, altering ecological conditions and interactions between animals and humans. As a result, a large RVF outbreak occurred in animals and humans. In 1997-1998, a major outbreak occurred in Kenya, Somalia and Tanzania following El Niño event and extensive flooding (Woods et al., 2002). Following infected livestock trade from the horn of Africa, RVF spread in September 2000 to Saudi Arabia and Yemen (Ahmad, 2000; Madani et al., 2003), marking the first reported occurrence of the disease outside the African continent and raising concerns that it could extend to other parts of Asia and Europe.

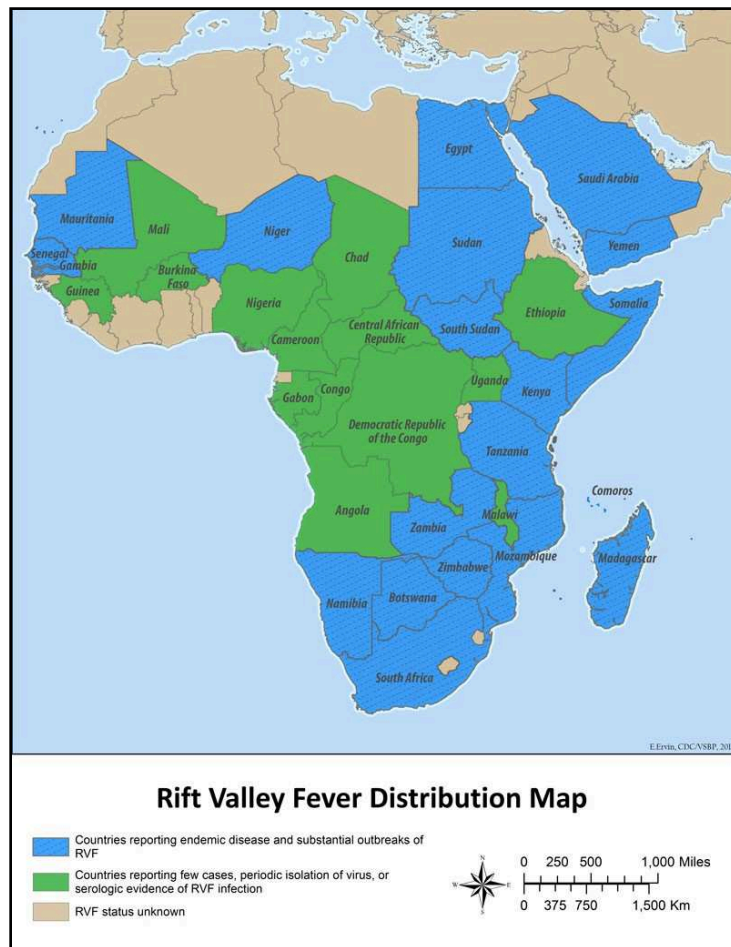


Figure 12. RVFV geographic distribution (CDC, 2017).

During this outbreak, 516 cases with 87 deaths of RVF were reported by the Ministry of Health of Saudi Arabia while the Ministry of Public Health in Yemen reported 1087 suspected cases including 121 deaths (Ahmad, 2000; CDC, 2017; Madani et al., 2003; WHO, 2017). The African continent remains the most affected. Outbreaks continue to be reported from different countries. In 2003 in Egypt, there were 148 cases including 27 deaths of RVF reported by the Ministry of Health of Egypt (CDC, 2017; WHO, 2017). Several other outbreaks occurred in Kenya (2006, 2007), Tanzania (2007), Somalia (2007), Sudan (2007), Madagascar (2008, 2009), and Republic of South Africa (2010) (CDC, 2017; WHO, 2017).

In October and November 2010, an outbreak of RVF was reported in the northern part of Mauritania (El Mamy et al., 2011). Cases in animals and humans seemed to occur shortly after intense and unusual rainfalls (Faye et al., 2014). Severe clinical signs and high mortality was described among camel. At least, 63 human cases were noted including 13 deaths (Faye et al., 2014). RVFV strains were isolated from humans and also from *Cx. antennatus* mosquitoes (Faye et al., 2014). In 2012, a large but unspecified number of ruminants were affected, together with more than 34 human cases, including 17 deaths, from six regions in Mauritania. Most of the human cases had a history of contacts with animals or sick animals (Sow et al., 2014). Then, from September 2013, domestic ruminants and humans from Senegal were tested positive for RVFV by RT-PCR in several regions (Sow et al., 2016). The outbreak coincided with preparations for the annual Muslim celebration (Sow et al., 2016). From September to November 2015, 31 hospitalized patients who presented severe clinical forms of RVF disease were subsequently tested positive for the virus. At least, eight people died (Boushab et al., 2016).

Most recently, in 2016 in Uganda, CDC localized an outbreak of three confirmed and two probable human cases. In addition, in August 2016, health authorities in Niger reported cases of

febrile illness, including hemorrhagic symptoms, in humans, as well as sickness and abortions in ruminants. Subsequent diagnostic testing confirmed 17 RVF human cases and 33 deaths (CDC, 2017).

3. Classification and lineages distribution

RVFV belongs to the Bunyaviridae family, *Phlebovirus* genus comprising 70 viruses that can be divided in 2 groups: the sandfly fever virus group and the Uukuniemi-like virus group (Elliott and Brennan, 2014). There are important differences between these groups; notably that the Uukuniemi-like viruses do not encode a nonstructural protein and they are all transmitted by ticks. The sandfly fever group are transmitted by dipterans (phlebotomines and mosquitoes). This group contains important human and animal pathogens, including RVFV and the sandfly fever viruses (Maclachlan and Dubovi, 2010).

A number of studies on the genetic diversity of RVFV have been published over the past years revealing an increasing number of lineages. These studies showed that the genetic diversity of RVFV across all lineages is limited, with maximum pair-wise differences for the S segment and M segments of 4% and 5.4% for the nucleotide sequence and 1% and 2.8% for the amino acid sequence, respectively.

Extensive analysis of the complete genomes (S, M, and L segments) of 33 diverse RVFV, via high-throughput technique, showed that this virus can be separated into 7 distinct genetic lineages (A to G) (Bird et al., 2007). RVFV strains with diverse geographic origins can be found in each lineage, which is indicative of widespread dispersal and movement of the virus genotypes throughout Africa.

Grobbelaar and collaborators (2011) analyzed five derived strains and 198 isolates of RVFV that have circulated over 67 year period. It showed that despite low genetic diversity, these isolates resolved into 15 lineages (A–O): Six lineages (B, D, F, J, O and I) contained isolates from South Africa, six other lineages (A, C, E, G, H and N) contained clusters of isolates associated with outbreaks in individual countries or regions and three lineages (K, L, and M) consisted of isolates having a common root node deriving from strains isolated from the same mosquito species (Grobbelaar et al., 2011).

A more recent study compared sequences from the three mini-genomic segments of viral strains from 18 countries across Africa plus Saudi Arabia. In this study, 11 RVFV lineages (A–K) were identified (Samy et al., 2017). The differences between this phylogeny and the previous ones could be the result of a larger dataset.

4. Virus description and genome structure

RVFV virions, as the majority of Bunyaviruses, are spherical, approximately 100 nm in diameter, and are composed of a lipid envelope with glycoprotein spikes (Figure 13). Inside it, there are three circular ribonucleoprotein (RNP) complexes comprised of individual genome RNA segments associated with the viral nucleoprotein (Maclachlan and Dubovi, 2010; Mansfield et al., 2015).

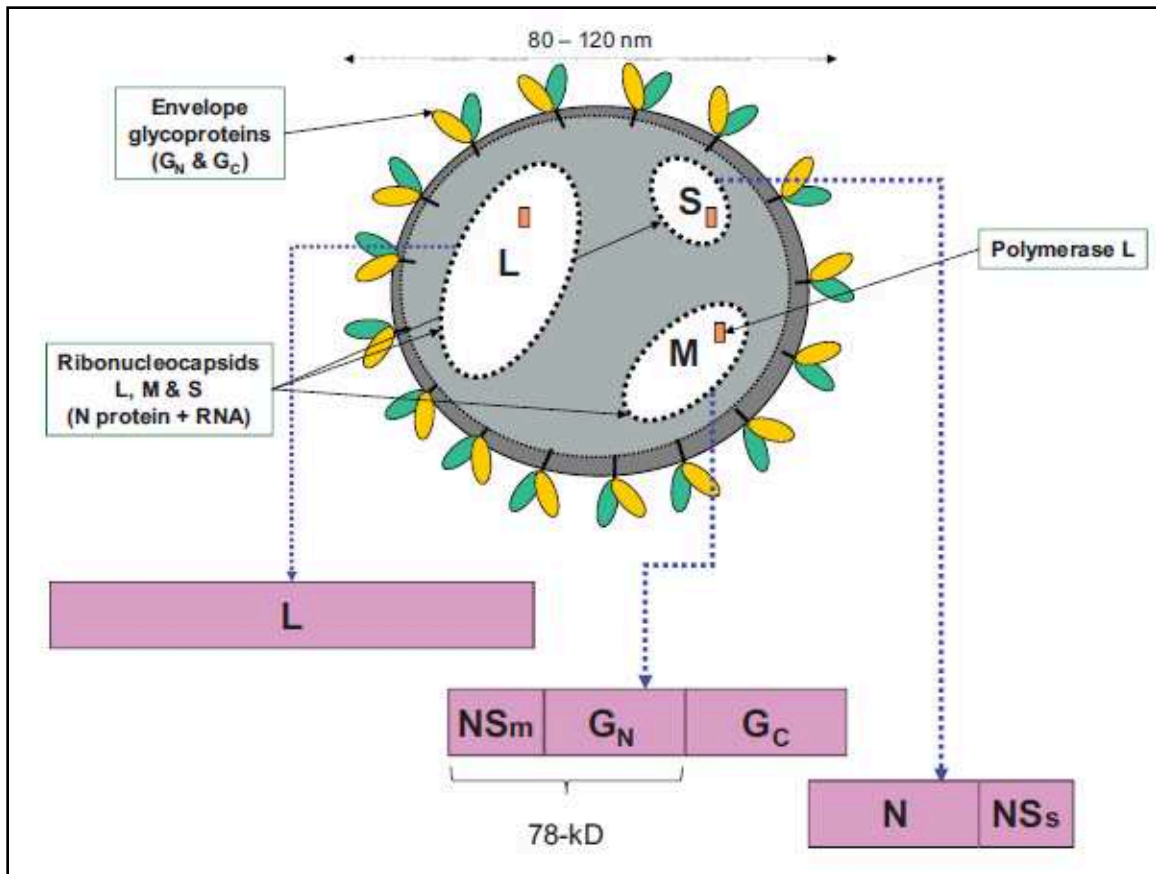


Figure 13. Schematic diagram of RVFV structure. The virion is enveloped by glycoproteins and comprises three segments (S, M and L) of RNA encoding for structural and nonstructural proteins (Pepin et al., 2010).

The genome of RVFV as all viruses belonging to the Phlebovirus group, is approximately 12 kb and consists of three segments of negative-sense (or ambisense), single-stranded RNA, designated large (L), medium (M), and small (S) (Figure 13). The RNA segments differ in size: L RNA segment is of 6.4 kb, the M RNA segment is of 3.2 kb, and the S RNA segment is of 1.7 kb (Maclachlan and Dubovi, 2010). The L RNA encodes a single large protein L, the RNA-dependent RNA polymerase (transcriptase). The M RNA encodes a polyprotein that is processed to form two glycoproteins (Gn and Gc) and a non-structural protein (NSm). The S RNA, an ambisense segment (single-stranded RNA containing both positive-sense and negative-sense sequences), encodes the nucleocapsid (N) protein and a non-structural (NSs) proteins (Maclachlan and Dubovi, 2010). The N and NSs proteins of RVFV are each translated from a

separate subgenomic mRNA. The non-structural NSs protein interferes with the innate host-cell antiviral response via inhibition of the cell signaling molecules leading to global suppression of the type I interferon response (Maclachlan and Dubovi, 2010; Mansfield et al., 2015).

RVFV, like other Phleboviruses, is sensitive to heat and acid conditions, and is easily inactivated by detergents, lipid solvents, and common disinfectants (Maclachlan and Dubovi, 2010).

5. Replication

RVFV, like most Bunyaviruses, replicates in Vero (African green monkey) cells, BHK-21 (baby hamster kidney) cells, and C6/36 mosquito (*Aedes albopictus*) cells.

Viral entry into its host cell occurs by receptor-mediated endocytosis; all subsequent steps take place in the cytoplasm. After penetration and uncoating, the virion RNA polymerase (transcriptase) is activated and RNA is transcribed to viral mRNAs from each of the three virion RNAs (the S, M and L segments) (Maclachlan and Dubovi, 2010). These proteins mature by budding into smooth-surfaced vesicles in or near the Golgi apparatus of the host cell to be eventually released.

6. Pathogenesis

Infected people remain mainly asymptomatic; otherwise, the disease begins after a very short incubation period (2 to 6 days) with mild fever, severe headache, chills, “back-breaking” myalgia, diarrhea, vomiting. Usually the clinical disease lasts 4 to 6 days, followed by a prolonged convalescence and complete recovery. In 1% to 2% of affected individuals, the disease can become more severe including fulminant hepatitis, encephalitis, retinitis, blindness,

or a hemorrhagic syndrome. Among severely affected persons, the case fatality ratio is approximately 10% to 20% (Madani et al., 2003).

Affected cattle, water buffaloes, sheep and goats often remain asymptomatic; but fever lasting 24 to 96 hours, dull coat, lacrimation, nasal discharge, excessive salivation, anorexia, weakness, bloody diarrhea, low milk yield, and high abortion rates in pregnant cows are common symptoms. In ruminant livestock, mortality rate reaches 100% in neonates and 10 to 20% in adults (Bird et al., 2009; FAO, 2017).

7. Diagnosis

RVF epidemics should always be strongly suspected when there is a sudden onset of large numbers of abortions in sheep, goats, cattle or camels and deaths in lambs, or calves (Maclachlan and Dubovi, 2010). Rapid diagnosis aiming for virus detection is achieved using RT-PCR assays, a very sensitive technique able to detect approximately 5 to 10 RNA copies/sample. Consequently, it allows for the detection of infection during the early phase of the disease. This technique is easily adapted to high-throughput use during epizootics and epidemics but can detect viral nucleic acids only during the viremic period, which is relatively transient.

Moreover, the presence of the virus can be confirmed by virus isolation in cell culture such as Vero E6 and BHK-21 cells. The virus is rapidly cytopathic and forms plaques (Maclachlan and Dubovi, 2010). In this case, nucleic acid sequencing is used to prove the identity of isolates. Virus culture requires high safety level setting and usually conducted in reference laboratories.

Serologic diagnosis of acute infection is commonly used to seek for the presence of immunoglobulin (IgM) capture on sera samples by enzyme immunoassays, neutralization or hemagglutination-inhibition assays on sera samples from surviving animals (Maclachlan and

Dubovi, 2010). However, the duration of detectable anti-RVF IgM antibody in these animals is limited (approximately 60 to 90 days) (Morvan et al., 1992). Tests for detection of total RVF virus-specific immunoglobulins (IgM and IgG) in cattle, sheep, and African buffaloes became commercially available (Paweska et al., 2005). Although this assay is unable to differentiate acute and convalescent phases of infection, it may be useful in emergency situations. The Virus Neutralization Assay is the golden standard assay that is recommended by the World Organization for Animal Health (OIE) for international animal trade. But, this technique can only be performed in adequate biosecurity facilities.

8. Treatment and vaccine development

There is no specific treatment for RVF. The most widely tested antiviral agent is the nucleoside analogue ribavirin along with its closely related chemical derivatives. However, the limited penetration of ribavirin across the blood-brain barrier may limit the effectiveness of this treatment in preventing delayed-onset neurologic disease.

To date, there is no licensed vaccines against RVF available to immunize humans but various strains for livestock and ruminants are licensed and commercially produced such as the live-attenuated Smithburn vaccine, Formalin-Inactivated vaccine, and Clone 13 (a form of freeze dried live attenuated virus) (Alhaj, 2016). Although, the first two vaccines are widely used in control, both of them were accompanied by significant concerns. The first one requires multiple doses for protection, causes abortion in pregnant ewes, and has teratogenic effects; and the second has a risk of causing abortion and fetal malformation in pregnant animals especially in European breeds (Botros et al., 2006). For Clone13 vaccine, it is based on an attenuated strain which contains a large deletion in the main virulence factor, the NSs gene (Muller et al., 1995).

This strain was shown to be highly immunogenic and experimentally safe. However, intranasal inoculation with this attenuated virus has been shown to cause severe neurological disease in mice (Dodd et al., 2014), implying that NSs-deleted viruses may still retain some virulence. Moreover, the use of this vaccine during the first trimester of gestation remains controversial and was associated with malformations and stillbirths (Alhaj, 2016).

A newly developed live-attenuated MP-12 vaccine in USA is in clinical trials. It has been developed for both animal and humans but was conditionally licensed for animal vaccination (Ikegami, 2017). The MP-12 vaccine was developed from the pathogenic ZH548 strain, it is well attenuated, and a single dose induces neutralizing antibodies in ruminants (Ikegami, 2017). This attenuated viral strain contains mutations in the three viral gene segments (Ikegami, 2017; Mansfield et al., 2015). Vaccination trials results have been encouraging with minimal teratogenic effects seen in ruminants and suitable immunogenicity in non-human primates (Ikegami, 2017).

Control is based primarily on livestock vaccination, but vector control via the use of larvicides and insecticides is also applied during outbreaks to kill mosquitoes. In addition, the management the livestock export during RVF epizootic periods is also mandatory.

Chapter 4

Vector-virus interactions

Arboviruses are dependent on intricate relationships with their arthropod vectors and vertebrate hosts, and these relationships are further shaped by environmental conditions.

1. Vector Capacity and vector competence

The inter-relationships between viruses, vectors and vertebrate hosts are complex and dynamic and shaped by several biotic (viral strain, vector genetics, host susceptibility) and abiotic (temperature, rainfall, human land use) factors (Kramer, 2016).

The Vector Capacity (VC) is the measure of a mosquito population's capacity to transmit an infectious agent to a new susceptible population (Kramer, 2016). This capacity integrates biotic and abiotic factors and is quantified by the following formula as reviewed by Kramer (2016):

$$VC = [ma^2 (I*T) p^n] / -\ln(p)$$

in which:

- m is the vector density in relation to the host
- a is the probability that a vector feeds on a host in one day
- p is the probability that a vector survives one day
- n is the duration of the extrinsic incubation period (EIP) in days
- $1/-\ln(p)$ is the duration of the vector's life in days after surviving the EIP
- I is the infection rate and T is the transmission rate, so I*T represents the vector competence.

This equation shows that the vector capacity implicitly depends on both, extrinsic factors such as the availability of the vertebrate host, its immune status, temperature and rainfall, the abundance of vectors; and intrinsic factors like vector survivorship, trophic preferences, the blood meal frequency and vector competence.

Vector competence is one aspect of vector capacity. It defines the ability of the mosquito to become infected, replicate and transmit the virus to a host following an infectious blood meal (Kramer, 2016). Vector competence is affected also by both extrinsic and intrinsic factors. Intrinsic factors include genetic characteristics of both the virus and the vector origins which determines the vector's receptivity to the pathogen, and extrinsic factors such as the viral dose ingested by the vector and the incubation temperature of vectors after ingestion of an infected blood meal (Hardy et al., 1983).

The study of the receptivity of mosquitoes to different arboviruses has shown that the virus-vector relationship could be very specific. Different species, subspecies or even populations of mosquitoes do not have the same vector competence to the same virus. For instance, *Culex pipiens* mosquitoes, collected from different areas in the Maghreb region, showed diverse viral transmission capacities when infected with WNV (Amraoui et al., 2012). Another study showed that *Culex pipiens* biotypes (*molestus*, *pipiens* and their hybrid) do not show similar WNV transmission rates in the same conditions (Vogels et al., 2016). In the other way around, vector competence of one mosquito species is different when tested with different viruses or different lineages of the same virus. For example, *Culex quinquefasciatus* (of the *Culex pipiens* complex) from Florida had different transmission rates with diverse WNV isolates (Richards et al., 2014). These observations show that viral and vector genotypes may affect vector competence, but most

importantly, the interaction between these two factors shapes the characteristics of viral infection, dissemination and transmission.

As already mentioned, vector competence is influenced, not just by intrinsic factors, but also by extrinsic factors such as incubation temperature. In fact, several studies have shown that a higher temperature leads to an increase in the rate of infection and a decrease in the extrinsic incubation period (EIP). This has been demonstrated for DENV (Watts et al., 1987), the RVFV (Brubaker and Turell, 1998), WNV (Dohm and Turell, 2001) and CHIKV (Mourya et al., 2004).

Experimental observations allow us to notice that the vector competence, in particular viral transmission, depends on complex interactions between the three factors: mosquito genotype, viral genotype and temperature. It has been demonstrated that some populations of *Ae. albopictus* better transmit some strains of CHIKV at 20°C than at 28°C, whereas this is not the case for other populations of *Ae. albopictus* infected with the same viral strains (Zouache et al., 2014). This three-way interaction between mosquito population, virus strain and temperature, is known as genotype-by-genotype-by-environment (G×G×E) interactions (Zouache et al., 2014).

