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CHARACTERIZATION OF WEST NILE VIRUS STRAINS ISOLATED IN ITALY

Presentata da: Dott.ssa Silvia Silenzi

Coordinatore Dottorato | **Relatore**

Prof. Lucio Cocco | **Prof.ssa Maria Carla Re**

Correlatore

Dott.ssa Giada Rossini

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INDEX

ABSTRACT 1
INTRODUCTION 2
1. INTRODUCTION TO THE ARBOVIRUSES 2
2. FLAVIVIRUS 5
3. WEST NILE VIRUS 8
3.1 Structure of WNV 9
3.2 Genome of WNV 10
3.2.1 Viral structural proteins 12
3.2.2 Viral non-structural proteins 13
3.3 WNV Replication cycle 17
3.4 Molecular Classification 20
3.4.1 Lineage 1 20
3.4.2 Lineage 2 21
3.4.3 Lineage 3 22
3.4.4 Additional proposed lineages 22
3.5 Transmission cycle 24
3.5.1 Vectors: mosquitoes and other arthropods 24
3.5.2 Birds 25
3.5.3 Humans, horses and other animals 26
3.5.4 Non-Vector-Borne Transmission 27
3.6 Epidemiology of WNV in humans 29
3.6.1 Worldwide WNV Epidemiology 29
3.6.2 Epidemiology of WNV in Italy 33
3.7 Pathogenesis 36
3.7.1 WNV propagation in the mosquito host 36
3.7.2 Initial infection, viral amplification and spread in humans 37
3.7.3 Neuroinvasion 40
3.8 Tropism 44
3.9 Clinical manifestation in humans 47
3.9.1 West Nile Fever (WNF) 47
3.9.2 West Nile Neuroinvasive Disease (WNND) 48
3.9.3 Host Risk factors 51
3.9.4 Viral Risk factors 55
3.10 Diagnosis 57
3.10.1 Nucleic acid based tests for WNV 58
3.10.2 Serologic diagnosis of WNV 58
3.11 Vaccines 59
4. INNATE IMMUNITY 61
4.1 Innate immunity to virus infection 61
4.1.1 RIG-I-like receptor signalling 62
4.1.2 Toll-like receptor signalling 64
4.1.3 NOD-like receptor signalling 67
4.1.4 Type I Interferon signalling 67
4.2 Innate immune evasion strategies of WNV 68
AIM 71
MATERIALS AND METHODS 73
1. Cells and Viruses 73
2. Virus Titrations 73
3. Growth curves 73

4. Virus strains susceptibility to Interferon-α (IFN-α) action 74
RESULTS 75
1. Growth properties of WNV strains on Vero cells 75
2. Growth properties of WNV strains on 1321N1 cells 77
3. WNV strains susceptibility to Interferon-α (IFN-α) action on Vero cells 80
4. WNV strains susceptibility to Interferon-α (IFN-α) action on 1321N1 cells 82
DISCUSSION 84
REFERENCES 88

ABSTRACT

West Nile virus (WNV) is a neurotropic flavivirus that is maintained in an enzootic cycle between mosquitoes and birds, but can also infect and cause disease in humans and other vertebrate species. Most of WNV infections in humans are asymptomatic, but approximately 20% of infected people develop clinical symptoms, although severe neurological diseases are observed in less than 1% of them. WNV is the most widely distributed arbovirus in the world and has been recently associated with outbreaks of meningo-encephalitis in Europe, including Italy, caused by different viral strains belonging to distinct lineages 1 and 2. The hypothesis is that genetic divergence among viral strains currently circulating in Italy might reflect on their pathogenic potential and that the rapid spread of WNV with increased pathogenicity within naïve population suggest that epidemic forms of the virus may encode mechanisms to evade host immunity. Infection with WNV triggers a delayed host response that includes a delay in the production of interferon-α (IFN-α). IFNs are a family of immuno-modulatory cytokines that are produced in response to virus infection and serve as integral signal initiators of host intracellular defenses. The increased number of human cases and the lack of data about virulence of European WNV isolates highlight the importance to achieve a better knowledge on this emerging viral infection. In the present study, we investigate the phenotypic and IFN-α-regulatory properties of different WNV lineage 1 and 2 strains that are circulating in Europe/Italy in two cell lines: Vero and 1321N1. We demonstrate that: Vero and 1321N1 cells are capable of supporting WNV replication where different WNV strains show similar growth kinetics; WNV lineage 2 strain replicated in Vero and 1321N1 cells as efficiently as WNV lineage 1 strains; and both lineages 1 and 2 were highly susceptible to the antiviral actions of IFN-α.

Keywords: West Nile virus, growth properties, Vero cells, astrocytes, Interferon-α

INTRODUCTION

1. INTRODUCTION TO THE ARBOVIRUSES

The term *arbovirus* is an acronym for *arthropod-borne virus* (Hubalek *et al*., 2014). It has no taxonomic significance but rather is an ecologic term used to define viruses that require hematophagous (blood feeding) arthropod vectors such as mosquitoes and other biting flies, and ticks for transmission between hosts (WHO, 1985; Gubler DJ., 2001; Weaver SC. and Reisen WK., 2010).