2. Arbovirus transmission by mosquito vectors: mechanism and barriers

The transmission of an arbovirus by the mosquito vector proceeds in several steps as shown in figure 14 (Franz et al., 2015; Hardy et al., 1983; Mellor, 2000). For the transmission to occur the vector should first be attracted and feed on a viremic host. The ingested virus within the blood meal must then rapidly penetrate into the epithelial cells of the intestine before it is altered by the digestive enzymes. Due to specific recognition by interaction of a viral protein with a receptor present on the surface of the digestive epithelium, the virus penetrates into the epithelial cell by endocytosis and replicates there (Figure 14, steps 1 and 2). After replication, newly

formed virions must cross the basal lamina which supports the digestive epithelium and then be released into the general cavity of the insect or hemocoel where circulates the hemolymph (arthropod circulatory fluid) (Figure 14, step 3). Once the virus has reached the circulatory system, it can be disseminated in various tissues and organs such as muscle tissues, nervous tissues, fatty body, reproductive organs and salivary glands (Figure 14, step 4). The dissemination to the salivary glands allows the excretion of the virus in the saliva and its potential transmission during a subsequent blood meal (Figure 14, step 5). Thus, the mosquito's gastrointestinal tract and salivary glands appear to be the two main physical barriers to the transmission of the virus by a mosquito (Franz et al., 2015).

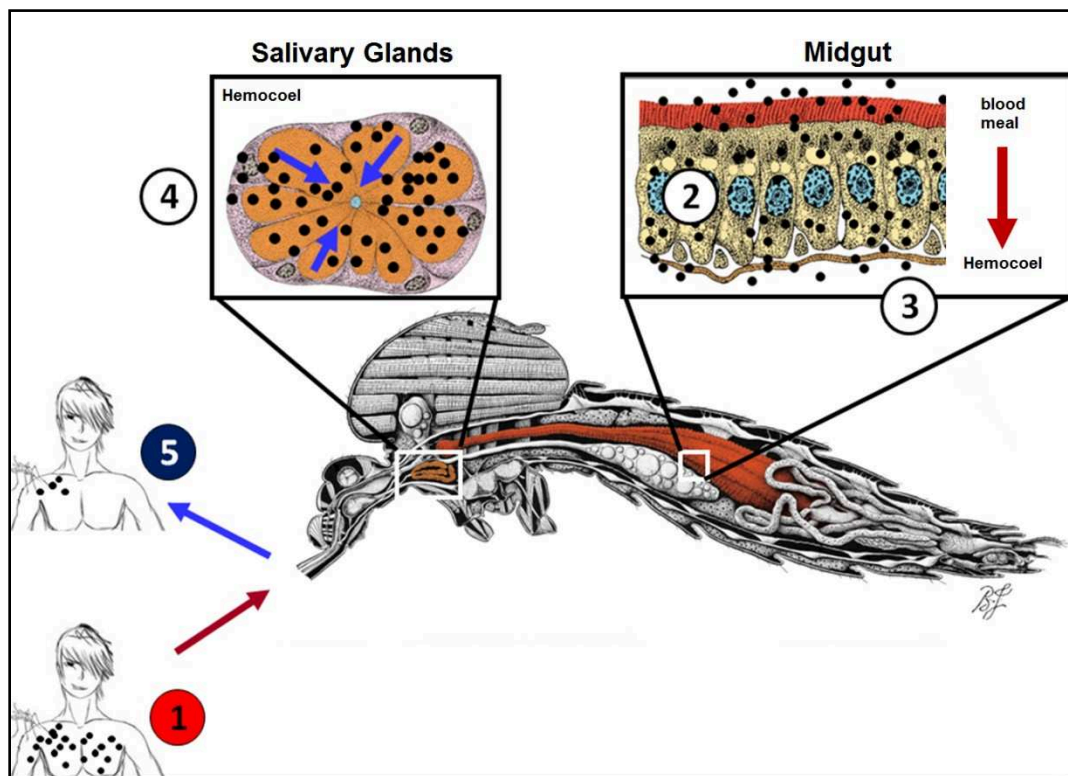


Figure 14. Steps of virus dissemination in the mosquito vector: 1. Ingestion of an infectious blood meal. 2. Viral replication and passage in the midgut cells. 3. Virions pass in the hemocoel. 4. The virus infects secondary organs such as salivary glands. 5. The virus is secreted in saliva and other hosts may be infected.

Adapted from AIV, IP.

It is largely known that the mosquito defense system is unable to clear arboviral infections but can effectively limit viral dissemination to a tolerable level (by the mosquito). This requires a complex interaction between the mosquito immune system and viruses which makes mosquitoes an ideal vector for virus transmission without being seriously affected.

The mosquito gut is the first-line barrier to limit viral dissemination. Studies conducted on *Ae. aegypti* suggest the existence of several pathways to restrict infections by arboviruses, including Flaviviruses: the RNA interference (RNAi) pathway, the Toll pathway, and the JAK–STAT pathway. Moreover, the gut microbial flora is also considered an important factor to control the midgut viral infection (Cheng et al., 2016).

In the hemocoel, the virus is immersed in a nutrient-rich environment that contains immune cells (innate cellular responses) and humoral response factors produced by hemocytes pericardial cells and fatty substances. Although mosquito hemocytes are permissive for the replication of arboviruses, humoral immunity in the hemolymph plays an important role in limiting systemic dissemination of these arboviruses (Xiao et al., 2014). The humoral components refer to soluble molecules in hemolymph such as pattern recognition receptors, antimicrobial peptides, and components of the phenoloxidase (PO) cascade that leads to the formation of melanin (Cheng et al., 2016; Hillyer, 2010). The role of melanization in eliminating bacterial and parasitic infections in invertebrates is relatively well elucidated. However, the antiviral role of melanization is poorly understood.

The salivary glands are apparently permissive for arboviral replication (Vega Rúa, 2015). Nevertheless, recent studies revealed that, at this level, immune reactions that limit viral replication do exist (reviewed in (Cheng et al., 2016). The salivary peptide cecropin (CEC) like (an antibacterial peptide) has been suggested to have a potent anti-DENV and anti-CHIKV

activity. This antiviral activity was validated in an *in vitro* cell culture system (Luplertlop et al., 2011) and reviewed in (Cheng et al., 2016)). The virus present in the salivary glands ensures horizontal transmission to a new vertebrate host by the saliva emitted by infected females (Hardy et al., 1983).

In some cases, the virus can disseminate to mosquito's reproductive organs. The presence of the virus in the ovaries and eggs can promote its vertical transmission (vertical transmission: from infected females to eggs). This rare phenomenon allows some arboviruses to persist in the absence of receptive hosts and during dry or cold periods, or during the absence or low density of mosquitoes that adversely affects horizontal transmission (Dohm and Turell, 2001; Dohm et al., 2002; Lequime and Lambrechts, 2014).

Viral infection is generally persistent in females. Therefore, once infected, the mosquito will be able to transmit the virus throughout its life. The time between the ingestion of the infectious meal and the time the mosquito becomes capable of transmitting the virus present in the saliva corresponds to the extrinsic incubation period (EIP) (Hardy et al., 1983). EIP varies according to the dose of virus infected in the mosquito, the viral genotype, the vector genotype, and external factors such as larval nutrition and temperature (Hardy et al., 1983). For effective transmission of a pathogen, the EIP must be shorter than the life of the vector. Thus, EIP is a key parameter of viral transmission.

3. Vector competence assessment

As previously noted, the determination of vector competence is essential for evaluating the potential for transmission of arboviruses by vectors. The vector competence is unique and characteristic of each mosquito-virus combination. Indeed, the evaluation of vector competence

makes it possible to predict the epidemiological risks posed by the introduction of an arbovirus in a certain environment where the populations of local mosquitoes can present a good receptivity to the viral infection (Hardy et al., 1983; Kramer and Ciota, 2015). This helps to identify localities in need of strengthening epidemiological surveillance (control and isolation of imported cases).

The evaluation of vector competence is carried out in the laboratory using experimental infections. Mosquitoes of the species and population of interest are raised under controlled conditions of nutrients, temperature, humidity and photoperiod. Females are starved 48 hours before being fed on an infectious mixture containing fresh rabbit blood and a viral suspension of known titer is prepared. Fully engorged females are kept for analysis. The viral dynamic within these females is monitored at different days during the post-infection incubation period (π) in order to assess the vector competence. In this perspective, the following parameters are usually evaluated at all kinetic points:

- Midgut infection rate: proportion of mosquito females with infected midgut among tested ones (alive and engorged at Day 0)
- Disseminated infection rate: proportion of females with infected head or legs or wings among females with infected midgut.
- Dissemination efficiency: proportion of mosquito females with infected head or legs or wings among tested ones.
- Transmission rate: proportion of females with infectious saliva among females able to disseminate the virus beyond the midgut barrier (with infected head or legs or wings).
- Transmission efficiency: proportion of mosquito females with infectious saliva among tested ones.

Thus, the disseminated infection rate and the dissemination efficiency allow the evaluation of the ability of the virus to cross the midgut barrier of the mosquito and to disseminate to other internal organs/tissues. While the transmission rate and the transmission efficiency indicate the capacity of the virus to cross the ultimate barrier of the salivary glands to be released into the saliva of the mosquito. Therefore, transmission rate and efficiency are the main parameters for assessing the epidemic risk associated with the transmission of a given virus by local mosquitoes.

To estimate the rate and efficiency of transmission, mosquito saliva should be recovered by forced salivation technique (Dubrulle et al., 2009). Briefly, after removing the legs and wings of the female mosquito, the proboscis is inserted gently into a tip containing Fetal Bovine Serum (FBS) and the mosquito is left to salivate for 30-45 minutes (Figure 15). The infectious status and the number of infectious viral particles in collected saliva are determined by viral titration on insect (C6/36) or mammalian (Vero) cells.



Figure 15. Forced salivation technique. After removing the legs and wings of the female mosquito, the proboscis is inserted gently into a tip containing Fetal Bovine Serum.

Chapter 5

Study Objectives

During this PhD research project, we aimed to assess the circulation of WNV in Lebanon and the capacity of local strains of *Cx. pipiens* to transmit, under experimental conditions, WNV and RVFV. Therefore, our project was divided into two parts:

The first part covers the epidemiology of WNV in Lebanon and aims to provide evidence of WNV circulation by screening mosquito populations for WNV RNA and by detecting WNV specific antibodies among human, horses and chicken sample populations. This was achieved by:

- Collecting mosquitoes from two different geographical areas in the country: the Bekaa Valley and North Lebanon. Collected samples were:
 - Morphologically identified using specific keys.
 - Screened for WNV RNA by molecular analysis (RNA extraction and RT-PCR) on identified mosquito pools.
- Collecting blood samples from human residents, horses and chicken in different areas in the country. The collected sera were then analyzed by serological techniques as follows:
 - Detection of IgG or IgY antibodies against WNV by in-house ELISA.
 - Confirmation of ELISA positive sera by PRNT and estimating the circulation rate of the virus among each population.
 - Observation of potential seroconversion in sentinel chickens in order to assess potential current WNV circulation. This was performed by detecting specific IgY level increase in these sentinels during the transmission season.

The second part of this study aimed to assess the vector competence of local populations of *Cx. pipiens* against both WNV and RVFV. For this, the following activities were performed:

- *Culex pipiens* egg rafts were collected.
- Mosquitoes were reared and maintained in BSL3 facilities.
- Females of *Cx. pipiens* were artificially fed with blood infected with WNV or RVFV.
- Estimation of viral infection, dissemination and transmission rates at different days after experimental infection with each virus.
- The vector competence of both *Cx. pipiens* biotypes was compared in order to detect any genetic influence on the virus transmission.

Part 2
Empirical Studies

Chapter 6

Survey of West Nile Virus in Lebanon

Introduction

WNV is an arboviral infection caused by a flavivirus that is transmitted from infected birds to humans by the bite of a broad range of mosquito species, but mainly members of the *Culex* genus. WNV infects mostly birds which constitute the main reservoir. Humans, just like horses, are “dead-end” hosts, meaning that they might be infected but do not spread the virus. The infection is usually asymptomatic in humans. WNV infections have been reported in many tropical and temperate countries in Africa, Europe, Asia, America and the Middle East. Recent outbreaks have affected Palestine in 2000, Greece in 2010 and 2011, and Turkey in 2010. In addition, evidence of virus circulation has been reported from Jordan and Egypt.

In Lebanon, human and animal cases caused by WNV have never yet been reported. However, we suspect the existence of this disease in the country. The introduction of this virus is likely to occur through the migratory birds that usually cross Lebanon during the migration season. Besides, *Culex pipiens*, the primary vector, is widespread in the country. This species is established in the coastal zone in addition to inland locations where the stagnant water systems are scattered. During this research project, we assessed the circulation of WNV in Lebanon by screening mosquito populations for this virus and by detecting specific antibodies among human, horse and chicken populations.

Material and Methods

1. Geographical description of Lebanon

Lebanon is a mountainous country located on the eastern side of the Mediterranean Sea. It is characterized by a typical Mediterranean climate. The landscape of the country is characterized by parallel alternation of plains and high lands creating four longitudinal geographic regions (Figure 16).



Figure 16. Lebanon landscape map.

The first region is a narrow coastal strip that becomes relatively broader at the Northern part of the country near Tripoli. This region is heavily urbanized and contains the major cities. The

agricultural life is limited to the north and the south of the country in which Citrus trees are planted. During the summer, and specially July and August, the weather is hot and steamy, and humidity can reach 90%, with temperatures over 30°C. The rain falls are mainly from December to March with relatively cold weather.

The second region is formed by the Mount Lebanon chain, running parallel to the coastline, and peaking at the northern part at 3083m. It has a typically Alpine climate with important snowfall and temperatures below zero on the mountains during winter.

The third region is the Bekaa plain, which is considered part of the Great Rift Valley that extends until South East Africa. It is an inland plain, situated 1000m above sea level. The northern part of this plain is arid with an average rainfall. The Bekaa Valley is shielded from the influence of the sea by the Lebanon Mountains. Therefore, the Bekaa is drier than the rest of the country with considerably less precipitation not exceeding 100 mm in its upper part (Hermel district) and a wider variation in daily and yearly temperatures. Summers are hot and dry, while winters are cold and frosty. It is a fertile and farming region in which two rivers originate: the Orontes River in the Northern district of Hermel and the Litani River that crosses the West Bekaa region. Moreover, the Bekaa region is relatively rich in stagnant water plans such as the Qaraoun artificial lake and Aammik natural swamps which provide a favorable environment for the breeding and proliferation of mosquitoes. Besides, this region is considered an important stopover for migratory birds (Figure 17).

The fourth region is formed by the Anti Lebanon Mountains that form the natural eastern border with Syria. The range's highest peak is Mount Hermon at 2814m.

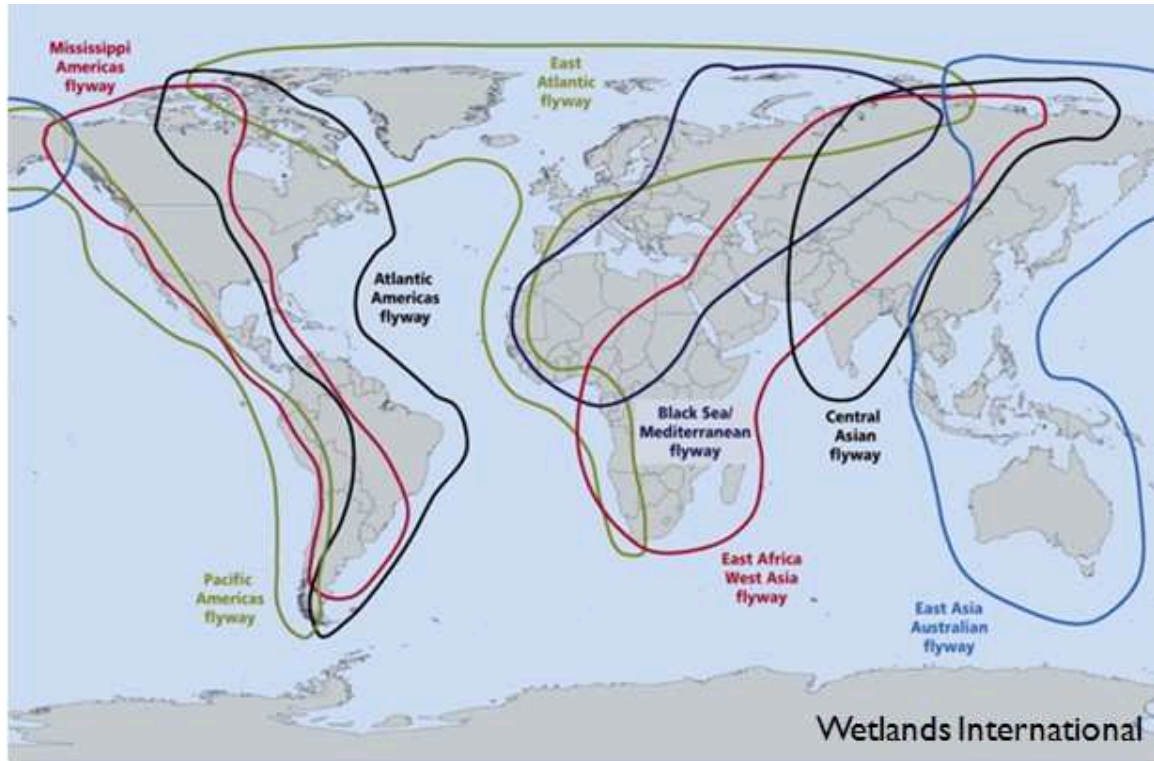


Figure 17. Flyways of migratory birds.

2. Entomological study part

a. Mosquito collections and processing

Adult mosquito specimens were collected during August and September 2014 from seven villages: 4 in the semi-arid district of West Bekaa, West of Lebanon, and 3 in Akkar district, a humid area North of the country (Figure 18).

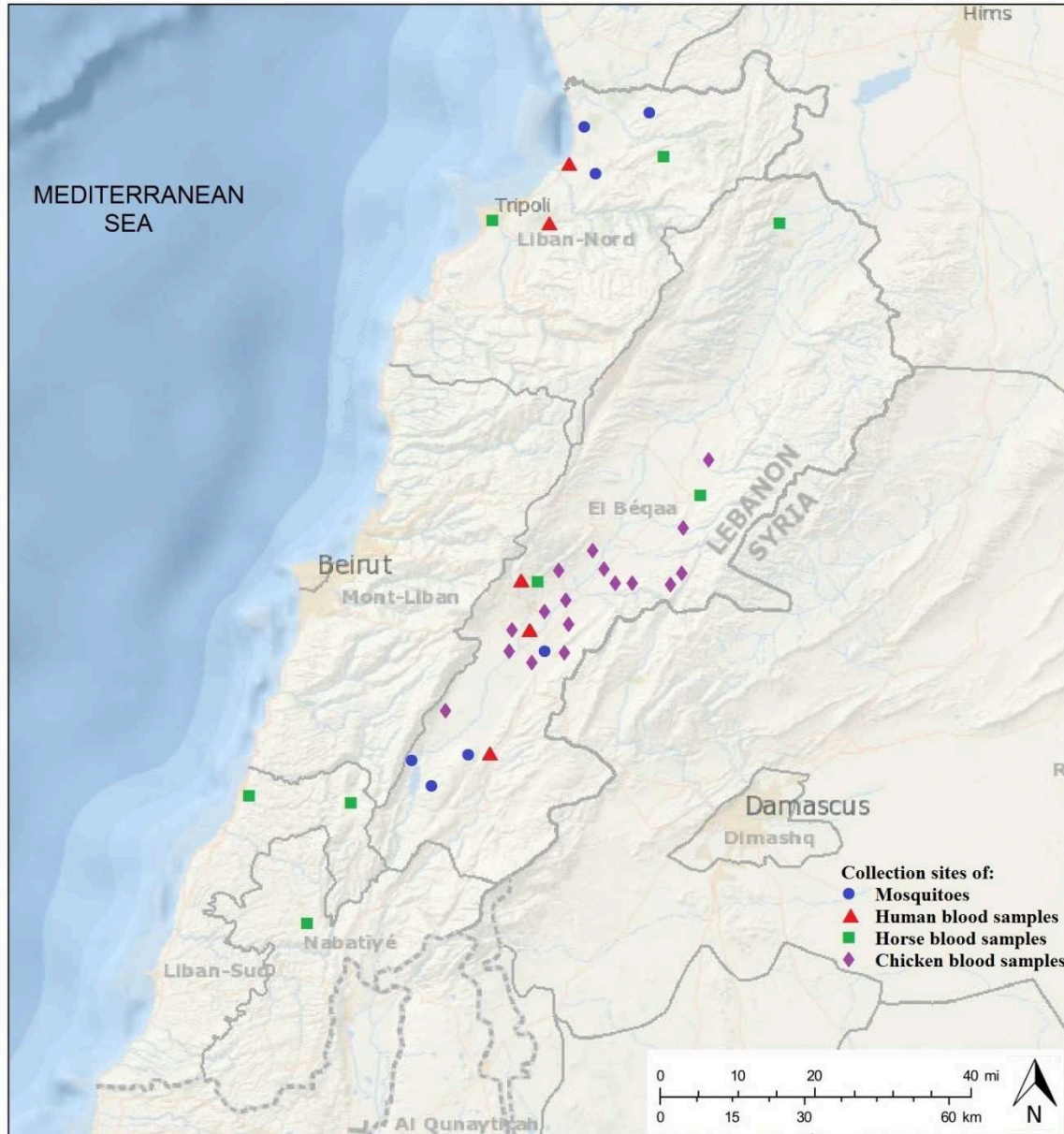


Figure 18. Collection sites of mosquitoes, humans, horses and chicken blood samples.

Collections were performed using two types of traps: CDC (Centers for Disease Control) light trap (Florida, USA), fitted or not with a CO₂ source (Figure 19A), and BG-sentinel traps (Biogents ®) using Bg-lure attractant containing lactic acid, ammonia and fatty acids, substances usually found on the human skin (Figure 19B). Traps were placed outdoor (mainly houses backyards) and in animal shelters (cattle and horses) from sunset till the next morning.

Collection bags were chilled in a freezer for one hour, mosquito specimens were then separated from other insects, placed in labeled cryotubes and stored in liquid nitrogen. Back to the laboratory, collection tubes were stored at -80°C until use.

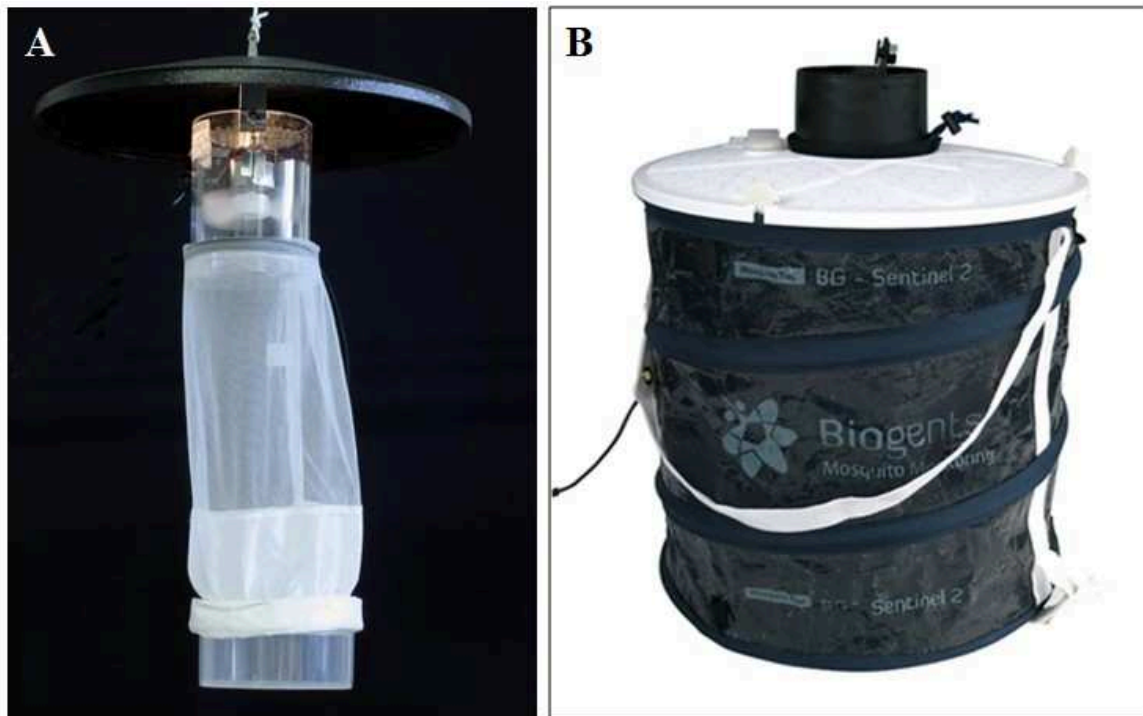


Figure 19. A. CDC light trap (BioQuip Products, 2011). B. BG sentinel trap (Biogents Products, 2017).

b. Mosquito identification

Mosquito specimens were identified under a stereomicroscope and placed in tubes containing RNA stabilizing solution (MCLAB, USA). The morphological identification was performed using a key for Mediterranean mosquitoes (Dubose and Curtin, 1965) in addition to the following digital identification keys: “The Mosquitoes of Europe” (IRD, 2001) and “The mosquitoes of the Mediterranean Africa” (IRD, 1999). Identified mosquitoes were pooled according to collection location, gender and species. Each pool contained up to 25 specimens. Mosquito pools were then preserved at -80°C for subsequent molecular analysis.

3. Screening for vector infection

Total RNA extraction followed by standard Reverse Transcription Polymerase Chain Reaction (RT-PCR) was performed on mosquito pools using WNV specific primers targeting the NS5 viral gene.

a. RNA extraction

RNA stabilizing solution was removed from the tubes, in which mosquitoes were pooled, before any manipulation. Total RNA was extracted using the NucleoSpin RNA isolation kit (Macherey-Nagel, Germany). Following the manufacturer's protocol, a first step of homogenization of the tissues was done by using the kit's Lysis Solution/2-Mercaptoethanol mixture that is required to fully inactivate RNases. Two sterile stainless steel beads (5mm diameter, Qiagen, USA) were added to each tube and tissues were grinded and crushed by vortex until no visible debris was observed (around two minutes). Note that after homogenization, two centrifugations were performed on supernatants of homogenates in order to get rid of mosquito debris that may block the filter/column during the RNA extraction process. After homogenization, proteinase K (Qiagen, USA) was added to the homogenate and incubated at 55°C for 10 min to effectively release RNA from fibrous tissues. RNA extraction process continued as per the manufacturer's protocol. RNA was collected and stored at -80°C. A dosage of the RNA products was performed using the NanoDrop Lite Spectrophotometer (Thermo Scientific, USA). This quantification was used to determine the volume of RNA extract needed for the reverse transcription (RT) step.

b. Reverse transcription

Subsequent to RNA dosage, the GeneAmp Gold RNA PCR Reagent Kit (Applied Biosystems, USA) was used to obtain a cDNA and perform a PCR. A Two-Step RNA PCR was done in order to keep the cDNA for any further studies. As per the manufacturer's protocol, in the Reverse Transcription (RT) step, 5 μ L RNA samples or templates of the mosquitoes' RNA pools, the positive control RNA (pAW109 RNA) provided by the kit and the negative control or blank were transcribed. This reverse transcription reaction was performed using the GeneAmp PCR System 9700, Applied Biosystems, Thermal Cycler (USA). Then the cDNA products were conserved at -20°C.

c. PCR amplification and viral characterization

The PCR mix was prepared using the Thermo Scientific (USA) PCR master mix kit. Specific selected WNV desalted primers (Integrated DNA Technologies IDT, USA) WNV NS5 WN 9794 and WNV NS5 WN 9483 were used to screen the pools for WNV RNA (Kauffman et al., 2016; Lanciotti et al., 2000). These primers amplify a 326 base pairs (bp) fragment within the Non Structural protein (NS5) gene which encodes for the viral RNA-dependent RNA polymerase (Lanciotti et al., 1999). This polymerase is the most highly conserved protein of WNV (and of Flaviviruses in general) (Brinton, 2002). As a consequence of the conserved nature of the region, chosen primers should readily bind to any potential strain of WNV. The sequences of the used primers are shown in table 2. In addition, Primer DM151 and Primer DM152, upstream and downstream respectively, were used to amplify the positive control gene (pAW109 RNA) for the reverse transcriptase reaction.

Table 2. WNV NS5 primer sequences (Kauffman et al., 2016; Lanciotti et al., 2000).

Description	Sequence (5'-3')	Size bp
WN 9483	CAC-CTA-CGC-CCT-AAA-CAC-TTT-CAC-C	326
WN 9794	GGA-ACC-TGC-TGC-CAA-TCA-TAC-CAT-C	

Primers were used at a final concentration of 0.5 μ M in a reaction volume of 50 μ L PCR mixture. 3 μ L cDNA was used for amplification. After optimization, PCR cycling parameters were as follows: Enzyme activation at 95°C for 10 min, PCR (denaturation, annealing, extension) with 35 cycles at 94°C for 45 s, 56°C for 45 s, 72°C for 1 min, PCR final step (elongation) at 72°C for 10 min. This amplification reaction was performed using the GeneAmp PCR System 9700, Applied Biosystems, Thermal Cycler (USA). WNV cDNA positive control was generously provided by Dr. Laura Kramer (Laboratory of Arboviruses, Slingerlands, New York, USA).

The obtained PCR products were run on a 1.5% agarose gel electrophoresis suspended in Tris Borate EDTA (1 \times TBE) running buffer (Appendix E: 10 \times TBE buffer Preparation). DNA fragments were colored by Gel Red Nucleic acid gel stain (Biotium, USA). Potential WNV amplicons are expected to appear at 326 bp position.

DNA purification was performed using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Germany). According to the manufacturer's protocol, the band of interest was cut from the gel and prepared in order to bind DNA on a silica membrane within the spin column. The eluted DNA was ready for immediate use or stored at -20°C. Purified DNA samples were submitted to sequencing using the same PCR primers. The obtained sequences were identified after their alignment with sequences of different strains published in GenBank.

4. Blood samples collection

The serological study was conducted on three types of hosts: humans, horses and chickens. Blood samples were collected in 5mL gel tubes. Human blood samples were all collected by certified laboratory technicians whereas horse and chicken blood samples, they were withdrawn by veterinarians.

a. Human blood collection

Two human populations were included in this seroprevalence study. The first population consisted of consenting donors from the Bekaa region, an area characterized by the presence of large water systems. All involved participants were outpatients attending the laboratories of three hospitals in the study area in order to conduct routine blood analysis: Tal Chiha Hospital in Zahle (June 2013), Bekaa Hospital in Taanayel, Chtoura (June-September 2013) and Farhat Hospital in Kamed El Lawz (July and August 2013) (Figure 18).

The sera of the second population were taken from a serum bank and belong to patients that were involved in a previous serosurvey on Sand fly fever virus. All patients were recruited in September 2012 from outpatients attending two laboratory facilities in North Lebanon district: the laboratory of health dispensary of the United Nations Relief and Works Agency (UNRWA) inside Nahr El Bared Camp (NBC) for Palestinian refugees in Akkar and the laboratory of the military clinic in Al Kobbah, Tripoli, North Lebanon (Figure 18).

All participants have signed a consent form and filled a questionnaire in Arabic language (Appendices A and B). Translated versions of both forms appear in Appendices C and D. The consent form notified each participant about the objectives of the study, the requirements and the confidentiality of results. The questionnaire aimed to collect information about the participant's

socio-economical factors (gender, place of residence, work nature, summer fever history, travel history...). Only patients below 9 and above 85 years old were excluded from this study.

b. Horse blood collection

Equine sera samples for the seroprevalence study part were collected from June to October 2015 from farms and riding clubs in rural areas of the following districts in Lebanon: Tripoli, Akkar, Baalbeck, Hermel, Zahle, West Bekaa, Nabatiye, Saida and Jezzine districts (Figure 18). Most of the collected samples belonged to horses but some were from ponies; the horses were of diverse age, breed and gender.

Blood was obtained from the jugular vein (Figure 20). Horses were checked for microchips using a microchip scanner. ID numbers, horse's name, age, gender, breed and medical history were registered, when available. None of the horses or ponies being tested has been vaccinated against WNV. Some farms and clubs employed pest control measurements on the horses and in the facility.



Figure 20. Blood withdrawal from the jugular vein of a horse (The Courier Journal, 2015).

c. Chicken blood collection

In order to estimate the seroprevalence of WNV, blood samples were collected from egg-laying hens that were raised in gardens of private houses in different villages of the Bekaa Plain. Blood collection was performed between September and December 2015. Samples were collected from 15 different locations in West Bekaa, Zahle, and Baalbek districts (Figure 18).

Moreover, and in order to assess the current circulation of WNV in the Bekaa area, a seroconversion study was conducted. Therefore, two groups of ten sentinel chickens each were placed in Younine (Baalbek) and El Marj (West Bekaa) villages (Figure 18). In each locality, five white and five red chickens (Leghorn) were marked with wing nodes made by ribbons of five different colors. These chickens were entrusted to a villager in each locality who placed them in his private garden (outdoor) during the study period. Blood samples were withdrawn from each of these chickens: one in September 2015 (coinciding with the beginning of bird migration period), one in October 2015 and the last one in December 2015. The aim of this study is to detect the appearance of specific antibodies against WNV in these chickens and to monitor their evolution over time. All Chicken blood samples were collected from the alary vein (Figure 21).



Figure 21. Blood withdrawal from the alary vein of a chicken.

5. Sera screening by ELISA

After blood collection, all samples of different origins were brought to the laboratory and centrifuged at 4000g for 10 min in order to obtain the sera. Aliquots from each serum were prepared under sterile conditions and stored at -20°C for further use.

All collected sera were tested for the presence of specific antibodies against WNV: IgG antibodies (in human and equine) and IgY antibodies (in chicken). The technique used was an in-house indirect Enzyme Linked Immunosorbent Assay (ELISA) based on the protocol performed at the Laboratory of Arboviruses (Slingerlands, New York State Department of Health, USA). A series of optimization tests were done to determine Coating Antigens' concentrations, secondary antibody dilutions, and buffers to be used in the laboratory conditions.

The coating antigens consisted of a normal slurry antigen (also called negative antigen) and another positive WNV antigen. The first is composed of the lysate of uninfected Vero cell culture. The latter consisted of the lysate of WNV (New York 1999 strain) cultured on Vero cell. Both antigens in addition the required human and horse positive and negative controls were generously provided by Dr. Laura Kramer's Laboratory. Chicken positive and negative controls were kindly provided by Dr. Sylvie Lecollinet from Maison Alford, Paris, France. All used controls belonged to sera confirmed positive or negative against WNV by PRNT.

The applied ELISA protocol was similar for human, equine and chicken sera. Briefly, 50µL of antigen diluted at 1:200 in fresh coating buffer (Appendix E: Coating buffer preparation) were applied to each 96 wells of Nunc-Immuno MicroWell ELISA plates (Sigma, USA). The normal slurry antigen was dispensed in the wells of the 1st, 4th, 7th and 10th columns while the positive antigen was distributed in the wells of the remaining columns of the plate (Appendix F: plate plan). The antigens were allowed to bind during an overnight incubation at 4°C.

The following day, the coat solution was discarded, the plate was washed three times with Phosphate Buffered Saline (1×PBS) 0.05% Tween 20 (PBS-T) (Appendix E: 10XPBS buffer preparation). 100µL blocking buffer (5% skimmed milk in PBS-T) was added, and the plates were left in a humid chamber at 37°C for 1 hour. Following incubation, blocking solution was discarded and plates were washed as above. 50µL of each serum sample, diluted 1:200 in PBS-T-BA buffer (1×PBS, 0.05% Tween 20, 0.5% Bovine Serum Albumin (BSA)), were dispensed to one negative and two positive antigen-containing wells. Plates were then returned to a humid chamber and incubated at 37°C for 1 h.

Afterward, plates were removed, washed as above, and 50µL of goat anti-human IgG (H+L) Horseradish Peroxidase (HRP) secondary antibody (Southern Biotech, USA), diluted 1:4000 in PBS-T-BA buffer, were added to each well. For horse and chicken sera screening, goat anti-equine IgG (H+L) HRP (Southern Biotech, USA) or goat anti-chicken IgY (H+L) HRP (Southern Biotech, USA) were respectively used at the same dilution. After incubation and washing as above, potentially formed immune complexes were revealed by the addition of 50µL of 3,3',5,5'-Tetramethylbenzidine (TMB) liquid substrate (Eurobio, France) for 10 min. The reactions were stopped with 50µL of 1:20 HCl, and the optical density (OD) of each well was read at 450 nm ($\lambda_{\text{read}} = 450 \text{ nm}$ and $\lambda_{\text{reference}} = 492 \text{ nm}$) using ELISA reader Multiskan FC (Thermo Scientific, USA). It is worth noting that in each plate, in addition to the control sera samples, blank (wells without serum and secondary antibodies) and secondary antibodies (wells without serum) controls were designated. For each serum, a ratio of OD obtained on the positive antigen (ODP) over the OD obtained on the negative antigen (ODN) was calculated. We considered a human serum as positive for WNV when the value of ODP/ODN was greater than or equal to 2. Whereas for horse and chicken sera samples, we considered suspected positive all

sera showing an ODP/ODN ratio greater than 1.2. These ratio cut-off values were chosen according to the number of tested sera samples, in a way not to miss any potential positive sample.

Some validation criteria were taken into consideration to validate observed results in each plate. These criteria depended mainly on the OD values of the positive and negative control sera, the secondary antibody and blank controls.

6. Confirmation by PRNT

The plaque-reduction neutralization test (PRNT) is considered the gold standard procedure to confirm arboviral infection especially when other serological techniques, such as ELISA and Immunofluorescence Assay (IFA), show important cross reactivity between closely related circulating viruses (in our case, other flaviviruses). PRNT is used to determine the titer of neutralizing antibodies for a virus in a serum sample. The assay is time consuming and laborious. For this reason, it is mainly performed in reference laboratories and in high security level settings.

All sera that turned to be positive for WNV by ELISA (at the fixed threshold), were tested by PRNT for confirmation at the Laboratory of Arboviruses (Wadsworth Center, New York State Department of Health, Slingerlands, New York, USA) as part of a scientific collaboration project with our laboratory (Laboratory of Immunology and Vector-Borne Diseases, Faculty of Public Health, Lebanese University). Here, we take this opportunity to thank Dr. Laura Kramer, head of the American partner laboratory, for her generous collaboration and precious advices that guided our project.

Fresh Vero cell suspension (2.0×10^5 cells/mL) is prepared and dispensed (3 mL/well) in 6 well plates. Plates are then incubated at 37°C and 5% CO₂. Test sera were serially diluted from 1:5 through 1:160 in BA-1 diluent (Medium-199H, 0.05M Tris pH 7.6, 1% bovine serum albumin, 0.35g/L sodium bicarbonate, 100 units/mL penicillin, 100 µg/ mL streptomycin, and 1 µg/mL fungizone) in a 96-well plate (100 µL/well). The WNV (strain NY-1999) was diluted to obtain 200 PFU/100µL. Afterward, the viral suspension was distributed on the plate wells and mixed with the existing 100µL of diluted sera. The plate was then incubated overnight at 4°C.

The following day, 100µL of each serum-virus mixture was added onto confluent monolayers of Vero cells (in the 6 well plates) and allowed to adsorb at 37°C for 1 h, after which an agarose overlay (3 mL/well) was added and incubation was continued. When virus plaques became visible, a second overlay of nutrient-agar (3 mL/well) containing Neutral Red (1mL of Neutral Red/100 mL of overlay) was added. Neutral red stains viable cells leaving white/cleared plaques where virus is present. The plates are then returned to the incubators.

In order to determine the neutralizing titer, a virus control (also called back-titration) was prepared. It consists of duplicate virus suspensions containing 200, 20, and 2 PFU/0.1 mL mixed with an equal volume of BA-1 diluent to contain 100, 10 and 1 PFU/0.1 mL.

Plaques were counted on the 3rd (WNV) day after the viral inoculation. The highest dilution of serum neutralizing 90% of the inoculum as determined by back-titration (control) was considered the neutralizing titer. WNV positive and negative control sera were used in this test.

7. Statistical analysis

All human serological results were statistically analyzed in terms of frequencies and percentages. For each qualitative variable, seropositivity against WNV, human participant

gender, place of residence and presence of domestic animals, frequencies and percentages were calculated. The chi-square test was used to compare the frequencies and percentages between groups of each qualitative variable. Fisher's exact test was used when indicated. Statistical Package for the Social Sciences (SPSS) software version 16.0 was used for the analysis of obtained data.

Results

1. Description of mosquito collections

Adult mosquito trapping in West Bekaa (Jebb Jennine, Qaraoun, Ein El Jawzi and El Marj villages), over the period of 6 days, yielded 2934 adult mosquitoes with a total of 78 trap-days. Identification of these specimens showed that 91.65% of collected mosquitoes were *Culex pipiens*. The remaining specimens (8.35%) belonged to four other species: *Culex martini*, *Culex perexiguus*, *Culiseta longiareolata* and *Orthopodomyia pulcripalpis* (Table 3).

Adult mosquito trapping in Aakar, North Lebanon, in three different locations, using CDC light traps and BG sentinel traps, over the period of 15 days, yielded 3912 adult mosquitoes with a total of 88 trap-days. Identification of these specimens showed that 83.90% of collected mosquitoes were *Culex pipiens*. In addition, other species were found in much lower percentages such as, *Culex martini*, *Culex perexiguus* and *Culiseta longiareolata* (Table 4).

Table 3. Species composition and description of collected mosquitoes in localities of West Bekaa during August 2014.