Being, by definition, biologically transmitted, *arboviruses* must replicate in the arthropod vector prior to transmission, as opposed to being mechanically transmitted, without replication in the vector, through contaminated mouthparts (Weaver SC., 1997; Weaver SC. and Reisen WK., 2010). Biological transmission can be vertical, involving the passage of the virus from an infected female vector to both male and female offspring. Horizontal transmission can be venereal, from a vertically infected male directly to a female vector, as well as oral from a female vector to a vertebrate host via the saliva during blood feeding. The latter horizontal mode of transmission is most common for the majority tract following a viremic bloodmeal, dissemination of the virus in the vector, and eventual virus replication in the salivary glands, followed by the injections saliva during blood feeding (Weaver SC. and Reisen WK., 2010). Thus, in general *arboviruses* require a minimum of two hosts to complete their life cycle: a vertebrate and an arthropod (WHO 1985; Gubler DJ., 2002). For most arboviruses (e. g. Usutu virus, West Nile virus, Japanese encephalitis virus) humans are often dead-end hosts, as they do not develop the high viremias needed to infect the arthropod vectors (Filipe A., *et al*., 1985; Dobler G., 1996; Gubler DJ., 2001; Jones KE., *et al*., 2008; Cleton N., *et al*., 2012). Therefore, humans are not necessary for virus maintenance and they represent just an accident during the biological transmission among vectors and hosts (Diaz LA. *et al*., 2013). Only a few viruses like Yellow fever, Chikungunya and Dengue virus have expanded their host range to include humans as an amplifying host (Cleton N., *et al*., 2012).

There are currently at least 530 viruses registered in the International Catalogue of Arboviruses: about 40% are known or probable arboviruses; another 54% are listed as possible arboviruses and about 6% are listed as definitely or probably not arboviruses (Fig. 1) (Karabatson N., 1985; Gubler DJ., 2002; Lequime S. and Lambrechts L., 2014). Most of the viruses listed in this catalogue are zoonoses or viruses that have vertebrate animals other than humans as their principal reservoir hosts (Gubler DJ., 2001) and of

the over 530 suspected arbovirus species more than 150 are documented to cause disease in humans (Karabatson N., 1985; Cleton N., *et al*., 2012; Lequime and Lambrechts, 2014). For most arboviruses (e. g. Usutu virus, West Nile virus, Japanese encephalitis virus) humans are often dead-end hosts, as they do not develop the high viremias needed to infect the arthropod vectors (Filipe A., *et al*., 1985; Dobler G., 1996; Gubler DJ., 2001; Jones KE., *et al*., 2008; Cleton N., *et al*., 2012). Therefore, humans are not necessary for virus maintenance and they represent just an accident during the biological transmission among vectors and hosts (Diaz LA. *et al*., 2013). Only a few viruses like Yellow fever, Chikungunya and Dengue virus have expanded their host range to include humans as an amplifying host (Cleton N., *et al*., 2012).

Arboviruses are included in different taxonomic families, the majority belonging to the *Flaviviridae*, *Bunyaviridae* or *Togaviridae* families, but a small number are member of the *Rhabdoviridae, Reoviridae*, and *Orthomyxoviridae* families (Fig. 2) (Dobler G., 1996; Claton N., *et al*., 2012; Go YY., 2014). Among them, four major viral genera account for the majority of arboviral disease: *Flavivirus* (e. g., Dengue, West Nile, Japanese encephalitis, and Yellow fever viruses), *Alphavirus* (e. g., Chikungunya, Eastern equine encephalomyelitis, Western equine encephalomyelitis and Venezuelan equine encephalitis viruses), *Orthobunyavirus* (e. g., California encephalitis and LaCrosse viruses) and *Phlebovirus* (e. g., Rift Valley fever and Sandfly fever viruses) (Lequime and Lambrechts, 2014).

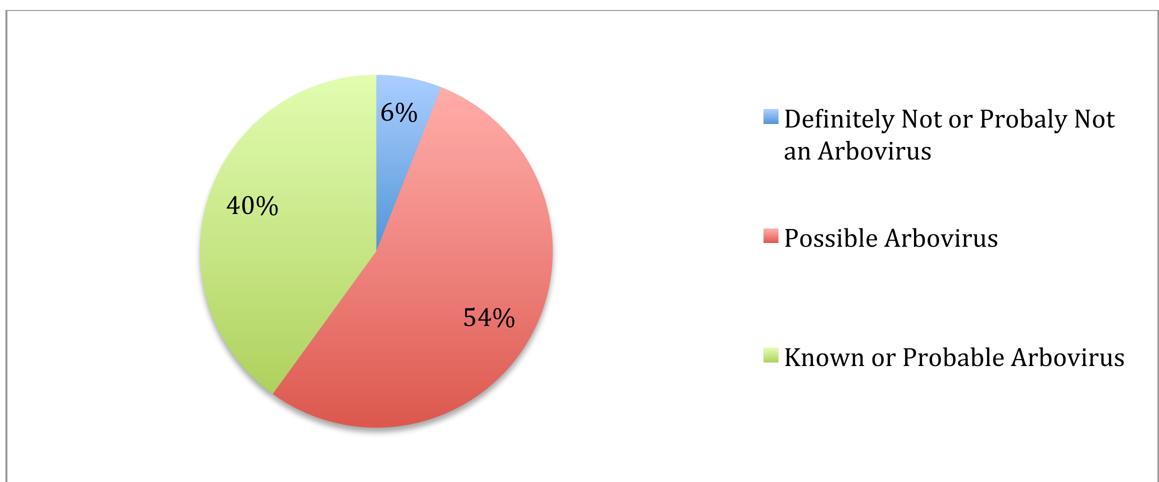


Figure 1: Arboviral status of viruses registered in the arbovirus catalogue (Gubler DJ., 2001).