Place	Coordinates		Altitude (m)	# of collection days	Trap type	Trap-days	<i>Culex pipiens</i>		<i>Culex perexiguus</i>		<i>Culex martini</i>		<i>Culiseta longiareolata</i>		<i>Orthopodomyia pulcripalpis</i>		Incomplete
	LAT	LONG					F	M	F	M	F	M	F	M	F	M	
Jebb Jennine	33°37' 58" N	35°46' 55" E	870	3	BG sentinel	8	27	58			2		3	3		2	4
					CDC	19	360	33	7		18		1	2		2	37
El Marj	33°45' 42" N	35°53' 10" E	870	2	BG sentinel	8	119	256					3	5		1	11
					CDC	19	1042	11	2		5	2	4	1		4	53
Qarao un	33°34' 10" N	35°43' 10" E	900	2	BG sentinel	6	37	73			3		4	2		1	7
					CDC	13	658	9	1		2		2	6			41
Deir Ein El Jawzi	33°35' 49" N	35°41' 29" E	1000	1	BG sentinel	2	1								2	2	
					CDC	3	5										
Total						78	2249	440	10	0	30	2	17	19	2	12	153
Total of specimens (%)							2689 (91.65%)		10 (0.34%)		32 (1.09%)		36 (1.23%)		14 (0.48%)		153 (5.21%)
Grand total							2934										

LAT: Latitude, LONG: Longitude, #: number, F: female, M: male

Table 4. Species composition and description of collected mosquitoes in localities of North Lebanon during September 2014.

Place	Coordinates		Altitude (m)	# of collection days	Trap type	Trap-days	<i>Culex pipiens</i>		<i>Culex perexiguus</i>		<i>Culex martini</i>		<i>Culiseta longiareolata</i>		Incomplete
	LAT	LONG					F	M	F	M	F	M	F	M	
Darine	34°36' 15" N	36°06' 25" E	74	1	BG sentinel	2	22	11							6
Klayaat	34°35' 10" N	35°59' 35" E	5	2	CDC	12	1			1					
El Bahsa Bebnine	34°30' 90" N	35°59' 25" E	70	12	CDC	74	2748	500	82		8		12		521
Total						88	2771	511	82	0	9	0	12	0	527
Total of specimens (%)							3282 (83.90%)		82 (2.10%)		9 (0.23%)		12 (0.31%)		527 (13.47%)
Grand total							3912								

LAT: Latitude, LONG: Longitude, #: number, F: female, M: male

2. Molecular screening of mosquitoes for WNV

Collected mosquito specimens were separated into pools according to collection site, species identity and gender. Mosquitoes collected from West Bekaa were divided into 66 pools, each contained up to 50 mosquitoes. Fifty seven of them were composed of females *Cx. pipiens* as shown in Appendix G. Similarly, mosquitoes collected in Aakar were divided into 141 pools. Each pool contained up to 25 mosquitoes; 119 of these pools were composed of females *Cx. pipiens* (Appendix G). Following DNA extraction, each pool was screened for the presence of WNV gene by a 2-steps RT-PCR. Screening results showed that all tested pools were negative for WNV (Figure 22).

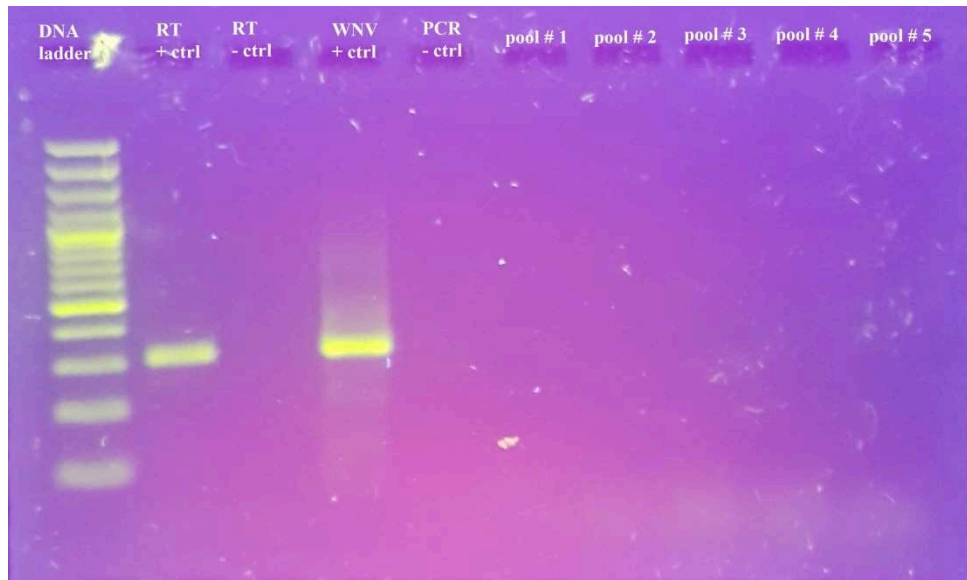


Figure 22. Gel electrophoresis picture of PCR products. Wells from left to right represent: DNA ladder, reverse transcription positive control band on 308 bp, reverse transcription negative control, WNV positive control band of 326 bp, PCR negative control, pools # 1 to 5 all negative for WNV.

3. Serological study of human population

a. Description of participants

For this study, we analyzed 803 serum samples of consenting outpatients attending laboratory facilities of three hospitals in West Bekaa: 168 from Tal Chiha Hospital in Zahle, 501 from Bekaa Hospital in Taanayel-Chtoura and 134 from Farhat Hospital in Kamed El Lawz. In addition, in Northern Lebanon, a total of 288 sera were collected from consenting participants recruited equally in the UNRWA dispensary at Nahr El Bared Camp, Akkar and in the laboratory of the military clinic of El Kobbeh Army Barrack, Tripoli (Table 5).

Table 5. Description of human sample population.

Area	Location	Sample population (N)	Females	Males	Median Age
Bekaa	Bekaa Hospital	501	298	203	36
	Tel Chiha Hospital	168	97	71	49
	Farhat Hospital	134	89	45	31
Bekaa total		803	484	319	39
Akkar	Nahr El Bared Camp	144	106	38	37.5
Tripoli	El Kobbeh Barrack	144	101	43	40.5
North total		288	207	81	40
Cumulative total		1091	691	400	39

Almost two third of the participants were females (63.34%) (Table 5). The age range of the population was between 9 and 85 years with an average age of 40.7, a median of 39, and a high representation of participants aging between 21 and 30 years old (Figure 23). Their occupations varied from indoor and outdoor types of jobs, to soldiers or students (Figure 24). Moreover, 499 female participants (72.21%) were either house wives or unemployed; and 97 male participants (24.25%) were either unemployed or retired. 143 male participants had outdoor jobs such as in agriculture, farms, construction or drivers or several other occupations. 104 male participants had

indoor or desk jobs in banks, administration of companies, or hospital staff or other occupations.

The remaining participants were soldiers and students (Figure 24).

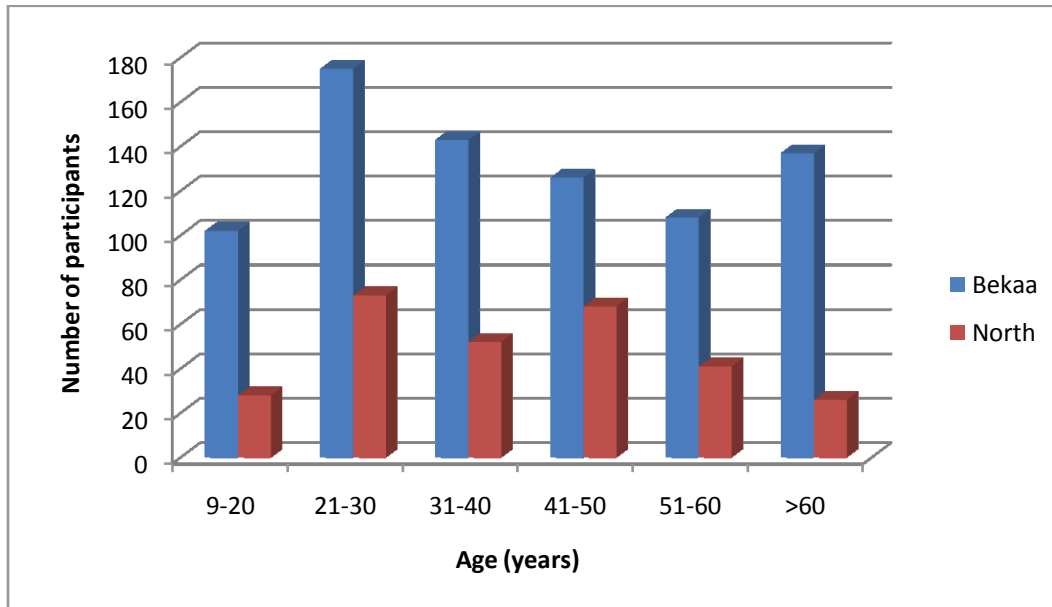


Figure 23. Bar chart representing the age distribution of the study participants.

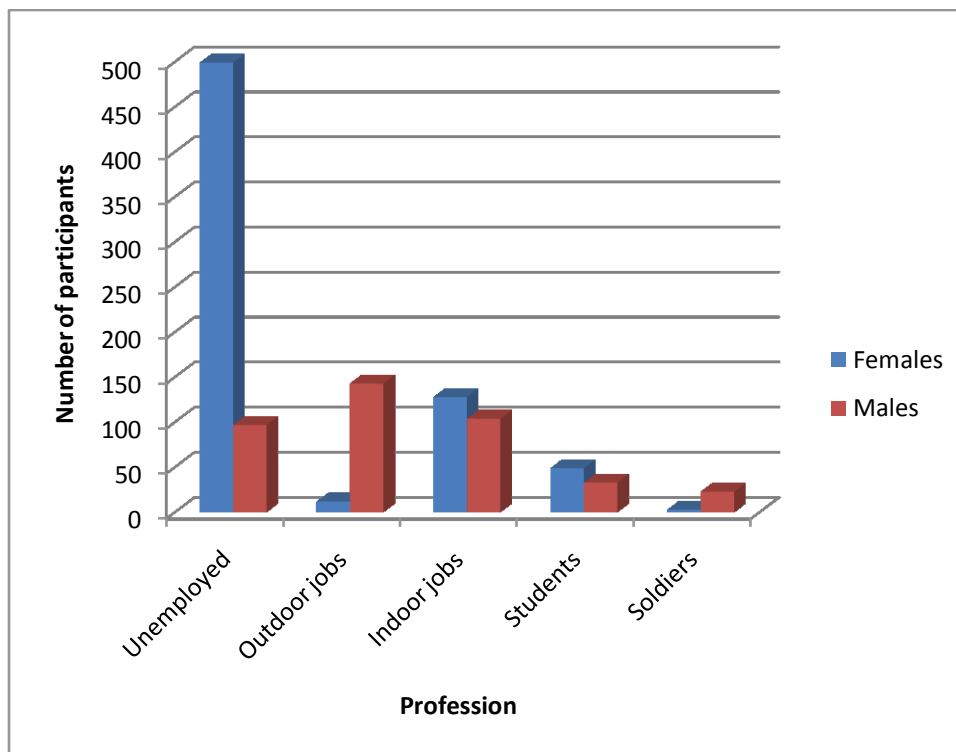


Figure 24. Bar chart showing the profession distribution of the study participants.

b. Seroprevalence results

Sera analysis using an in-house ELISA technique was performed on all collected samples, and those showing an OD ratio above 2 were considered as suspected positive. Results revealed that 74 individuals (9.22%, N=803) from Bekaa region and 2 participants (0.69%, N=288) from North area were seropositive against WNV (Figures 25 and 26). This brings the seroprevalence rate for both regions to 6.97% (76 sera out of 1091).

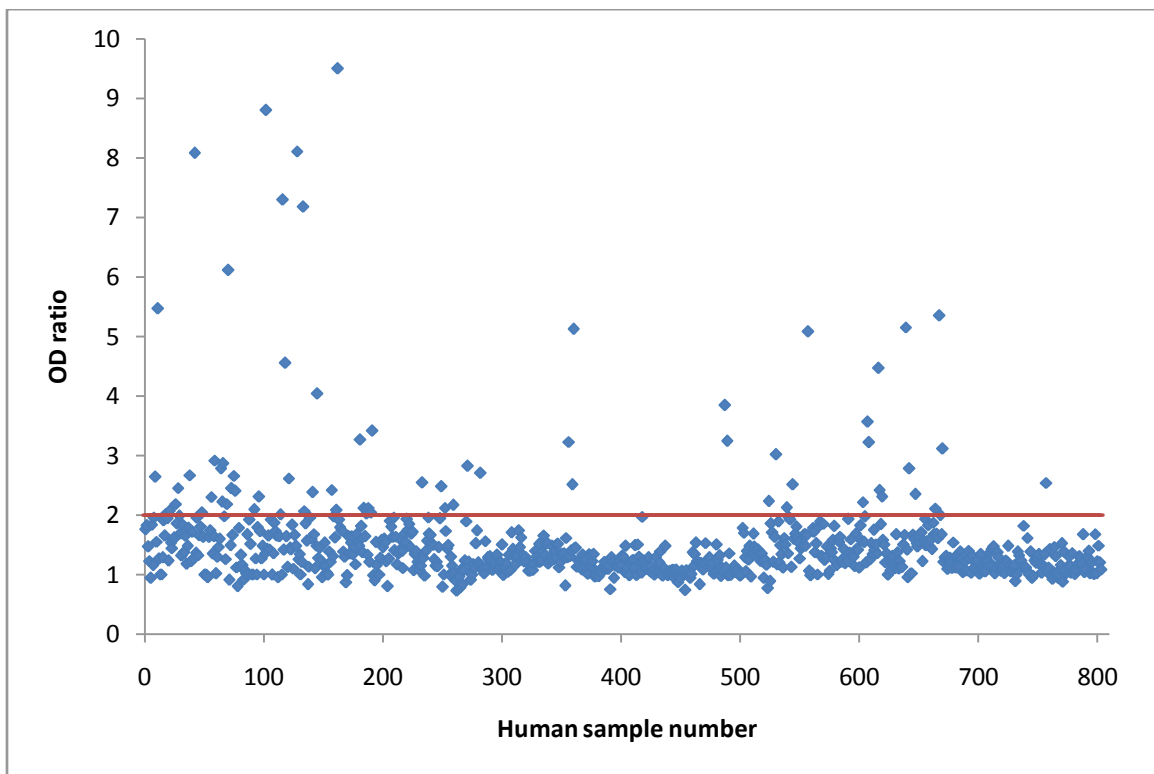


Figure 25. Scatter graph showing OD ratio obtained by ELISA on human sera samples collected from Bekaa region. The red line represents the fixed threshold (OD ratio = 2).

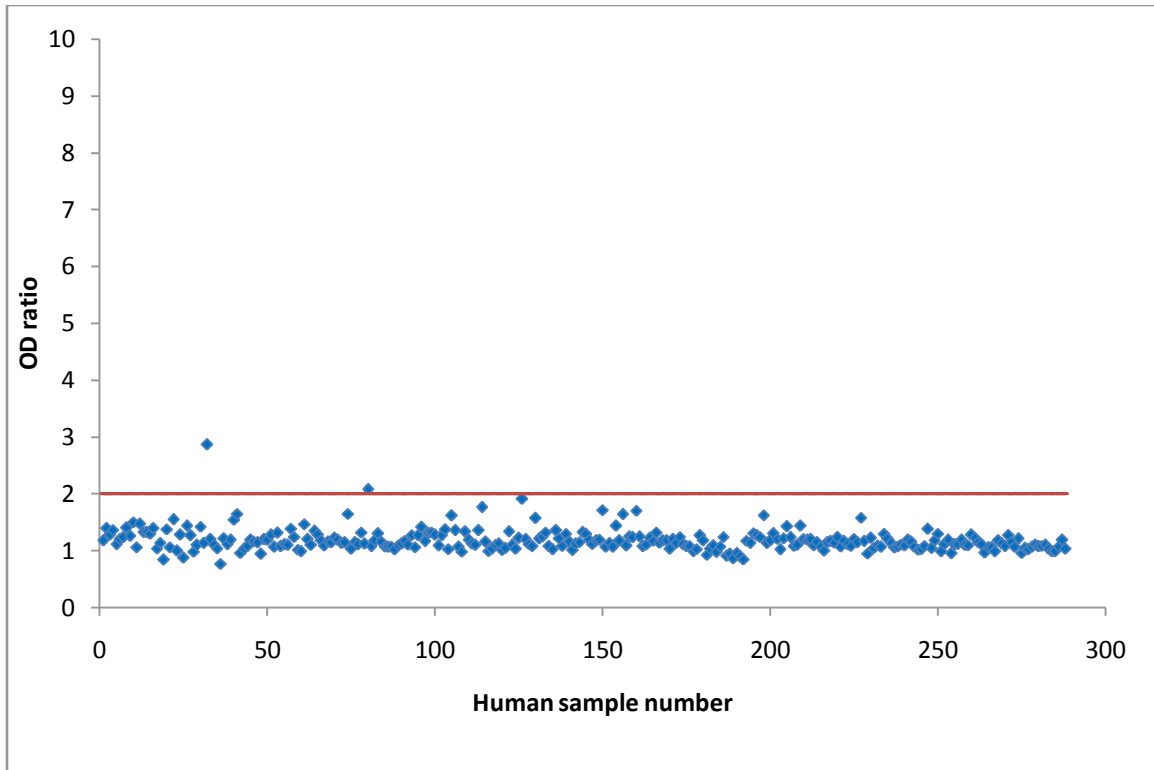


Figure 26. Scatter graph showing OD ratio obtained by ELISA on human sera samples collected from North Lebanon. The red line represents the fixed threshold (OD ratio = 2).

In order to confirm seropositivity after ELISA, PRNT was performed on suspected sera (OD ratio ≥ 2). Nevertheless, for the North area, and since the number of suspected positive samples is limited (only 2), we included in the seroneutralization test those sera having OD ratio ≥ 1.7 in order to avoid missing any potential positive specimen (Figure 26). Results showed that only 11 samples from the Bekaa region had neutralizing antibodies at a titer of 40 or higher whereas no sera from North Lebanon had WNV specific neutralizing antibodies at the fixed titer. This brings the seroprevalence rate of WNV to 1.37% in the Bekaa area and 0% in North Lebanon area (1.01% in both regions together). It is worth noting that three and four samples, from Bekaa and North regions respectively, had neutralizing antibodies at a titer of 20 or below (Table 6).

Table 6. Summary of human serology results. Participants included in this table have PRNT titer ≥ 10 .

Area	Serum #	ELISA OD ratio	PRNT titer result	Travel history to endemic area	Age	Gender	Profession
Bekaa	B9	2.64	40	No	75	Female	Unemployed
	B11	5.47	≥ 320	No	70	Female	Unemployed
	B42	8.08	≥ 320	Canada, 2008	83	Male	Unemployed
	B75	2.66	10	No	45	Female	Agriculture
	B116	7.30	≥ 320	No	80	Male	Unemployed
	B118	3.24	10	No	43	Male	Unemployed
	B121	2.61	10	Venezuela, 2003 & Syria, 2010	51	Female	Unemployed
	B133	5.00	160	Egypt, 2013	31	Male	Private company
	B162	5.22	≥ 320	No	65	Female	Unemployed
	12	2.83	≥ 320	No	85	Male	Unemployed
	235	3.85	160	Brazil, 2003 & Syria, 2010	66	Female	Unemployed
	237	3.25	160	Syria, 2013	73	Male	Unemployed
	T57	5.08	≥ 320	USA	76	Female	Unemployed
	T167	5.35	≥ 320	USA	82	Male	Unemployed
North	25	1.71	10	No	74	Male	Unemployed
	35	1.70	10	No	68	Female	Unemployed
	U91	1.95	20	Germany, 2009	67	Female	Unemployed
	U284	2.86	20	No	81	Male	Unemployed

Ten (5 females and 5 males) of the confirmed seropositive samples had more than 65 years old. All of them were either unemployed or housewives. One male seropositive participant is 31 years old and works indoor in a private company. When analyzing travel history of seropositive participants, only four of them had travelled to countries endemic to WNV (Egypt, USA and Canada). The remaining seropositive participants, either had never travelled out of Lebanon (2 patients) or had travelled to Syria or to other countries non-endemic for WNF (Table 6). These observations constitute a strong evidence of local circulation of WNV and local exposure of human population to this virus.

4. Serological study of horse population

a. Description of horse sample population

According to the Lebanese ministry of agriculture, the estimated number of horses in Lebanon is around 4000 horses. In this study, 202 horse samples were collected from 16 different farms/stables in eight districts in Lebanon (Figure 27). Seven of them were ponies and the others belonged to different breeds such as Arabian, Thoroughbred, Dutch, Belgian Draft, Turkish, and the local breed called Alef which was the most represented breed in the study population (144 samples, 70.59%) (Figure 28). More than two thirds of the horse samples were females (140 samples, 69.31%). The age of included horses ranged from 4 months to 22 years with an average of 8.74 years (Figure 29).

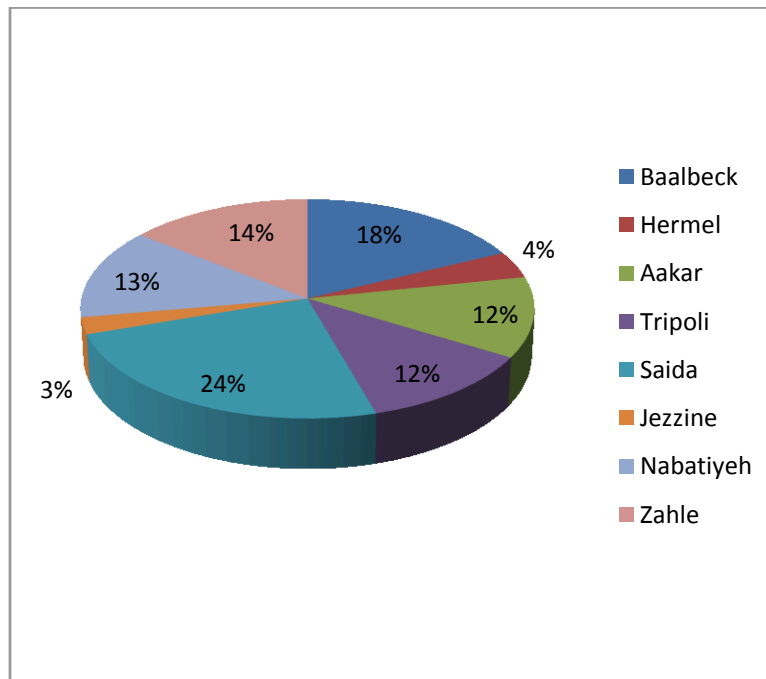


Figure 27. Pie chart showing the horse population distribution by district.

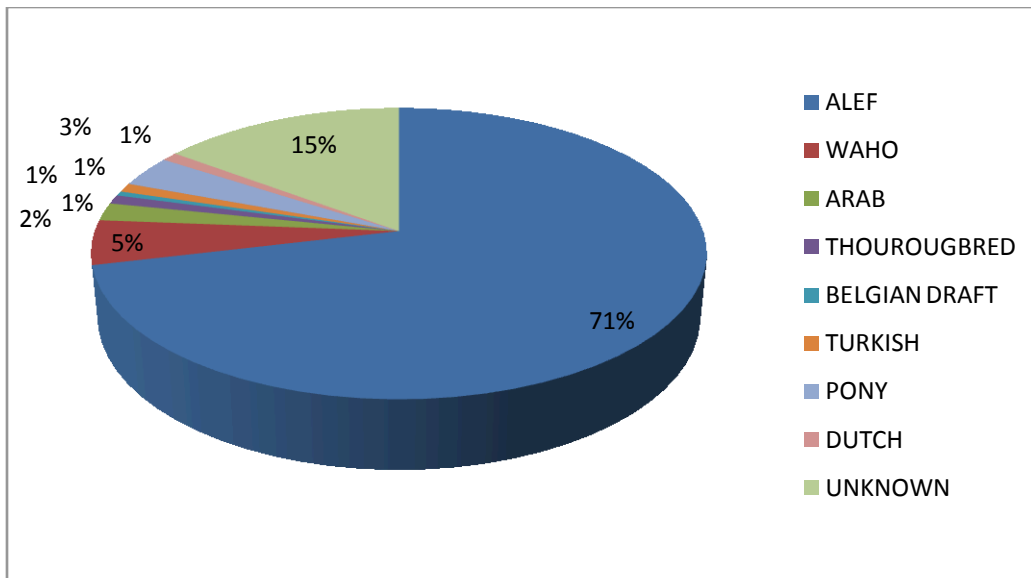


Figure 28. Pie chart showing the horse sample population breed distribution.

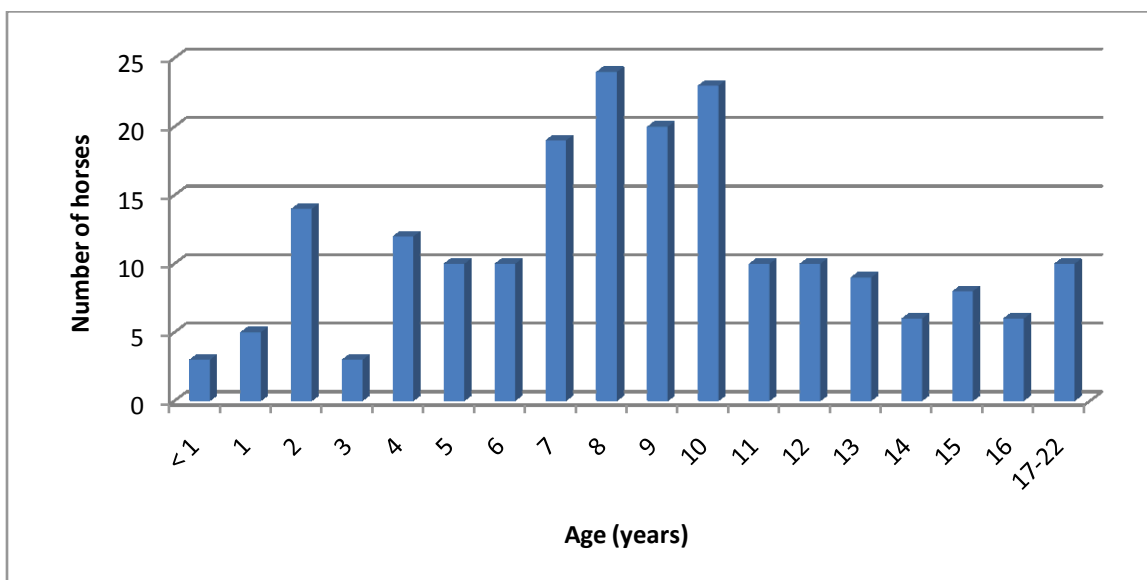


Figure 29. Bar chart representing the age distribution of the horse population.

b. Seroprevalence results

ELISA testing showed that 10 sera samples had an OD ratio above 1.2 (Figure 30). Thus the seroprevalence rate is 4.95 % for the fixed threshold. PRNT was performed on these samples. It revealed that only four had neutralizing antibodies for WNV at a titer of 40 or higher (Table 7). Thus, the seroprevalence rate dropped to 1.98%. Only one horse had specific antibodies at titer

of 10. This serum was considered negative as it is below the fixed threshold. All positive horses were of Alef breed with no cross border movement history. They were recruited from farms in Tripoli, Aakar and Nabatiyeh districts.

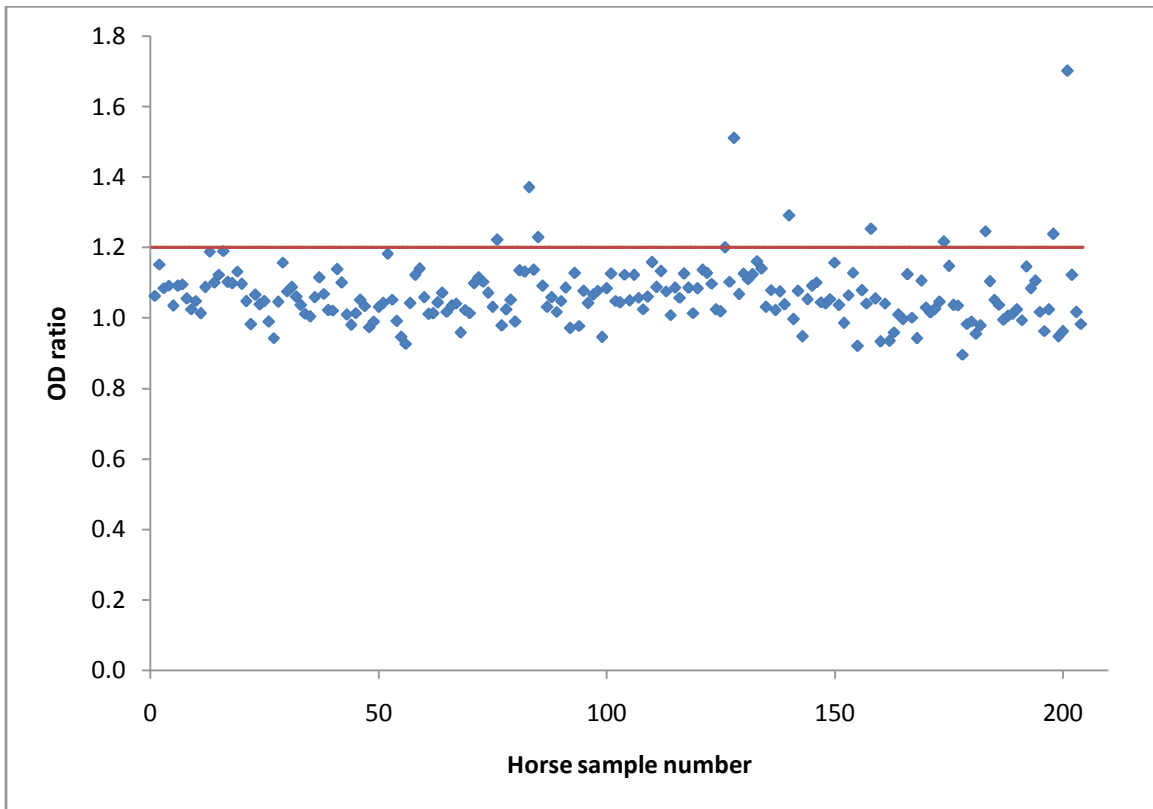


Figure 30. Scatter graph showing OD ratio obtained by ELISA on horse sera samples. The red line represents the fixed threshold (OD ratio = 1.2).

Table 7. Summary of horse serology results. Samples included in this table have PRNT titer ≥ 10 .

Serum #	ELISA OD ratio	PRNT titer result	Breed	Age	Gender	Location
83	1.371	160	Alef	7	Male	Tripoli
128	1.511	160	Alef	7	Female	Aakar
183	1.246	10	Alef	20	Female	Nabatiyeh
199	1.238	40	Alef	10	Female	Nabatiyeh
202	1.701	320	Alef	7	Female	Nabatiyeh

5. Serological study of chicken population

a. Description of the chicken study population

In order to estimate the seroprevalence rate among chicken population, 163 chicken sera were sampled between September and December 2015 from 15 different locations. These chickens are of Leghorn breed and belonged to house poultries of residents and farmers in the Bekaa valley in West Bekaa, Zahle, and Baalbek districts. The majority (106 chickens, 65%) of the collected chicken originated from Zahle district (Figure 31).

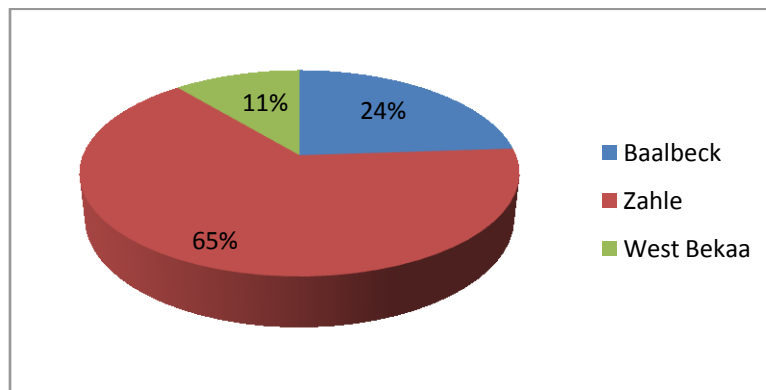


Figure 31. Pie chart representing the chicken sample population distribution by district.

Similarly, the 20 chickens (10 white and 10 red) used for the seroconversion study part were of Leghorn breed.

b. Seroprevalence results

ELISA screening of chickens for WNV specific IgY did not reveal any clear positive serum. In fact, the OD ratios observed were all less than 1.2, except one serum that had an OD ratio of 1.26 (Figure 32). Two chicken sera (those having the highest OD ratio value) were further tested by PRNT against WNV and both turned to be negative. Thus, in the present study, the observed seroprevalence rate against WNV among chicken is null.

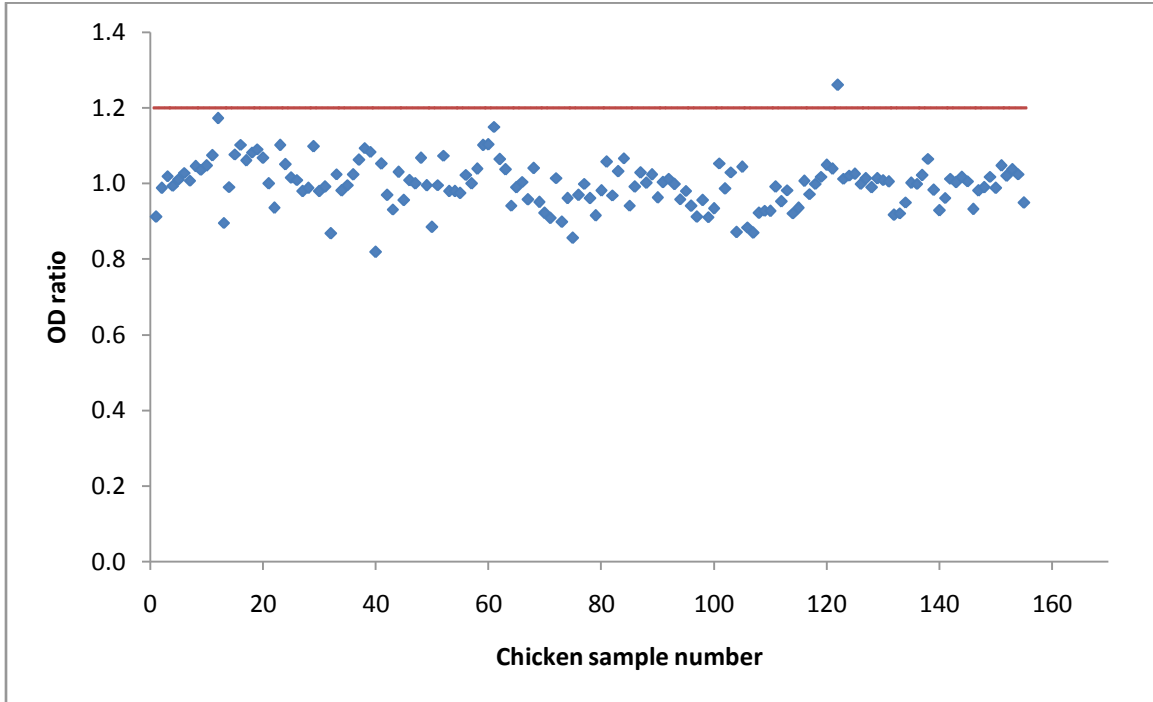


Figure 32. Scatter graph showing OD ratio obtained by ELISA on chicken sera samples. The red line represents the fixed threshold (OD ratio = 1.2).

c. Seroconversion results

As for the temporal analysis of chicken sera by ELISA technique, results did not show any onset or increase of WNV specific antibody levels in any of the selected individuals in either locations. The observed OD ratios remained comparable in the three samples taken for each chicken (Figure 33). These results show that during the study period and in the Bekaa area, there was no detectable circulation of WNV and therefore, the tested chickens were not exposed to infectious bites of the mosquito vector.

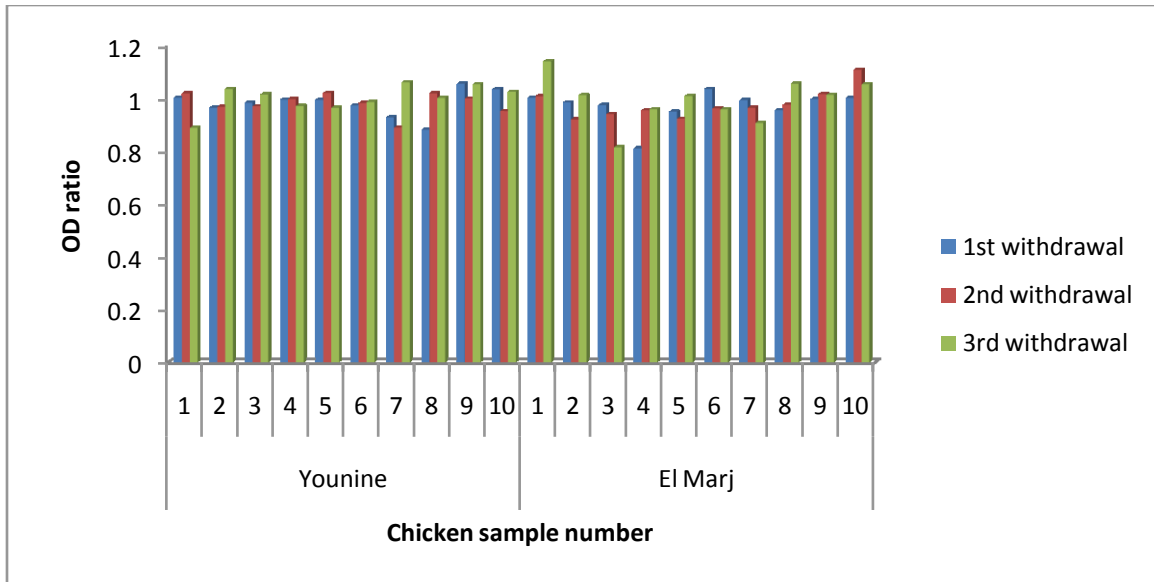


Figure 33. Bar chart showing OD ratios of chicken samples of the seroconversion study.

6. Statistical analysis

Among serology results of human sample populations, no statistical significant differences were observed between seropositivity against WNV and any factor for human (sex, profession...).

Discussion and Conclusion

Lebanon is considered non-endemic and non-enzootic for WNV. To date, no WNF cases were officially reported. Our study brings a strong evidence of its occurrence in the country. The detection of relatively high titer of neutralizing antibodies IgG (≥ 320) in participants who never travelled out of the country confirms a local exposure to this virus. Moreover, the fact that all seropositive human cases (n=11) originated from the Bekaa area suggests that this region is the primary introduction site of WNV in the country. This area is considered a stopover zone for migratory birds. It harbors several water plans (Aammik swamps and Qaraoun lake) and important agriculture activities, which make it suitable for the introduction and local spread of the WNV. The detection of confirmed positive sera (by PRNT) among horses is an additional evidence of WNV circulation as all seropositive horses were of local breed with no record of cross-border transfer. However, their movement within the country did not allow us to conclude on the exposure area to WNV.

The absence of WNV specific antibodies (IgY) in the poultry study population from the Bekaa and the absence of seroconversion in sentinel chickens suggest that there is no active current circulation of WNV in this region. Apparently, the circulation of WNV in Lebanon, when it occurs, seems to happen at low rate, which explains why human and animal cases have not yet been reported in the country.

In a previous study, conducted by Gallian and collaborators (2010), among 639 Lebanese blood donors, six were WNV seropositive by ELISA among which only 3 (0.47%) were confirmed positive by PRNT at titers higher than 1/10. Seropositive participants lived in South

Lebanon, Beirut, and the Bekaa however the authors did not specify whether these participants had any travel history or not (Gallian et al., 2010).

The observed seroprevalence rate in our study, although relatively low, is comparable to what is usually reported from geographically close endemic countries such as in Greece where the seroprevalence rate was 1.02% following confirmation by PRNT (Papa et al., 2010). In the neighboring Turkey, the WNV seroprevalence rate, as also confirmed by PRNT, have been reported to be as low as 0.8% in blood donors (Ayturan et al., 2011), and as high as 17% in Mardin province (near the Syrian border) among human population following the appearance suspected encephalitis cases (Karakoç et al., 2013). In another study conducted in the Manisa province on the Aegean Sea, West of Turkey, 4.3% of tested sera of healthy rural residents were seropositive following ELISA screening and confirmation by IIFA (Gazi et al., 2016). Similarly, in Egypt, WNV neutralizing antibodies (by PRNT) were found in humans and the rate varied from 1% (in Northern Sinai) to 35% (in Upper Egypt) (Soliman et al., 2010). In Jordan, that, like Lebanon, is considered non-endemic for WNV, the seroprevalence rate was estimated at 8% in care center population following screening by ELISA (Batieha et al., 2000).

In Israel, a country at the Southern border of Lebanon, WNV has been responsible for several outbreaks. In the year 2000, hundreds of cases were reported from this country, mainly from the central and northern regions, close to Lebanon (Weinberger et al., 2001). A cross-sectional study, involving more than 3100 healthy individuals recruited between 2011 and 2014, showed that 11% of subjects had specific antibodies IgG for WNV following screening by ELISA and confirmation by PRNT. This study revealed that the Coastal Plain, the Inland Plain and the Great Rift Valley, which is a geographic extension of the Bekaa valley, had the highest seroprevalence

of WNV antibodies (Bassal et al., 2017). These observations suggest that WNV has a lower prevalence in Lebanon than in Israel.

The serosurvey among horses revealed a 4.95% seropositive rate for WNV by ELISA which dropped to 1.98% after PRNT. All confirmed positive horses (n=4) came from different geographical locations (Tripoli, Aakar and Nabatiyeh). These results represent the first record of equine exposure to WNV in Lebanon. The observed seroprevalence rate is considered low compared to that observed in neighboring or distant endemic countries. In Turkey, following the WNV outbreak in 2011, the seroprevalence rate among horses was 21% after confirmation by PRNT (Ozkul et al., 2013). In South of France, following an outbreak among horses in 2000, a serosurvey conducted among equines revealed a seroprevalence rate of 8.5% by ELISA (Durand et al., 2002). Moreover, in Ukraine, a similar study was conducted in 2013 following limited WNV outbreak among humans in 2011 and 2012 and showed a seroprevalence rate of 13.5% by PRNT (Ziegler et al., 2013). Nevertheless, the seroprevalence observed in our study is comparable to what was reported from Iran where the endemicity of WNV is considered relatively low among both human and horses. Following screening by ELISA technique, WNV seroprevalence rate among horses (N=315) was 2.8% (Chinikar et al., 2013). Interestingly, in Jordan and Algeria, the seroprevalence rates among horses using ELISA technique were 24.9% and 26.8% respectively (Abutarbush and Al-Majali, 2014; Lafri et al., 2017). In the absence of confirmation by PRNT, these high rates should be carefully interpreted since they can reflect the circulation of not only WNV, but also other Flaviviruses.