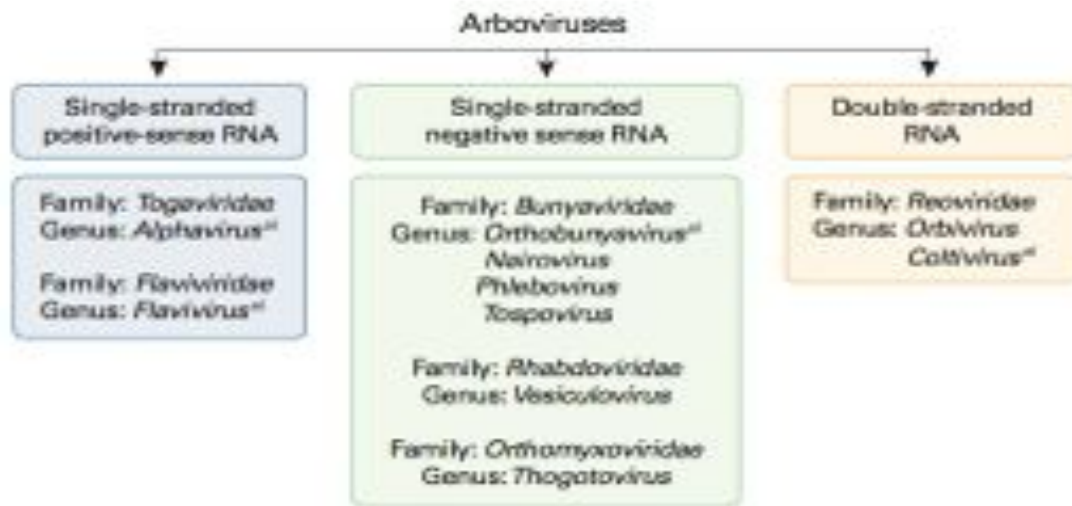


Figure 2: Classification of arboviruses. Arboviruses are included in six different taxonomic virus families. [a)] Arboviruses that cause human encephalitides belong to four genera in four virus families (Go YY., *et al*., 2014).

Arboviruses as a group have a worldwide distribution and that of each *arbovirus* is restricted by the ecological parameters governing its transmission cycle (Gubler DJ., 2001; Gubler DJ., 2002). The majority of them were first isolated in tropical areas such as Africa, South America and in some Asian countries where climate conditions permit year-round transmission by cold-blooded arthropods (Karabatsos N., 1985; Gubler DJ., 1996; Gubler DJ. and Roehrig JT., 1998; Go YY., *et al*., 2014). However, the geographic distribution and frequency of epidemic outbreaks of arboviral diseases have expanded dramatically across the world in the past several decades and they are responsible for significant global public health problems (Gubler DJ., 1996; Gubler DJ., 2001). In general, several factors such as environmental disturbs from anthropogenic activities (Vasconcelos P. *et al*., 2001), climatic changes affecting vector and host population fluctuations (Weaver SC. and Reisen WK., 2010), human movements through airplanes, animal trade and migration (Pfetter M. and Dobler G., 2010), and changes in viral genetics (Go YY., *et al*., 2014) facilitated expansion and transmission of *arboviruses* resulting in emergence/reemergence of arboviral disease outbreaks in new regions in the world (Diaz LA., *et al*., 2013). Introduction of West Nile virus (WNV) into the New World and the emergence of Japanes encephalitis virus (JEV) in Australia are few prominent examples of recent unexpected emerging/reemerging zoonotic disease (Hanna JN., *et al*., 1996; Hanna JN., et al., 1999; Go YY., *et al*., 2014).

2. FLAVIVIRUS

Flaviviruses are a group of arboviruses belonging to the family *Flaviviridae* (Pastorino B., *et al*., 2010). The genus *Flavivirus* consists of more than 70 positive-sense single-stranded RNA viruses (Tyler S., *et al*., 2011). Several members of this genus are the most clinically important arboviruses world-wide, that cause serious human and animal disease and constitute major international health problems. These include West Nile virus (WNV), Dengue virus (DENV), Japanese encephalitis virus (JEV), Yellow fever virus (YFV), tick-born encephalitis virus (TBEV), Murray Valley encephalitis virus (MVEV), and St. Louis encephalitis virus (SLEV) (Tab. 1) (Mackenzie JS., *et al*., 2004; Gubler DJ., *et al*., 2007; Gould EA. and Solomon T., 2008; Cleton N., *et al*., 2012) and they are transmitted by mosquitoes (DENV, YFV, JEV, WNV) or ticks (TBEV) (Kuno G., *et al*., 1998; Gaunt MW. *et al.*, 2001).

The *Flaviviruses* can be grouped by pathogenicity, geographic distribution, antigenic complex and subcomplexes based on classic serological criteria or into clusters, clades, and species, according to molecular phylogenetics (Calisher CH. and Gould EA., 2003; Lindenbach BD., *et al.,* 2007; Ye J., *et al*., 2013). Generally, they can be divided in three distinct groups: mosquito-borne viruses, tick-borne viruses, and viruses with unknown vectors (Cook S., *et al*., 2012). Mosquito-borne viruses infect a variety of animal species and humans. They can be further subdivided into *Culex* and *Aedes* clades, which also differ in their vertebrate hosts and pathogenesis. *Culex*-clade viruses have bird reservoirs, are neurotropic, and frequently cause meningo-encephalitis, while *Aedes*-clade viruses have primate reservoirs, are non-neurotropic, and mainly result in hemorrhagic diseases (Solomon T., *et al*., 2000; Gaunt MW., *et al*., 2001; Beck C., *et al*., 2013). The tick-borne viruses also form two groups: one group circulates among seabirds, while the other, the tick-borne encephalitis group, is primarily associated with rodents. This latter group generally produces encephalitic disease, although Omsk Hemorrhagic Fever virus (OHFV) and Kyasanur Forest Disease virus (KFDV) also cause hemorrhagic deseases in humans (Beck C., *et al*., 2013). The mosquito-borne and tick-borne groups, although distinct, appear to have evolved via a common ancestral line that diverged from nonvector borne viruses (i.e., for which no arthropod vectors are known) (Lindenbach BD., *et al*., 2007). Moreover, tick-borne flaviviruses seems to evolve at slower rate than mosquito-borne flaviviruses, probably as a results of a slower virus replication rate in tick and longer generation times of their tick hosts (Gould EA., *et al*., 1997). The salient features of *Flavivirus* taxonomy are illustrated in Figure 3.

Because of their evolution and epidemiology is largely determined by ecological needs of their invertebrate and vertebrate hosts (Fig. 3), *Flaviviruses* have distinct geographical distributions. The viruses have evolved to use whichever animal host and insect vector are present in a particular area. In general, mosquito-borne viruses tend to occur in warm climates, whereas the tick-borne viruses are more important in cooler climates (Solomon T., and Mallewa M., 2001). For example, YFV is endemic in tropical and subtropical regions in Africa and South-America and the endemic regions of DENV, geographically, overlap with those of YFV in Africa and South-America. However, DENV extends not only to Middle America and southern parts of North America but also to large parts of South-East Asia, where YFV is not found (Vasilakis N., *et al*., 2011). In Europe, many *Flaviviruses* are endemic (West Nile, Usutu, tick-borne encephalitis viruses) or occasionally imported (dengue, yellow fever viruses) (Beck C., *et al*., 2013).

Virus	Main clinical syndromes*	Main vectors	Natural hosts
Mosquito-borne viruses			
Dengue virus (serotypes 1–4)	FAR, HF	*Aedes Aegypti*	Humans, (macaque monkeys in Africa)
Yellow fever virus	Hepatitis, HF	*Aedes* and other species	Primates (monkeys, chimpanzees, baboons), humans
Japanese encephalitis virus	CNS	*Culex tritaeniorhynchus*	Waterfowl (egrets, herons), chickens, pigs
West Nile virus	FAR, CNS	*Culex pipiens*	Waterfowl and other birds
St Louis encephalitis virus	CNS	*Culex pipiens, tarsalis, nigripalpus*	Birds (pigeons, sparrows)
Murray Valley encephalitis virus	CNS	*Culex annulirostris*	Waterfowl, rabbits, marsupials
Kunjin virus	FAR	*Culex annulirostris*	(?Waterfowl, chickens)
Rocio virus	CNS	*Psorophora* species	Wild birds
Tick-borne viruses			
Tick-borne encephalitis virus	CNS	*Ixodes* species†	Forest rodents (mice, hedgehogs)
Louping ill virus	CNS	*Ixodes ricinus*	Sheep, shrews, field mice, grouse
Powassan virus	CNS	*Ixodes* species	Rodents (shrews, rats, squirrels), land birds, bats
Omsk haemorrhagic fever virus	HF	*Dermacentor* species‡	Rodents (muscrats, voles)
Kyasanur forest disease virus	HF	*Haemophysalis* species	Rodents, birds, bats, monkeys

*FAR = fever arthralgia rash syndrome, HF = haemorrhagic fever, CNS = central nervous system infection.
† Also transmitted via infected milk; ‡ also transmitted by direct contact with infected carcasses.

Table 1: Medically important *Flaviviruses* (Solomon T. and Mallewa M., 2001).

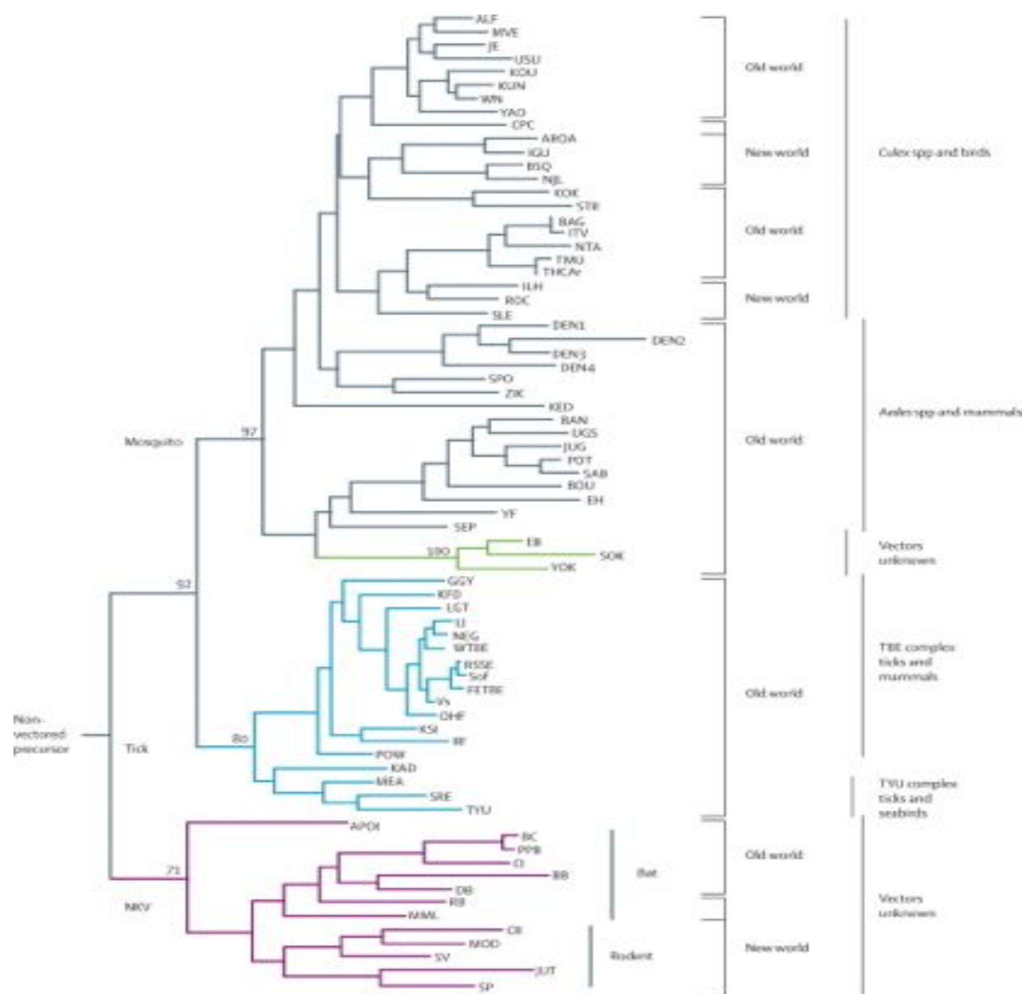


Figure 3: Phylogenetic tree showing the association of the groups of related viruses with their invertebrate vectors, vertebrate hosts, and geographic distribution.