The ELISA technique is considered a screening method to test a relatively large number of sera. This technique is very sensitive but can lead to the detection of false positive sera due to the cross-reaction with other Flaviviruses (such as Dengue Virus, Zika virus, Usutu virus, or other

viruses) that might be circulating in the region without being noticed. Previous studies in Lebanon confirmed arbovirus circulation in 1945-1946, in which an epidemic of Dengue fever was reported from Beirut region and affected more than 100,000 individuals (Hitti and Khairallah, 1946). Another serological survey conducted during 1962-1963 showed a seroprevalence for DENV of 61.9% using hemagglutination-inhibition test (Garabedian et al., 1971). Besides, the tiger mosquito (*Aedes albopictus*) is well spread in Lebanon (Haddad et al., 2007). Lebanese strains of this mosquito appear to be able to transmit Dengue virus under experimental conditions (Haddad et al., 2012). Moreover, the tiger mosquito is a possible vector of Zika virus (Wong et al., 2013). The exposure of Lebanese population to this virus was never assessed. Thus, and in order to increase the specificity of sera screening, all ELISA positive samples were confirmed by PRNT, a clearly more specific technique than other serological methods. It is worth noting that 65 sera samples had high OD ratio by ELISA but did not have neutralizing antibodies against WNV. This observation suggests the circulation of other Flaviviruses which should be identified.

According to the Society for the Protection of Nature in Lebanon (SPNL) and Bird Life Lebanon, there are more than 300 bird species in the country. Migratory birds in Lebanon include Cranes, Pelicans, Common Buzzard, Lesser Spotted Eagle, and White Storks (SPNL, 2017). Some of these species of wild and domestic birds were seropositive against WNV in endemic countries such as Israel (Di Sabatino et al., 2014), Greece (Valiakos et al., 2011), Hungary (Erdélyi et al., 2007), and USA (Nash et al., 2001; Steele et al., 2000). Moreover, in Germany, during 2005-2009, a study detected WNV neutralizing antibodies in 10 species of migratory birds, but not in resident birds (Seidowski et al., 2010). Migratory birds are very difficult to monitor. It was proven that chickens are very sensitive to WNV infection but they

usually do not develop clinical symptoms. Besides, they develop relatively low viremia that is insufficient to infect mosquito vectors (Langevin et al., 2001). Nevertheless, following infections, these birds develop detectable neutralizing antibodies. Consequently, chickens are largely used during WNV surveillance programs as sentinel animals to detect the circulation of the virus. In order to assess WNV circulation, we screened Leghorn chickens for viral exposure. These chickens have a life span of 1 to 6 years. Therefore, their serological monitoring is useful to detect relatively recent WNV infection. In our study, we were not able to detect WNV exposure. To our knowledge, no other studies attempted to assess the circulation of WNV neither in domestic nor in migratory birds in Lebanon.

Our entomological surveys in Bekaa and Akkar revealed high presence of *Culex pipiens*, the known primary vector of WNV. Moreover, another potential vector, *Culex perexiguus*, was also present but in limited numbers. These findings are in concordance with previous studies (Knio et al., 2005) and entomological surveys conducted by our laboratory (unpublished data). Molecular analysis results revealed that none of the mosquito pools was found naturally infected with WNV. This is not very surprising if we consider the low circulation of the virus in the country as suggested by the observed low seroprevalence rates among human and horses. Therefore, a much higher number of mosquitoes should have been screened in order to increase the chances of virus detection in the potential vectors.

In the EMR, WNV was naturally isolated from different *Culex* species in endemic countries in which the seroprevalence rates among human and horses were much higher than those observed in the present study. In Turkey, after an outbreak in 2012, WNV was isolated from 1.9% of collected mosquito pools (Ergunay et al., 2013). Whereas in Israel, analysis of 277186 mosquitoes collected during 15 years (1985-2000), allowed the detection of WNV lineages 1 and

2 in 336 mosquito pools (Lustig et al., 2016). The virus was isolated from 2.39% and 9.22% of *Cx. pipiens* and *Cx. perexiguus* pools respectively (Lustig et al., 2016). Lustig and collaborators' observations in 2016 highlighted the role of this latter species in the spread of the virus. This mosquito was previously associated with WNV infection. Following molecular screening of almost 430,000 mosquitoes collected during eight consecutive years (2000-2007), the majority of WNV infections were found in the three most common species: *Cx. pipiens*, *Cx. perexiguus*, and *Ae. caspius*. The WNV infection rate of *Cx. perexiguus* (2.6%) was significantly higher than those of the first and the third species (0.5% and 0.8% respectively) (Orshan et al., 2008). In the same study, the authors noticed that in the year 2004, when a sharp decline of the number of human cases occurred, only *Cx. perexiguus* was found infected. In Spain, Munoz and collaborators (2012) analyzed the feeding pattern of different potential WNV vectors belonging to the *Culex* genus. They noticed that the feeding behavior of *Cx. perexiguus* is in favor of WNV transmission from bird to bird and from bird to horse, this mosquito species being then the primary candidate for enzootic (bird-to-bird) transmission and for epizootic transmission to horses (Muñoz et al., 2012). The authors noticed that *Culex pipiens* is also an enzootic vector, but has only a low risk for epizootic transmission to humans. In Lebanon, the ecology of both vectors should be further investigated in order to better understand the pattern of transmission of WNV in the country.

In conclusion, during this study, we were able to confirm that WNV circulates in Lebanon. This circulation was more obviously detected in the Bekaa region where the seroprevalence rate for humans was 1.37%. Besides, and for the first time, we detected seropositive horses in the country (1.98%). We assume that the exposure to WNV is not recent as no seroconversion in sentinel chickens and no WNV particles in mosquito populations were detected. *Culex pipiens*,

the most abundant mosquito species, is the suspected vector. Besides, *Cx. perexiguus*, a known WNV vector in the East Mediterranean Region, was also present but in limited numbers. The feeding behavior of both mosquitoes should be analyzed in order to determine their implication in the transmission cycle. We recommend conducting similar surveys for several consecutive years in order to detect potential introduction of WNV to Lebanon.

Chapter 7

Experimental transmission of West Nile Virus and Rift Valley Fever Virus by *Culex pipiens* from Lebanon

In this chapter, we present the submitted article to *Plos Neglected Tropical Diseases* journal. Peer reviewing is under process.

Experimental transmission of West Nile Virus and Rift Valley Fever Virus by *Culex pipiens* from Lebanon

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Introduction

West Nile virus (WNV) and Rift Valley fever virus (RVFV) are two important emerging mosquito-borne zoonotic agents transmitted by *Culex pipiens*, a complex of sibling species that includes *Cx. pipiens s.s.*, *Cx. quinquefasciatus* and possibly *Cx. australicus* (Harbach, 2012; Turell, 2012). *Cx. pipiens s.s.* includes two biotypes or subspecies: *Cx. pipiens pipiens* and *Cx. pipiens molestus* (Fonseca et al., 2004; Harbach, 2012). The first biotype is primarily a bird-feeding mosquito present in temperate areas while *Cx. pipiens* biotype *molestus* feeds on mammals (mainly human) and thrives in sewers in temperate and sub-tropical regions (Bahnck and Fonseca, 2006; Fonseca et al., 2004). Because morphological identification of these biotypes is not possible, they can only be distinguished using molecular techniques (Bahnck and Fonseca, 2006; Fonseca et al., 2004; Price and Fonseca, 2015).

WNV is a member of the Flavivirusgenus (Flaviviridae family) and was first isolated in Uganda in 1937 (Smithburn et al., 1940). Usually, only 20% of infected individuals develop symptoms and less than 1% of infected people develop serious and potentially fatal neurological illnesses such as meningitis and encephalitis. Birds are considered the main reservoir of the virus and *Cx. pipiens* is recognized as the primary enzootic vector (Andreadis, 2012). WNV infections have been reported in many tropical and temperate countries in Africa, Europe, Asia and America. The Middle East and North Africa (MENA) region has been long considered as a WNV-endemic area (Marka et al., 2013; Murgue et al., 2001a). Locally acquired cases have been recently reported in Israel (Paz and Semenza, 2013), Greece (Papa, 2017), Turkey (Failloux et al., 2017), and Italy (Calistri et al., 2010). In addition, evidence of WNV circulation has been reported in Jordan (Batieha et al., 2000) and Egypt (Soliman et al., 2010). The introduction of

WNV into the United States in 1999, which constitutes a turning point in WNV epidemiology, is thought to have originated from Israel following introduction from Africa (Giladi et al., 2001; Lanciotti et al., 1999).

RVFV belongs to Phlebovirus genus (Bunyaviridae family). It was first identified in Kenya in 1931 (Daubney et al., 1931). This virus usually affects livestock and causes abortion. The main enzootic vectors belong to the *Aedes* genus (Tantely et al., 2015). However, several *Culex* species, including *Cx. pipiens* are considered secondary vectors and contribute to the transmission of WNV to humans (Tantely et al., 2015). Infected people can be asymptomatic or develop a mild febrile disease. In less than 10% of cases, people may develop more severe symptoms such as encephalitis and hemorrhagic fever. RVFV was responsible for numerous outbreaks among animals and humans in Sub-Saharan Africa (Himeidan et al., 2014) up to Mauritania (El Mamy et al., 2011) but also in Egypt (Meegan, 1979). In the Middle East, epizootics were reported in Saudi Arabia and Yemen (Ahmad, 2000).

In Lebanon, *Cx. pipiens* is a predominant mosquito species besides another vector of arboviruses, *Aedes albopictus* (Haddad et al., 2007; Knio et al., 2005). *Cx. pipiens* colonizes urban and rural habitats whereas *Ae. albopictus* is mostly present in the densely populated coastal fringe. Local *Ae. albopictus* are competent to transmit Chikungunya virus and to a lesser extent, Dengue virus (Haddad et al., 2012). The vector competence of local populations of *Cx. pipiens* to transmit WNV and RVFV has never been evaluated. Diseases caused by these two viruses have never been reported in Lebanon. Nevertheless, a serological study conducted in a main hospital in the capital city of Beirut, confirmed the presence of neutralizing WNV antibodies in blood donors (Gallian et al., 2010). In fact, Lebanon is situated in a WNV-endemic area and located on the flyways of migratory birds with potential introduction of the virus into

the country. Moreover, Lebanon is geographically close to Yemen and Saudi Arabia, regions where RVFV had circulated actively. Intensive livestock trade between Lebanon and these countries increases the risk of RVFV introduction.

Here, we assess the vector competence of local populations of *Cx. pipiens* towards WNV and RVFV. For each *Cx. pipiens* biotype, we estimate viral infection, dissemination and transmission at different days after experimental infections.

Material and Methods

Mosquito collections

Culex egg rafts were sampled in June 2015 in Bab Mareh, in the West Bekaa district, a sub-humid, agricultural area in east of Lebanon with large stagnant water systems (Fig. 34). Egg rafts were collected on the water surface in an artificial basin and placed individually in a tube containing 30mL of water collected from the breeding site.



Figure 34. Map showing the geographic location of Lebanon East of the Mediterranean basin. This map indicates the site of mosquito egg collections in West Bekaa region. The map was modified using PowerPoint from <http://landsatlook.usgs.gov/> (Credit: U.S. Geological Survey Department of the Interior/USGS --U.S. Geological Survey). Photo: breeding site (N. Haddad).

Mosquito preparation for vector competence study

Collected egg rafts were shipped to the Laboratory of Arboviruses and Insect Vectors (AIV) at the Institut Pasteur, Paris. They were reared until the adult stage. Adults emerging from each raft were morphologically identified and only *Cx. pipiens* species were retained for this study.

Viruses and blood meal preparation

Two viruses were used in this study: WNV lineage 1 strain isolated from a horse in Camargue (France) in 2000 (Murgue et al., 2001b) and an avirulent RVFV strain Clone 13 isolated from a human case in Bangui (Central African Republic) in 1974 (Muller et al., 1995). After passages on Vero (E6) cells (ATCC cell lines), both viruses were produced on C6/36 mosquito cells. Viral stocks were stored at -80°C until use. The infectious blood meal was composed of a viral suspension (1:3) diluted in washed rabbit erythrocytes (New Zealand White rabbit, Charles River) collected at the day of mosquitoes infection. A phagostimulant (ATP) was added at a final concentration of 5mM. Virus titer in the blood meal was 1.6×10^8 plaque forming units (PFU)/mL for WNV and 1.33×10^7 PFU/mL for RVFV.

Artificial feeding of female mosquitoes

The susceptibility of Lebanese *Cx. pipiens* mosquitoes to WNV and RVFV was tested on F0 and F1 generation respectively. Ten-to-twelve day-old female *Cx. pipiens* mosquitoes were left to starve for 48 h in Biosafety Level 3 (BSL3) insectary at $28 \pm 1^{\circ}\text{C}$ with 80% relative humidity and a 16h:8h photoperiod. Females were then allowed to feed for one hour through a chicken skin membrane (obtained from a commercially purchased chicken) covering the base of a capsule of the feeding system (Hemotek) containing the blood-virus mixture maintained at 37°C . Fully engorged females were sorted, then transferred in cardboard containers and maintained with 10% sucrose at $28 \pm 1^{\circ}\text{C}$ until examination.

Saliva collection

Around 20 female mosquitoes were tested at 3, 7, 14 and 19 days post-infection (dpi). For each mosquito, saliva was collected using the forced salivation technique (Dubrulle et al., 2009). Briefly, mosquitoes were chilled, their legs and wings removed and the proboscis was inserted into 20 μ L tip filled with 5 μ L of Fetal Bovine Serum (FBS). After 45 min, medium containing the saliva was expelled into 0.2mL tube containing 45 μ L of Dulbecco's MEM (DMEM) medium. Collected saliva and the remaining mosquito bodies were conserved at -80°C for further analysis.

Viral detection in mosquitoes

In order to assess the ability of both viruses to invade and cross the midgut barrier, the infection rate (IR) and the dissemination efficiency (DE) were determined. IR reflects the proportion of female mosquitoes with infected bodies (thorax and abdomen including the midgut) among tested specimens while DE is the proportion of female mosquitoes with infected head (detection of the virus having succeeded to reach the mosquito general cavity) among tested ones. Thus, heads and bodies were separated and ground each in 300 μ L DMEM supplemented with 3% FBS. After centrifugation, the supernatant of each homogenate was conserved at -80°C. Then, 20 μ L of each sample were diluted in 180 μ L DMEM supplemented with 2% FBS and distributed in serial dilutions from 10^{-1} to 10^{-3} in duplicates on Vero cell monolayers ($3 \cdot 10^5$ cells/well) in 96-well plates. After incubation at 37°C for 6 days, inoculum was removed and the cells were fixed and stained using a crystal violet solution (0.2% in 10% formaldehyde and 20% ethanol). After washing, the presence or absence of cytopathic effect was noted.

The capacity of the WNV and RVFV to cross the salivary glands barrier was evaluated by determining the transmission efficiency (TE) which corresponds to the proportion of female mosquitoes that secrete infectious saliva among tested specimens. The number of infectious particles within collected saliva samples was estimated on Vero cell culture and expressed as PFU/saliva. Briefly, 20 μ L of each saliva were diluted in 280 μ L DMEM 2% FBS. The total volume was inoculated on a monolayer of Vero cells (8.10⁵cells/well) in six-well plates. Cells were incubated at 37°C for 6days under an overlay consisting of DMEM, 2% FBS, 1% antibiotic-antimycotic mix and 1% agarose. The lytic plaques were counted after staining with a crystal violet solution.

Statistical Analysis

Proportions (IR, DE and TE) were compared using Fisher's exact test and sample distributions (number of viral particles) with the Kruskal-Wallis test. Statistical analyses were conducted using the Stata software (StataCorp LP, Texas, and USA). P-values <0.05 were considered significant.

Results

Artificial feeding

Collected egg rafts were hatched in laboratory conditions and provided 480 adult females of *Culex pipiens* (F0 generation); of those only 174 (36.25%) had successfully fed on a WNV-infected blood. A batch of 600 F1 female mosquitoes was used for the RVFV infection assay. Of those, only 91 (15.16%) had successfully fed on infected blood.

Viral infection

Infection rate (IR) for each virus was estimated by determining the number of infected bodies (abdomen and thorax) among all engorged female mosquitoes examined at each dpi (3, 7, 14 and 19) (Fig. 35A). For WNV, IRs were very high (94.7-100%) from 3 to 19 dpi. For RVFV, IRs were much lower: at 3 dpi, the IR was 44% \pm 12.05 (mean \pm SE) and increased gradually to reach 65% (\pm 10.9) at 14 dpi and 64.3 (\pm 13.3) at 19 dpi.

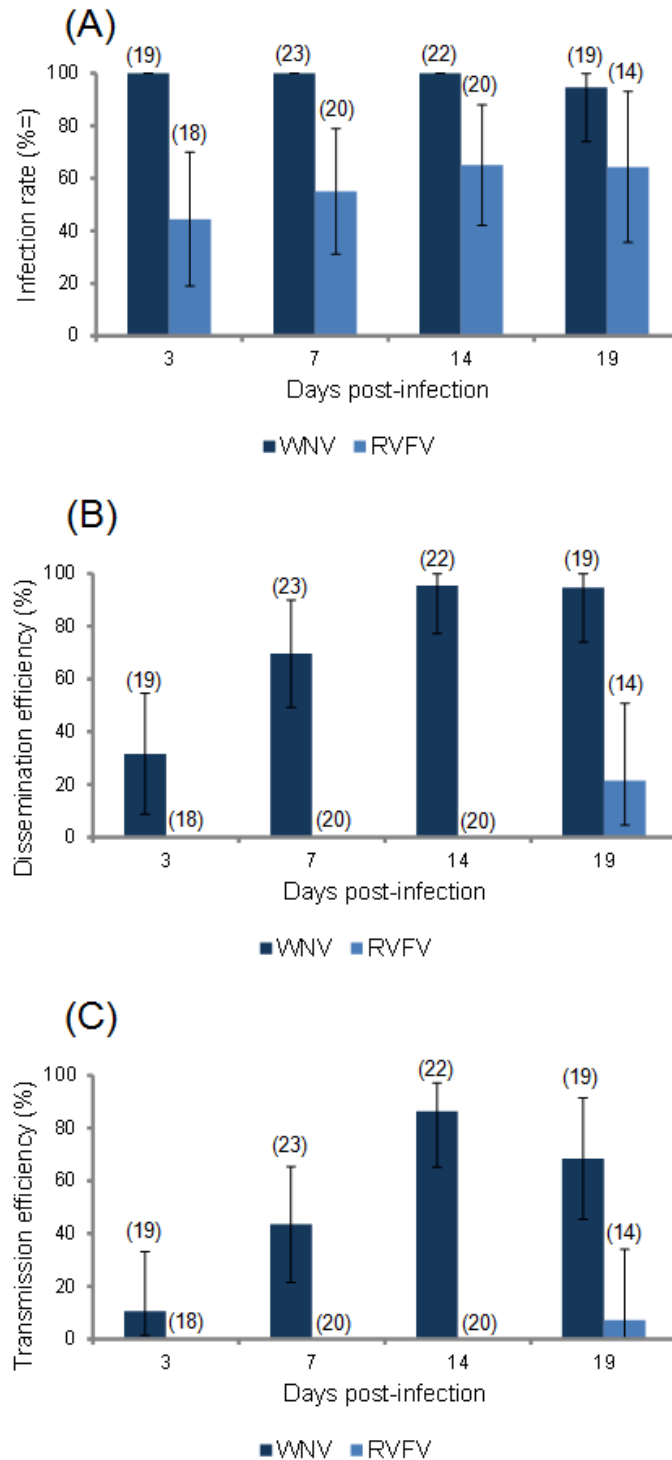


Figure 35. Infection (A), Dissemination (B) and Transmission (C) of *Culex pipiens* at different days after ingestion of an infectious blood meal containing WNV or RVFV. *Culex pipiens* F0/F1 mosquitoes were orally challenged with WNV at a titer of 1.6×10^8 PFU/mL and RVFV at titer of 1.33×10^7 PFU/mL using an artificial feeding system (Hemotek). At 3, 7, 14, and 19 dpi, homogenates of mosquito bodies and heads, and saliva collected from females were tested for the presence of virus on Vero cells to estimate infection rate, dissemination efficiency, and transmission efficiency, respectively. In brackets, the number of tested mosquitoes. Error bars show the confidence interval (95%).

Viral dissemination

The detection of viral particles in mosquito heads allowed estimating the ability of the virus to disseminate from the midgut to internal organs. For WNV, dissemination efficiency (DE) increased from 31.6% (3 dpi) to 94.7% (19 dpi) (Fig. 35B). For RVFV, virus was only detected at 19 dpi with a DE of 21.4% (± 10.9) (Fig. 35B).

Viral transmission

The ability of mosquitoes to transmit the virus was measured by detecting viral particles in saliva expectorated by mosquitoes. With WNV, transmission efficiencies were much higher than with RVFV (Fig. 35C). At 3 dpi, 10.5% (± 7.0) of mosquitoes had WNV particles detected in their saliva. TE increased gradually to reach 86.4% (± 7.3) at 14 dpi and slightly dropped to 68.4% (± 10.9) at 19 dpi. For RVFV, viral particles were only detected in saliva at 19 dpi with a TE of 7.1% (± 6.9) (Fig. 35C).