ALF=Alfuy. MVE=Murray Valley encephalitis. JE=Japanese encephalitis. USU=Usutu. KOU=Koutango. KUN=Kunjin.WN=West Nile. YAO=Yaounde. CPC=Cacipacore. ARO=Aroa. IGU=Iguape. NJL=Naranjal. KOK=Kokobera. STR=Stratford. BAG=Bagaza. IT=Israel Turkey meningoencephalomyelitis virus. TMU=Tembusu. THCAr=strain of Tembusu. ILH=Ilheus. ROC=Rocio. SLE=St Louis encephalitis. DEN=dengue. SPO=Spondweni. ZIK=Zika forest. KED=Kedougou. UGS=Uganda S. JUG=Jugra. POT=Potiskum. SAB=Saboya. BOU=Bouboui. EH=Edge Hill. YF=yellow fever. SEP=Sepik. EB=Entebbe bat. SOK=Sokoluk. YOK=Yokose. GGY=Gadgets Gully. KFD=Kyasanur Forest disease. LGT=Langat. LI=Louping ill. NEG=Negishi. Sof=Sofj in. FETBE=far eastern TBE. Vs=Vasilchenko. OHF=Omsk haemorrhage fever. KSI=Karshi. RF=Royal Farm. POW=Powassan. KAD=Kadam. MEA=Meaban. SRE=Saumarez Reef. TYU=Tyuleniy. APOI=Apoi. BC=Batu Cave. PPB=Phnom Penh bat. CI=Carey Island. BB=Bukalasa bat. DB=Dakar bat. RB=Rio Bravo. MML=Montana myotis leucoencephalitis. CR=Cowbone Ridge. MOD=Modoc. SV=Sal Vieja. JUT=Jutiapa. SP=San Perlita. TBE=tick-borne encephalitis. WTBE=Western European TBE. RSSE=Russian spring and summer encephalitis. NKV refers to viruses with no known vector (Gould EA and Solomon T., 2008).

3. WEST NILE VIRUS

West Nile virus (WNV) is a mosquito-borne neurotropic pathogen, enveloped positive-strand RNA virus that belongs to the family Flaviviridae, genus Flavivirus (Andreson *et al*., 1999; Lanciotti *et al*., 1999; Cho H. and Diamond MS., 2012; Qian F., *et al*., 2014). Within the genus Flavivirus, WNV has been serologically classified within the Japanese encephalitis (JEV) antigenic complex, which includes the human pathogens JEV, Murray Valley encephalitis (MVE), St Louis encephalitis (SLE), and Kunjin (KUN) viruses. WNV is maintained in a mosquitoes-bird-mosquito transmission cycle (Work TH., *et al*., 1955), whereas humans and horses are considered dead-end hosts (De Filette M., *et al*., 2012). WNV is endemic in parts of Africa, Europe, the Middle East, and Asia (Dauphin G., *et al*., 2004), and since 1999 has spread to North America, Mexico, South America, and the Caribbean (Lim SM., *et al*., 2011).

The WNV has been reported in dead or dying birds of at least 326 species (CDC Database). In birds, the clinical outcome of infection is variable: some species are resistant to disease, while others are particularly susceptible (De Filette M., *et al*., 2012).

In humans, WNV was first isolated in 1937 from the blood of a woman with an undiagnosed febrile illness in the West Nile district of northern Uganda (Smithburn KC., *et al*., 1940). It was not observed again until the 1950s, when WNV was shown to be widespread in the Middle East and India and caused outbreaks of human disease in Israel. Moreover, sporadic epidemics were reported in southern France and Russia in the early 1960's and in South Africa, Belarus, and Ukraine in the 1970's. However, until the mid-1990's, WNV was rarely seen and was considered as a minor importance to public health because it only appeared sporadically (Karabatsos N., 1985; Hayes C., 1989; Gubler DJ., 2002; De Filette M. *et al*., 2012). In the 1990's, the epidemiology of infection apparently changed. Epizootic and epidemics of severe neurologic disease in horses, birds, and humans began to occur with increasing frequency and severity compared to previous outbreaks (Hubalek Z. and Halouzka J., 1999). The first human cases of WNV in its lethal encephalitis form were reported in Algeria in 1994. In 1996 severe outbreaks with a high incidence of neurological disease and death were reported in Marocco, Tunisia, Italy, Russia, Israel and France (Zeller HG. and Schuffenecker I., 2004). In the late 1990's, the virus became more virulent and expanded its geographical range to the Western Hemisphere (Rossi SL. *et al*., 2010). Since its first incursion in New York city, in the 1999 (Hayes CG., 2001), it has rapidly spread throughout the continental United States where it has been estimated to cause more than 4 million infections, resulting in over 780.000 illnesses, 38.000 clinically confirmed cases, and

1.600 deaths between 1999 and 2014 (Petersen LR., *et al*., 2012; CDC 2013; Suthar MS. and Pulendran B., 2014) becoming a major public health in many parts of the world and veterinary concern (De Filette M. *et al*., 2012).

In humans, the clinical manifestations range from asymptomatic (approximately 80% of infections) to meningo-encephalitis/paralysis and death (less than 1% of infections) (Hayes EB. and Gubler DJ., 2006; Rossi SL., *et al*., 2010; Brandler S. and Tangy F., 2013). Despite the ongoing risk to public health, there are still no specific therapy or vaccine approved for use against WNV infection in humans (Lim SM., *et al*., 2011; Cho H. and Diamond MS., 2012).

3.1 STRUCTURE OF WNV

The structure of WNV particles, specifically New York 99, the strain responsible for the outbreak in the United States, have been elucidated by Mukhopadhay *et al*. in 2003 (Fig. 4) (Mukhopadhay S. *et al*., 2003; Kaufmann B., *et al*., 2010). Electron microscopy and image reconstruction techniques established that mature WNV virion is a small spherical icosahedral with a 50 nm diameter, with no surface projections or spikes. The outermost layer contains the highest density and corresponds to the viral envelope (E) and membrane (M) transmembrane proteins that are embedded in a lipid bilayer forming the envelope of the virion (Adams SC., *et al*., 1995; Berthet FX., *et al*., 1997; Mukhopadhay S. *et al*., 2003; Kramer LD., *et al*., 2007; Kramer LD., *et al*., 2007; Rossi SL., *et al*., 2010; Colpitts TM., *et al*., 2012; De Filette M., *et al*., 2012). This outer shell is constituted by 180 copies of M protein and an equal number of copies of the E glycoprotein disposed as 90 anti-parallel homodimers arranged in three distinct symmetry environments, thus resulting in a particle of icosahedral symmetry (Mukhopadhay S. *et al*., 2003; Kaufmann B., *et al*., 2010). Inside the envelope is the nucleocapsid core, which contains multiple copies of the capsid (C) protein and the genome RNA (Kramer LD., *et al*., 2007). The C proteins, located inside virions, have no discernible nucleocapsid symmetry and no contacts between C proteins and either E or M on the inner side of the virion envelope have been observed (Zhang W., *et al*., 2003). Although nucleocapsid particles consisting of multiple copies of the C protein and genome RNA are observed after removal of the virion envelop with nonionic detergent, capsid dimers can be dissociated from these structures by treatment with high salt (Kiermayr S., *et al*., 2004). C protein dimers have a very high charge, with half of the basic residues located on the face and conserved hydrophobic region that forms an apolar surface on the opposite face (Ma L., *et al*., 2004). It is thought that the apolar surface of the C dimer interacts with

the inner side of the virion envelop while the basic residue surface of the capsid dimer interacts with the genomic RNA (Brinton MA., 2014).

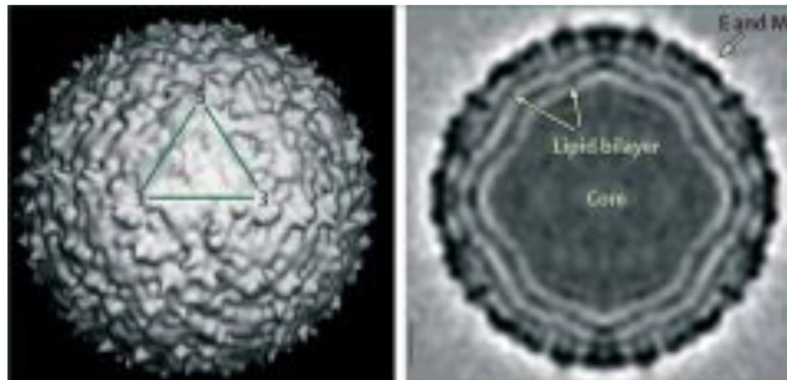


Figure 4: West Nile virion. The virus structure as reconstructed by cryo-electron microscopy. One asymmetric unit of the icosahedron is indicated by the triangle on the surface shaded view. The central section of the reconstruction shows the concentric layers of mass density. Reproduced with permission from the American Association for the Advancement of Science. (Mukhopadhyay S., *et al.*, 2003; Kramer LD., *et al.*, 2007).

3.2 GENOME OF WNV

The WNV genome is linear and is constituted by a single-stranded RNA molecule of positive polarity (Fig. 5). This RNA molecule of approximately 11.000 nucleotides (nts) in length, encodes a polyprotein in a single open reading frame (ORF) that is flanked by 5' and 3' untranslated regions (UTR). These form extensive secondary structures, which are important for replication, transcription, translation, and packaging (Shi PY., *et al.*, 1996; Khromykh AA., *et al.*, 2001; Friebe P. and Harris E., 2010; Martin-Acebes MA. and Saiz JC., 2012). The 5' UTR of the WNV genome is 96 nts in length, while the length of the 3' UTR varies from 337 to 649 nts. The 5' end contains a type 1 cap structure (m7GpppAmp) that is added by NS5 during genome transcription (Brinton MA., 2014). The variable region of the 3'UTR is located just 3' of the coding region stop codon (Beasly DW., *et al.*, 2001). The 3' end of the genome RNA does not contain a poly A tract but instead terminates with a conserved CU_{OH} (Rice CM., *et al.*, 1985; Brinton MA., *et al.*, 1986; Wengler G., *et al.*, 1991). Proper methylations of the cap structure at guanine N-7 and ribose 2'-OH positions of the first transcribed adenine are necessary for optimal infectivity of WNV RNA. Viruses defective in the N7 methylation mechanism are non-replicative, and recently the 2'-OH

methylation has been related to evasion of innate immunity by evading certain components of interferon response, therefore WNV defective in this methylation mechanism can replicate but is attenuated *in vivo* (Dong H., *et al*., 2008; Daffis S., *et al*., 2010).