Intensity of transmission

Mosquitoes were able to deliver an average of 550 (± 450) PFU/saliva at 3 dpi with WNV, which increased to reach 1004 (± 442) PFU/saliva at 7 dpi (Fig. 36). Despite a decrease at 14 dpi, the viral load remained high at 19 dpi with 1028 (± 405) PFU/saliva. For RVFV, only one female had infectious particles in saliva at 19 dpi with a viral load of 42 PFU (Fig. 36).

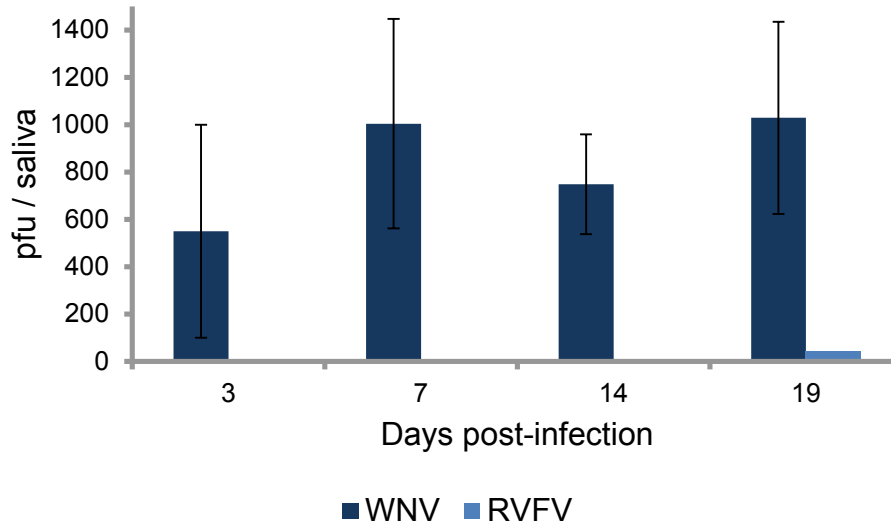


Figure 36. Mean titer of infectious viral particles secreted in saliva of *Culex pipiens* at different days after ingestion of an infectious blood meal containing WNV or RVFV. The number of infectious particles in saliva was estimated by inoculation of collected saliva on Vero cells and expressed as pfu/saliva. In brackets, the number of tested mosquitoes. Error bars refer to the standard error.

Vector competence of *Cx. pipiens* biotypes

Mosquitoes previously examined were then analyzed according to their biotype. When infected with WNV, the three biotypes presented similar IR, DE and TE at each dpi (Fisher's exact test: $p > 0.05$) (Fig. 37A-C). *Cx. pipiens molestus* started to transmit WNV from 3 dpi while *pipiens* biotype from 7 dpi and hybrids from 14 dpi. When infected with RVFV, IR, DE and TE were slightly similar at each dpi whatever the biotype (Fisher's exact test: $p > 0.05$) (Fig. 38A-C). RVFV was only detected in saliva of *Cx. pipiens* biotype *pipiens* from 19 dpi. No virus was detected in saliva of *molestus* and hybrid biotypes. For these results, it is important to consider the low number of individuals analyzed.

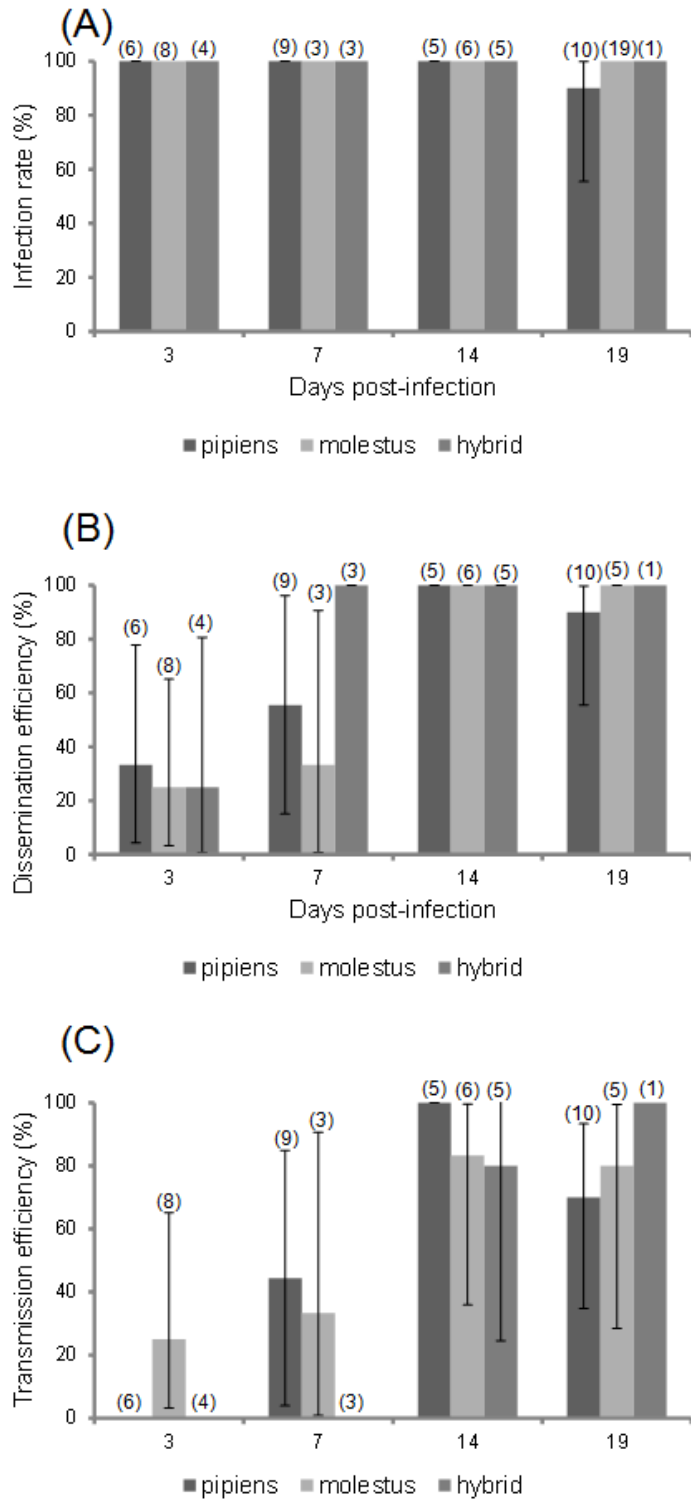


Figure 37. Comparison between *Culex pipiens* biotypes challenged with WNV: infection rate (A), dissemination efficiency (B) and transmission efficiency (C). Mosquito legs or wings were used to amplify the CQ11 gene of *Cx. pipiens*, a diagnostic gene to distinguish the two *Cx. pipiens* biotypes and their hybrids. IR, DE and TR were calculated for each biotype. In brackets, the number of tested mosquitoes.

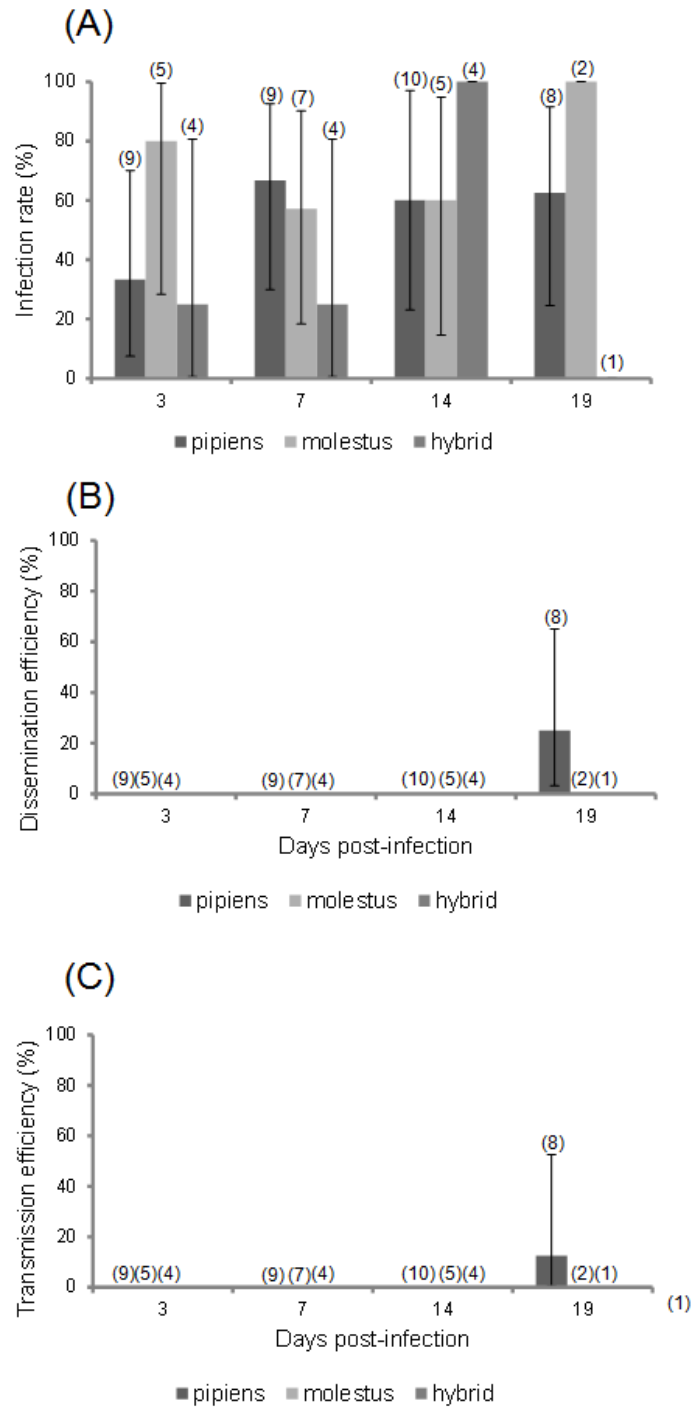


Figure 38. Comparison between *Culex pipiens* biotypes challenged with RVFV: infection rate (A), dissemination efficiency (B) and transmission efficiency (C). Mosquito legs or wings were used to amplify the CQ11 gene of *Cx. pipiens*, a diagnostic gene to distinguish the two *Cx. pipiens* biotypes and their hybrids. IR, DE and TR were calculated for each biotype. In brackets, the number of tested mosquitoes.

When comparing the amount of virus expectorated by the two *Cx. pipiens* biotypes and their hybrids, they delivered more than 500 viral particles from day 3 for *molestus* biotype, day 7 for *pipiens* biotype and day 14 for hybrids (Fig. 39A). Only one *Cx. pipiens* biotype *pipiens* delivered 42 particles of RVFV in saliva at 19 dpi (Fig. 39B).

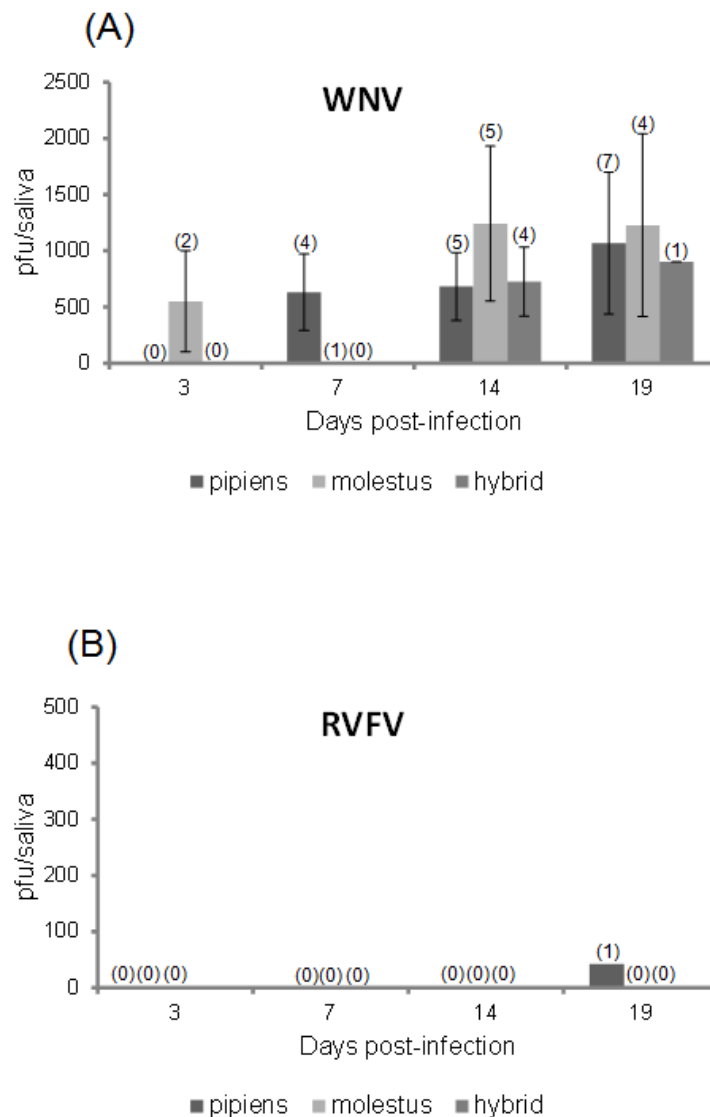


Figure 39. Mean number of viral particles of WNV (A) and RVFV (B) in saliva of the biotypes of *Cx. pipiens* examined at different days after ingestion of an infectious blood meal provided. Blood meals were provided at a titer of 1.6×10^8 PFU/mL for WNV and 1.33×10^7 PFU/mL for RVFV. The number of infectious particles in saliva was estimated by inoculation of collected saliva on Vero cells and expressed as PFU/saliva. In brackets, the number of tested mosquitoes. Error bars refer to the standard error.

Discussion

Culex pipiens is the most widely distributed mosquito species in Lebanon and is suspected to transmit WNV and RVFV in several countries (Andreadis, 2012; Tantely et al., 2015). Using experimental infections, we showed that *Cx. pipiens* populations collected from West Bekaa, Lebanon were susceptible to infection by these two viruses and ensured efficient transmission of WNV and to a lesser extent, RVFV.

When exposed to an infectious blood-meal containing WNV, *Cx. pipiens* was capable to ensure viral infection, dissemination and transmission starting from 3 dpi. Most mosquitoes exposed to the infectious blood-meal were infected as IRs reached 100% at the 4 dpi examined (3, 7, 14 and 19 dpi). Dissemination and transmission were slightly lower suggesting that not all infected mosquitoes were able to transmit WNV. Mosquitoes delivered more than 500 viral particles in saliva from 3 dpi. Based on these results, *Cx. pipiens* can be qualified as a competent vector of WNV. On the other side, infections with RVFV present different patterns: lower IR, DE and TE. 44 to 64% of mosquitoes remained infected from 3 to 19 dpi. Only 21% of mosquitoes were able to ensure viral dissemination at 19 dpi and 7% were able to transmit at 19 dpi. This suggests a significant role of the midgut and the salivary glands as respective barriers to the release of viruses into the body cavity and their excretion in saliva (Kramer and Ebel, 2003). In this manner, *Cx. pipiens* was less susceptible to RVFV than to WNV.

Overall, *Cx. pipiens* can transmit experimentally both viruses but the time interval between the ingestion of the viremic blood-meal and the ability to transmit the virus termed the extrinsic incubation period (EIP) was 3 days for WNV and 19 days for RVFV. *Cx. pipiens* from Tunisia showed similar EIP of 3 days with WNV and a much shorter EIP of 3 days with RVFV (Amraoui et al., 2012) underlining the significant role of mosquito genotype in specific

interactions between mosquito and virus genotypes; these interactions promoting adaptation of viral lineages to specific mosquito vector genotypes influence the outcome of transmission (Fansiri et al., 2013). In addition, when viral dose increases in blood meals, transmission efficiency also increases suggesting that hosts presenting a high viremia may infect more mosquitoes (Martin et al., 2010). Animals susceptible to RVFV can develop very high viremia, higher than $10^{10.1}$ MIPLD50 (mouse intraperitoneal 50% lethal dose/mL) in lambs (Easterday et al., 1962). Then, at higher titers of blood meal, RVFV may infect more mosquitoes.

Our study showed that different biotypes of *Cx. pipiens* exhibit different vector competence to WNV and RVFV. *Cx. pipiens* biotype *molestus* transmits earlier WNV than *pipiens* and hybrid biotypes. Therefore, *Cx. pipiens* biotype *molestus* has the shortest EIP under our experimental conditions. Mosquitoes of the *molestus* biotype persist all year long (not able to diapause during winter), are autogenous (lay the first batch of eggs without taking a blood-meal) and stenogamous (mate in confined spaces) mainly in underground areas in cities (Harbach et al., 1985). Mosquitoes of this biotype feed mainly on mammals and then can potentially transmit pathogens such as WNV to humans. It can be suggested that the *molestus* biotype predominant in urban settings could intervene in an inter-human transmission of WNV. It has been shown that transmission of WNV by *molestus* was not affected by temperature (Vogels et al., 2016); the colonization of hypogenous sites (where temperatures are more stable) may lead to select genetically differentiated mosquito populations more adapted to this environment with presumably effects on their phenotypic characteristics including vector competence for arboviruses.

With RVFV, the biotype *pipiens* was alone capable of transmitting the virus at 19 dpi. While the infection was efficient from 3 dpi for the three biotypes, only *Cx. pipiens* biotype

pipiens was able to disseminate the virus at 19 dpi. This result indicates that the midgut plays a significant role of barrier to the release of the virus into the mosquito general cavity. Moreover, the RVFV strain Clone 13 which is a naturally attenuated virus characterized by a deletion of 70% of the NSs gene encoding for a virulence factor (Billecocq et al., 2004; Le May et al., 2004) replicates differently in mosquitoes. Viral dissemination was higher when mosquitoes were exposed to a virulent RVFV such as ZH548 (Moutailler et al., 2008). In our study, only one *Cx. pipiens* biotype *pipiens* was able to transmit the virus at 19 dpi with 42 viral particles detected in saliva. This viral load was more than 14 times lower than the dose detected in *Cx. pipiens* mosquitoes from the Maghreb region (i.e. 620 viral particles) (Amraoui et al., 2012), but probably enough to infect hosts, animals and humans.

In conclusion, the predominant *Cx. pipiens* mosquito in Lebanon is susceptible to both viruses, WNV and RVFV. The biotype *molestus* can transmit WNV earlier than *pipiens* and hybrid biotypes whereas the biotype *pipiens* acts as the sole efficient experimental vector of RVFV, i.e. capable of transmitting at late days post-infection. As Lebanon is located in a region where WNV and RVFV can be potentially introduced (respectively through migratory birds and animal trades), local health authorities should establish an active surveillance to detect any new human cases in addition to reinforce the entomological surveillance allowing an early viral detection in field-collected mosquitoes.

Acknowledgments

We are grateful Anubis Vega-Rua for technical advice.

Chapter 8

General Discussion and Perspectives

WNV and RVFV are mosquito-borne viruses. Their introduction to a given country usually occurs following the introduction of viremic hosts. The vector capacity of the local vectors is the determinant factor that would allow the transmission of these viruses in the country. This capacity does not depend only on the receptivity of the vector to the virus but also on several extrinsic factors such as attractiveness to the host and vector density. *Culex pipiens* is a potential vector for both viral species. In the Middle East, high human population density and human-induced habitat changes creating potential mosquito breeding sites favor the presence of this mosquito (Conley et al., 2014).

The transmission of WNV occurs in Lebanon as we were able to demonstrate the exposure of local hosts (humans and horses) to this virus. The Bekaa plane is the probable introduction region of the virus. Besides, *Culex pipiens* of this region showed to be receptive to WNV. The biotype *molestus* which is known to feed on mammals including humans and also on birds (Harbach, 2012), showed a shorter EIP than the *pipiens* biotype under our experimental conditions. Therefore, *molestus* biotype is highly competent to ensure transmission of WNV to humans. The observed low endemicity of this virus, compared to what is usually observed in the neighboring Israel, that is located on the same flyway of migratory birds as Lebanon, is intriguing. The climatic conditions of the Bekaa plane, which is a plateau of 800-900 m of altitude, during the bird migration season, could explain this difference. Indeed, during spring (northward migration) and fall (southward migration), the local temperatures in the Bekaa valley are not high enough and consequently, mosquito population densities are not very important.

WNV does not seem to be the only flavivirus circulating in Lebanon as shown in the important cross-reactivity with WNV antigens by the In-House ELISA testing. Several avian flaviviruses, occurring in Africa, are likely to occur in the EMR, including Lebanon, through bird migration as one of their major routes from Africa passing through the Middle East (Figure 17). Bagaza virus (BAGV), which was recently considered a synonym of Israel Turkey Menigoencephalitis virus (Fernández-Pinero et al., 2014), is one of these avian flaviviruses that can potentially occur in the EMR. This virus affects mainly birds; however, specific antibodies were detected in patients suffering from encephalitis in India (Bondre et al., 2009). Usutu Virus (USUV) is another avian flavivirus, with more zoonotic potential, that can be spread to the EMR. This virus was described in South Africa and was introduced to Europe where it caused sudden death in birds and affected several immune-compromised persons in Italy during the year 2009 (Becker et al., 2012; Savini et al., 2011). Both BAGV and USUV can be transmitted by *Cx. pipiens* (Braverman et al., 2003; Calzolari et al., 2010).