The single open reading frame (ORF) of 10.301 nts in most WNV isolated, is translated as a single polyprotein of approximately 3000 amino acids that is post- and co-translationally cleaved by cellular and viral proteases into ten proteins: three structural proteins (C, premembrane or membrane, and envelope) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, and NS5) (Fig. 5). The three viral structural proteins are encoded within the 5' portion of the ORF and are mainly involved in viral particle formation, whereas non-structural proteins are encoded within the 3' portion and their function consists in viral replication, virion assembly, and evasion of host innate response (Kramer LD., *et al*., 2007; Lindenbach BD., *et al.,* 2007; Rossi SL., *et al*., 2010; Brinton MA., *et al*., 2014). The viral polyprotein contains multiple transmembrane domains that determine whether individual mature viral proteins are located on the cytoplasmic or luminal side of the endoplasmic reticular (ER) membrane after cleavage from the polyprotein (Lindenbach BD., *et al*., 2013). The C, NS3 and NS5 proteins are located on the cytoplasmic side while the PrM, E, and NS1 proteins are in the lumen and, with the exception of short regions between transmembrane domains, the NS2A, NS2B, NS4A and NS4B proteins are located within the ER membrane bilayer (Lindenbach BD., *et al*., 2013; Brinton MA., *et al*., 2014).

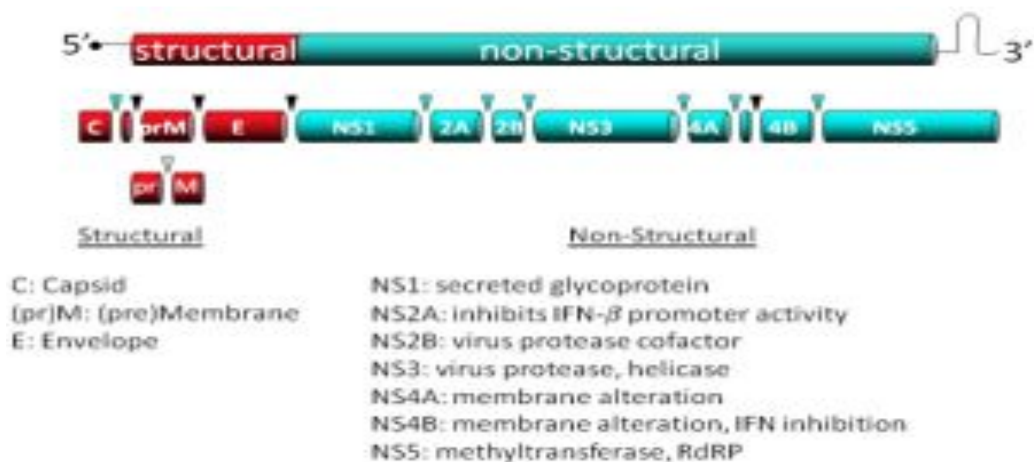


Figure 5: Schematic of WNV genome. A representation of the WNV genome including the 3 structural proteins that make up virion particle and the 7 non-structural proteins necessary for virus replication and immune evasion (Rossi SL., *et al*., 2010).

3.2.1 VIRAL STRUCTURAL PROTEINS

Capsid (C): The capsid, or core, (C) protein is a highly basic protein of ≈11 kd that contains a large number of scattered charged amino acids (Dokland T., *et al*., 2004; Lindenbach BD., *et al*., 2007) and is implicated in viral assembly and replication (Schrauf S., *et al*., 2009). The N- and C-termini parts of the protein are intrinsically disordered regions and may play a role in RNA folding during viral replication by conferring RNA chaperoning activity to the C protein (Ivanyi-Nagy R., *et al*., 2008). The central part of the C protein is a hydrophobic region that mediates membrane association (Ma L., *et al*., 2004; Lindenbach BD., *et al*., 2007). Nascent C (anchC) also contains a C-terminal hydrophobic anchor that serves as a signal peptide for ER translocation of prM. This hydrophobic domain is cleaved from mature C by the viral serine protease (Lobigs M. and Lee E., 1993). The protein dimerizes and tetramerizes to build the nucleocapsid that, together with viral RNA, forms the electron-dense core of the virion that is enveloped by the lipid bilayer. In WNV-infected cells, capsid protein can be detected in the cytoplasm, nuclei and the nucleolus of the cell, and it has been related to the induction of apoptosis (Yang MR., *et al*., 2008). Nuclear location of the C protein is mediated by a bipartite nuclear location signal and requires specific interaction with cellular importins (Bhuvanakantham R., *et al*., 2009). The capsid protein also interacts with other cellular factors, as the inhibitor of the serine/threonine phosphatase PP2A, I (2) (PP2A), Hsp70 and Jab1 (Oh WK. and Song J., 2006; Oh WK., *et al*., 2006; Hunt TA., *et al*., 2007). The phosphorilation status of the protein and Jab1 can regulate nuclear location and RNA binding activity (Oh WK., *et al*., 2006; Cheong YK. and Ng ML., 2011; Bhuvanakantham R., *et al*., 2010). The C protein has been also implicated in degradation of claudin proteins and disruption of epithelia barrier, thus helping to virus dissemination (Medigeshi GR., *et al*., 2009; Martin-Acebes MA., *et al*., 2012).