Non-Avian Flaviviruses could also circulate in Lebanon. Dengue virus was historically spread in the EMR. Since the elimination of its vector, *Ae. aegypti* at that time, the country is considered Dengue free. Nevertheless, the currently established *Ae. albopictus* can ensure the transmission of this disease (Haddad et al., 2012) as it occurred in some Mediterranean countries (Gjenero-Margan et al., 2011; La Ruche et al., 2010; Marchand et al., 2013). In addition, Zika virus can be also transmitted by *Ae. albopictus* as suggested by field and laboratory based observations (Chouin-Carneiro et al., 2016; Grard et al., 2014; Wong et al., 2013). The involvement of *Cx. pipiens* in the transmission of this virus is unlikely (Amraoui et al., 2016).

The potential introduction of RVFV to Lebanon depends on the livestock trade with countries in Africa and even in the Arabian Peninsula, where the virus occurs. *Culex pipiens*

from Lebanon, did not seem to be very competent to transmit RVFV. However, the exposure of this dominant mosquito to a highly viremic animal could lead to an efficient transmission of the virus. Moreover, the possibility of this virus to be locally transmitted by other mosquitoes, especially those of the *Aedes* genus, should be considered. Based on field observation, *Aedes albopictus*, is the most dominant species of this genus in Lebanon (Haddad et al., 2012). The role of this species in transmitting RVFV was suggested by Turell and collaborators (1988). In a vector competence study conducted on *Ae. albopictus* strains from Texas (USA), 15% of the orally infected mosquitoes, with a disseminated infection, transmitted the virus. This rate is similar to what was observed in *Aedes mcintoshi* that has been implicated in RVFV transmission in Africa (Turell et al., 1988). The vector competence of the Lebanese tiger mosquito toward this virus needs to be assessed.

RVF epidemics are more and more frequent in Africa (CDC, 2017) and in some countries of the Middle East, probably in relation with climatic changes and intensified livestock trade (Chevalier et al., 2010). Therefore, the probability of spread of RVF outside the historically known endemic area remains a possibility especially to regions with large population of livestock. Therefore, control measures especially on imported animals should be reinforced.

Current and previous entomological observations confirmed the dominance of two important vectors: *Cx. pipiens* and *Ae. albopictus* species. Both species are competent vectors to several arboviruses that are likely to be introduced to Lebanon as the result of globalization and climate change. Consequently, vector control programs that have ceased since 1975, at the beginning of the Lebanese war, should resume in order to prevent a potential local spread of these pathogens.

Our study gave evidence, for the first time, of WNV circulation in human and horses in Lebanon. Although the circulation seems to appear at a low pace, we observed a 1.01% and

1.98% seroprevalence rates against WNV among human and horses respectively (after PRNT). In addition, experimental infections of *Cx. pipiens* from Lebanon showed that these local mosquitoes are competent vectors of WNV and to a lower extent, RVFV. In this study, we were able to identify *molestus* and *pipiens* biotypes and their hybrid in the country. Both biotypes were competent to transmit WNV, while the *molestus* biotype had a shorter EIP and therefore can ensure faster transmission of this virus.

In this project we were able to describe important elements related to the epidemiology of WNV in Lebanon, however we failed to characterize the identity of the virus certainly because of the observed low endemicity. Nevertheless, this study highlighted the potential circulation of other flaviviruses in the country. Therefore, and in order to gather more epidemiological information, we suggest to perform the following studies:

- Repeat serosurveillance study on larger numbers of chickens and use more sentinel birds in order to pick up potential future circulation of WNV.
- Carry out additional entomological studies and screen by molecular tools greater numbers of mosquitoes for WNV or even for flaviviruses using generic primers.
- Conduct molecular screening for flaviviruses on cerebrospinal fluids of patients with aseptic meningitis in order to detect neuroinvasive flaviviruses.
- Conduct serological and entomological studies in new geographical areas such as in the South that border with the endemic Israel.
- Establish a surveillance system to conduct regular monitoring of horses to detect any potential exposure to WNV.

- Study blood preferences and feeding behaviors of *Culex pipiens* and other *Culex* species (such as *Cx. perexiguus*) in order to understand their potential involvement in WNV transmission cycle.

Finally and based on all the above observations, we invite local authorities to start an integrative surveillance system that include entomological monitoring and control programs in addition to human and animal serological screening programs in order to detect the introduction and prevent the spread of potential arboviruses.

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Appendix A: Informed Consent Form in Arabic

نموذج الموافقة المسبقة عن علم

عزيزي المواطن،

يقوم حالياً فريق البحث التابع لمختبر علم المناعة في الجامعة اللبنانية ، بالتعاون مع جامعة نيويورك ، بدراسة تهدف إلى تحديد مستوى المناعة لدى اللبنانيين تجاه "فيروس حمى غرب النيل" وفيروسات أخرى تنتقل من الطيور الموبوءة إلى الإنسان عن طريق لدغات البعوض. غالباً ما تكون الإصابات بهذا الفيروس من دون عوارض، وأحياناً قد تتسبب بحمى نادراً ما تؤدي إلى إصابة الجهاز العصبي والموت.

الهدف الرئيسي لهذه الدراسة هو تحديد نسبة تعرض السكان لهذا الفيروس بالبحث عن الأجسام المضادة لها في عينات مصلية تؤخذ من السكان. توضع نتائج هذه الدراسة بتصريف السلطات الصحية المختصة لرسم سياسة وقائية مناسبة من أجل الحد من الاصابات المستقبلية.

بغية المشاركة في هذه الدراسة، يرجى التالي:

- ملء الاستمارة المرفقة من أجل اجراء الدراسات الاحصائية الوبائية.

- التبرع ب 5 مل من الدم.

نود إحاطتكم علماً بأن:

- التجارب والفحوصات المخبرية هي مجانية. يتم تغطية نفقاتها بمنحة بحثية.

- نتائج الاختبارات تبقى سرية، وتقدم للمتبرع عند الطلب.

- عينات الدم ستستخدم فقط لغرض هذه الدراسة.

ونشكر لكم مشاركتكم!

لقد قرأت وفهمت المعلومات المذكورة أعلاه، وأقبل بحرية المشاركة في هذه الدراسة.

التاريخ:

الاسم والشهرة:

التوقيع:

Appendix B: Questionnaire in Arabic

الاستمارة

رقم الاستمارة: المكان: التاريخ:

الاسم والشهرة:

1. الجنس: ذكر أنثى

2. سنة الولادة: مكان الولادة:

3. نوع العمل: لا يعمل

4. هل تعمل في مكان مغلق (داخل مبنى) أو خارجي؟

داخلي: أقل من ساعة يومياً خارجاً خلال دوام العمل

خارجي: أكثر من ساعة يومياً خارجاً خلال دوام العمل

5. مكان الإقامة الحالية:

6. مكان السكن الثاني (إن وجد) :

7. هل أصبت، خلال السنوات الثلاثة الماضية، بحمى شديدة مرتبطة بصداع حاد، وآلام في العضلات والمفاصل؟

نعم كلا

في حال نعم،

- متى؟ السنة الحالية السنة الماضية ما قبل السنة الماضية

- ما كان سبب الحمى؟

لم تتم أية فحوصات مخبرية التحاليل التي اجريت لم تكشف عن العامل المسبب

تم تحديد العامل المسبب وهو:

8. هل تتعرض للدغات البعوض حيث تقيم؟

9. هل يوجد شبك مانع للبعوض حول نوافذ منزلك؟ نعم كلا

10. هل يوجد أي مستنقع أو مصدر مياه ثابتة بالقرب من مكان سكنك (100 م)؟ نعم كلا

11. هل يوجد أي قن دجاج أو بيت حمام بالقرب من مكان سكنك (100 م)؟ نعم كلا

12. هل أقمت خارج لبنان؟ نعم كلا أين؟ سنة؟

معلومات إضافية

Appendix C: Informed Consent Form in English

Dear Participant,

The research team of the Laboratory of Immunology at the Lebanese University, in collaboration with the University of New York, is conducting a study to evaluate the immune status of Lebanese population against West Nile Virus. This virus is transmitted from infected birds to humans by the bite of a mosquito. In most of the cases, the infection remains asymptomatic. Otherwise, it causes acute febrile illness, and rarely leads to encephalitis, causing long term disability or even death.

The main objective of this study is to search for the presence of specific antibodies in human which is indicative of previous exposure to the virus. The obtained results will be submitted to the Lebanese Health authorities to take necessary preventive measures.

In order to participate in this study, you are kindly requested to:

- fill out the attached questionnaire that will be used for statistical and epidemiological analysis.
- donate 5 ml of blood for laboratory analysis.

We would like to inform you that:

- The laboratory tests are free of charge. Their costs will be covered by a research grant.
- All results and provided personal information will remain secret and be treated in complete anonymity. The blood analysis results of each donor can be provided to him upon his request.
- The blood sera will be solely used for the purpose of this current study.

Thank you for your participation

I have read and understood the above mentioned information. I freely accept to participate to this study.

Date:

Name:

Signature:

Appendix D: Questionnaire in English

Questionnaire number: Place: Date:

Name:

1. Gender: Male Female
2. Year of Birth: Place of Birth:
3. What is your work type? Don't work
4. Do you work inside a building or outside?
 - Inside: less than one hour outside during work time
 - Outside: more than one hour outside during work time
5. Place of current residence:
6. Place of other residence (if present):
7. Have you suffered, in the past three years, from a febrile illness associated with severe headache, and important muscle and joint pains? Yes No
 - If yes: - When? this year last year years before
 - What was the reason? the causative agent was:
 - no performed tests performed tests didn't reveal the causative agent
8. Do you get bitten by mosquitoes at your residence?
9. Do your house windows have nets for mosquitoes? Yes No
10. Is there any lake or stagnant water near your place of residence (100m)? Yes No
11. Is there any chicken coop or pigeon house (loft or dovecote) near your place of residence (100m)? Yes No
12. Did you live outside Lebanon (abroad)? Yes No

Where? When?

Additional information

Appendix E: Coating buffer, 10X PBS Buffer and 10X TBE Buffer

Preparations

1. Coating buffer (pH 9.6)

Combine 0.3975g of Na_2CO_3 (0.015M) (Uni-Chem, MW 105.99), 0.7325g of NaHCO_3 (0.035M) (Sigma Aldrich, MW 84.01) and 250mL ddH₂O. Stir until dissolved, check for correct pH and adjusted it then store the solution at 4°C.

2. Preparation of 1L of 10X TBE Buffer

The following salts were dissolved in 800ml deionized water: 108g of Tris Base (Sigma Aldrich) and 55g of Boric Acid (Amresco). Then 40ml of prepared 0.5M EDTA (Scharlou) (pH=8) was added to the mixture and the volume was completed to 1L with deionized water.

3. Preparation of 5L of 10X PBS Buffer

The following salts were dissolved and stirred in 5L deionized water until all the salts were well dissolved: 340g of NaCl (Sigma Aldrich), 139.5g of $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ (Sigma Aldrich) and 21.5g of KH_2PO_4 (Sigma Aldrich) (pH of 10X PBS between 7 and 7.4). After dilution, 1× PBS was obtained with a pH between 6.8 and 7.

Appendix F: ELISA Plate Plan

	Neg Ag 1	WNV Ag 2 3		Neg Ag 4	WNV Ag 5 6		Neg Ag 7	WNV Ag 8 9		Neg Ag 10	WNV Ag 11 12	
A	C + (1)			S1			S9			S17		
B	C + (2)			S2			S10			S18		
C	C + (3)			S3			S11			S19		
D	C - (1)			S4			S12			S20		
E	C - (2)			S5			S13			S21		
F	C - (3)			S6			S14			S22		
G	Sec. Ant.			S7			S15			S23		
H	Blank			S8			S16			S24		

C +: positive control, C -: control negative, Sec. Ant.: Secondary Antibody, S1: serum 1, ..., S24: serum 24.

Appendix G: Pools details

Pool #	Pool content	Place of collection	Pool #	Pool content	Place of collection
1	30 ♀ <i>Cx. pipiens</i>	Jebb Jennine Bekaa	45	46 ♀ <i>Cx. pipiens</i>	Jebb Jennine Bekaa
2	30 ♀ <i>Cx. pipiens</i>	El Marj Bekaa	46	50 ♀ <i>Cx. pipiens</i>	Jebb Jennine Bekaa
3	27 ♀ <i>Cx. pipiens</i>	Qaraoun Bekaa	47	42 ♀ <i>Cx. pipiens</i>	Jebb Jennine Bekaa
4	30 ♀ <i>Cx. pipiens</i>	El Marj Bekaa	48	45 ♀ <i>Cx. pipiens</i>	Qaraoun Bekaa
5	31 ♀ <i>Cx. pipiens</i>	El Marj Bekaa	49	45 ♀ <i>Cx. pipiens</i>	Qaraoun Bekaa
6	31 ♀ <i>Cx. pipiens</i>	El Marj Bekaa	50	47 ♀ <i>Cx. pipiens</i>	Qaraoun Bekaa
7	31 ♀ <i>Cx. pipiens</i>	El Marj Bekaa	51	47 ♀ <i>Cx. pipiens</i>	Qaraoun Bekaa
8	30 ♀ <i>Cx. pipiens</i>	El Marj Bekaa	52	46 ♀ <i>Cx. pipiens</i>	Jebb Jennine Bekaa
9	30 ♀ <i>Cx. pipiens</i>	El Marj Bekaa	53	6 ♀ <i>Cx. pipiens</i>	Deir Ein El Jawzi Bekaa
10	30 ♀ <i>Cx. pipiens</i>	El Marj Bekaa	54	49 ♀ <i>Cx. pipiens</i>	Jebb Jennine Bekaa
11	30 ♀ <i>Cx. pipiens</i>	El Marj Bekaa	55	23 ♀ <i>Cx. pipiens</i>	Jebb Jennine Bekaa
12	30 ♀ <i>Cx. pipiens</i>	El Marj Bekaa	56	36 ♀ <i>Cx. pipiens</i>	El Marj Bekaa
13	30 ♀ <i>Cx. pipiens</i>	Jebb Jennine Bekaa	57	37 ♀ <i>Cx. pipiens</i>	Qaraoun Bekaa
14	30 ♀ <i>Cx. pipiens</i>	Jebb Jennine Bekaa	58	10 ♀ <i>Cx. martini</i>	Jebb Jennine Bekaa
15	30 ♀ <i>Cx. pipiens</i>	Jebb Jennine Bekaa	59	9 ♂ <i>Cx. pipiens</i>	Jebb Jennine Bekaa
16	30 ♀ <i>Cx. pipiens</i>	Qaraoun Bekaa	60	7 ♀ <i>Cx. perexiguus</i>	Jebb Jennine Bekaa
17	30 ♀ <i>Cx. pipiens</i>	Qaraoun Bekaa	61	7 (6 ♀ + 1 ♂) <i>Cx. martini</i>	El Marj Bekaa
18	30 ♀ <i>Cx. pipiens</i>	Qaraoun Bekaa	62	3 ♂ <i>Cx. pipiens</i>	El Marj Bekaa
19	30 ♀ <i>Cx. pipiens</i>	Qaraoun Bekaa	63	2 ♀ <i>Cx. perexiguus</i>	El Marj Bekaa
20	30 ♀ <i>Cx. pipiens</i>	Qaraoun Bekaa	64	5 ♀ <i>Cx. martini</i>	Qaraoun Bekaa
21	30 ♀ <i>Cx. pipiens</i>	Qaraoun Bekaa	65	6 ♂ <i>Cx. pipiens</i>	Qaraoun Bekaa
22	30 ♀ <i>Cx. pipiens</i>	Qaraoun Bekaa	66	1 ♀ <i>Cx. perexiguus</i>	Qaraoun Bekaa
23	30 ♀ <i>Cx. pipiens</i>	Qaraoun Bekaa	67	22 ♀ <i>Cx. pipiens</i>	Darine Aakar
24	30 ♀ <i>Cx. pipiens</i>	Qaraoun Bekaa	68	11 ♂ <i>Cx. pipiens</i>	Darine Aakar
25	30 ♀ <i>Cx. pipiens</i>	Qaraoun Bekaa	69	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
26	30 ♀ <i>Cx. pipiens</i>	Qaraoun Bekaa	70	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
27	30 ♀ <i>Cx. pipiens</i>	Qaraoun Bekaa	71	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
28	30 ♀ <i>Cx. pipiens</i>	Qaraoun Bekaa	72	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
29	47 ♀ <i>Cx. pipiens</i>	El Marj Bekaa	73	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
30	47 ♀ <i>Cx. pipiens</i>	El Marj Bekaa	74	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
31	45 ♀ <i>Cx. pipiens</i>	El Marj Bekaa	75	21 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
32	45 ♀ <i>Cx. pipiens</i>	El Marj Bekaa	76	20 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
33	45 ♀ <i>Cx. pipiens</i>	El Marj Bekaa	77	20 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
34	45 ♀ <i>Cx. pipiens</i>	El Marj Bekaa	78	20 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
35	45 ♀ <i>Cx. pipiens</i>	El Marj Bekaa	79	16 ♂ <i>Cx. pipiens</i>	El Bahsa Aakar
36	45 ♀ <i>Cx. pipiens</i>	El Marj Bekaa	80	23 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
37	45 ♀ <i>Cx. pipiens</i>	El Marj Bekaa	81	21 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
38	45 ♀ <i>Cx. pipiens</i>	El Marj Bekaa	82	21 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
39	45 ♀ <i>Cx. pipiens</i>	El Marj Bekaa	83	21 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
40	45 ♀ <i>Cx. pipiens</i>	El Marj Bekaa	84	21 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
41	45 ♀ <i>Cx. pipiens</i>	El Marj Bekaa	85	21 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
42	45 ♀ <i>Cx. pipiens</i>	El Marj Bekaa	86	21 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
43	50 ♀ <i>Cx. pipiens</i>	El Marj Bekaa	87	21 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
44	41 ♀ <i>Cx. pipiens</i>	Qaraoun Bekaa	88	21 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar

Pool #	Pool content	Place of collection	Pool #	Pool content	Place of collection
89	21 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar	133	28 ♂ <i>Cx. pipiens</i>	El Bahsa Aakar
90	21 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar	134	28 ♂ <i>Cx. pipiens</i>	El Bahsa Aakar
91	21 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar	135	17 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
92	21 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar	136	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
93	20 ♂ <i>Cx. pipiens</i>	El Bahsa Aakar	137	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
94	20 ♂ <i>Cx. pipiens</i>	El Bahsa Aakar	138	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
95	21 ♂ <i>Cx. pipiens</i>	El Bahsa Aakar	139	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
96	20 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar	140	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
97	20 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar	141	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
98	20 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar	142	19 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
99	20 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar	143	24 ♂ <i>Cx. pipiens</i>	El Bahsa Aakar
100	14 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar	144	7 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
101	20 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar	145	30 ♀ <i>Cx. perexiguus</i>	El Bahsa Aakar
102	20 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar	146	30 ♀ <i>Cx. perexiguus</i>	El Bahsa Aakar
103	20 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar	147	21 ♀ <i>Cx. perexiguus</i>	El Bahsa Aakar
104	22 ♂ <i>Cx. pipiens</i>	El Bahsa Aakar	148	20 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
105	22 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar	149	20 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
106	22 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar	150	20 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
107	22 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar	151	12 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
108	22 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar	152	22 ♂ <i>Cx. pipiens</i>	El Bahsa Aakar
109	22 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar	153	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
110	22 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar	154	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
111	22 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar	155	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
112	22 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar	156	22 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
113	22 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar	157	20 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
114	22 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar	158	20 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
115	20 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar	159	20 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
116	20 ♂ <i>Cx. pipiens</i>	El Bahsa Aakar	160	20 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
117	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar	161	20 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
118	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar	162	20 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
119	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar	163	25 ♂ <i>Cx. pipiens</i>	El Bahsa Aakar
120	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar	164	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
121	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar	165	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
122	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar	166	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
123	21 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar	167	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
124	25 ♂ <i>Cx. pipiens</i>	El Bahsa Aakar	168	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
125	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar	169	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
126	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar	170	30 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
127	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar	171	23 ♂ <i>Cx. pipiens</i>	El Bahsa Aakar
128	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar	172	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
129	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar	173	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
130	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar	174	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
131	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar	175	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
132	15 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar	176	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar

Pool #	Pool content	Place of collection
177	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
178	12 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
179	23 ♂ <i>Cx. pipiens</i>	El Bahsa Aakar
180	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
181	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
182	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
183	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
184	30 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
185	11 ♂ <i>Cx. pipiens</i>	El Bahsa Aakar
186	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
187	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
188	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
189	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
190	17 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
191	22 ♂ <i>Cx. pipiens</i>	El Bahsa Aakar
192	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
193	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
194	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
195	16 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
196	21 ♂ <i>Cx. pipiens</i>	El Bahsa Aakar
197	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
198	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
199	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
200	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
201	18 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
202	25 ♂ <i>Cx. pipiens</i>	El Bahsa Aakar
203	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
204	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
205	25 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
206	24 ♀ <i>Cx. pipiens</i>	El Bahsa Aakar
207	30 ♂ <i>Cx. pipiens</i>	El Bahsa Aakar