prM/M: The prM/M is a short transmembrane glycosylated protein associated to the lipid bilayer of the virion. The glycoprotein precursor of M protein, prM (≈26 kd), is translocated into ER by C-teminal hydrophobic domain of C. However, signal peptidase cleavage is delayed until the viral serine protease cleaves upstream of this region sequence to generate the mature form of C protein (Lobigs M. and Lee E., 1993; Amberg Sm., *et al*., 1994; Yamshchikov VF. and Compans RW., 1994). In addition, E protein expression influences the rate of this signalase cleavage (Lorenz IC., *et al*., 2002). The cleavage of this protein by a furin-like protease occurs within the *trans*-Golgi network and is necessary for particle maturation (Brinton MA., 2002). The conversion of immature virus particles to mature virions occurs in the secretory pathway and coincides with cleavage of prM into pr and M

fragments by the Golgi-resident protease furin enzyme (Stadler K., *et al*., 1997). This protein protects virions from fusion inside acidic vesicles of the Golgi complex (Martin-Acebes MA., *et al*., 2012). The furin-like protease cleaves the prM/M membrane protein, enabling a conformational rearrangement in the viral particle from immature particles (Zhang Y., *et al*., 2007) to mature ones (Mukhopadhyay S., *et al*., 2003). Modulation of the proportion of prM/M cleavage can also modulate the sensitivity of antibody-mediated neutralization (Nelson S., *et al*., 2008).

The envelope (E): The envelope (E) is a transmembrane protein anchored to the lipid envelope by a C-terminal α-helical hairpin. It is the most immunogenic protein of the virus and the target for most neutralizing antibodies. The protein is glycosylated on position 154 on most WNV strains (Beasley DW., 2005a). Glycosylation is important for efficient transmission in mosquito and birds (Moudy RM., *et al*., 2009; Murata R., *et al*., 2010) and may be related to neuroinvasiveness (Shitato K., *et al*., 2004). E protein contains 12 cysteines involved in the formation of intramolecular disulfide bonds and the production of homodimers. E glycoprotein is organized in three domains: DI, DII, and DIII. DI links domains II and III (Nybakken GE., *et al*., 2006). DII contains a conserved region of 13 hydrophobic amino acids that form an internal fusion loop necessary for virus fusion. DIII is an immunoglobulin-like domain that is thought to be involved in the interaction between virions and host cells to enable the virus entrance, moreover it contains multiple epitopes that are recognized by neutralizing antibodies. Upon acid exposure, the E glycoprotein undergoes conformational rearrangements and changes from dimers to trimers, exposing the fusion loop to enable viral fusion of the virion with cellular endosomal target membranes. For other flaviviruses, as tick-borne encephalitis virus, this process is triggered by protonation of an individual His residue on E glycoprotein (Fritz R., *et al*., 2008) that should act as a critical pH sensor. However, this hypothesis has not been validated for WNV (Nelson S., *et al*., 2009), although point mutations can modulate the fusion threshold (Martin-Acebes MA. and Saiz JC., 2011).

3.2.2 VIRAL NON-STRUCTURAL PROTEINS

Although the functions of the WNV non-structural proteins have not yet been completely characterized, all seven are directly or indirectly involved in viral RNA synthesis and additional functions for some of these proteins have been identified. Little is known about the interactions between the viral non-structural proteins or between viral non-structural proteins and cell proteins that are required for remodelling the cell environment and for appropriately regulating active viral RNA replication complexes at different phases of the

virus life cycle (Brinton MA., 2014).

NS1: This is a versatile non-structural viral glycoprotein that has a molecular weight of 46-56 kDa, depending on its glycosylation status. NS1 exists in multiple oligomeric forms: monomers, dimers (the primary form) and hexamers, and this seems to be related to its cellular retention or secretion stage (Brinton MA., 2002; Beasley DW., 2005a). NS1 is found at different cellular locations: either cell-membrane-associated (mNS1), in vesicular compartments within the cell or on the cell surface, and as a secreted lipid-rich, extracellular (nonvirion) species (sNS1) (Smith and Wright, 1985; Westaway and Goodman, 1987; Winkler *et al*., 1988; Mason, 1989; Gutsche *et al*., 2011). Intracellular NS1 acts as an essential cofactor for viral replication, it co-localizes with dsRNA and other components of replication complex (Mackenzie *et al*., 1996; Westaway *et al*., 1997), and inhibits Toll-like receptor 3 (TLR3) signalling (Wilson JR., *et al*., 2008). Whereas, cell surface and secreted NS1 antagonize complement activation, are highly immunogenic, and both the proteins and the antibodies it elicits have been implicated in disease pathogenesis (Chung KM., *et al*., 2006; Avirutnan P., *et al*., 2010; Muller DA. and Young PR., 2013). Recently, a larger NS1-related protein (termed NS1'), produced by a ribosomal frameshift near the beginning of the NS2A gene, has been detected in infected cells and related to neuroinvasiveness (Melian MB., *et al*., 2010).

NS2A: This is a small hydrophobic transmembrane protein involved in the biogenesis of virus-induced membranes, which have a vital role in virus assembly (Leung JY., *et al*., 2008). In fact, NS2A has been detected by immunogold labelling primarily within vesicle packets (VP), associated with labelled dsRNA (Mackenzie JM., et al., 1998). Moreover, NS2A has been reported to have an immunomodulatory role because it inhibits interferon-β promoter activation (Liu WJ., *et al*., 2004), and it has reported that mutations in this protein result in viral attenuation *in vivo* (Liu MJ., *et al*., 2006; Rossi SL., *et al*., 2007). Recently, an ER membrane topology model for flavivirus NS2A was reported (Xie X., *et al*., 2013): the N-terminal amino acids are located in the ER lumen while the C-terminal tail is in the cytosol. The first of five transmembrane regions located in the middle part of NS2A contains two helicase separated by the "helix-breaker" amino acids P85 and R84. Mutation of each of these amino acids in both a replicon and an infectious clone showed that R84 but not P85 was functionally important. Interestingly, an R48E mutation attenuated both viral RNA replication and virion production while an R84 mutation had no effect on viral RNA synthesis but inhibited the production of infectious virions (Brinton MA., 2014).

NS2B: It is also a small hydrophobic protein that interacts with the NS3 C-terminal protease

domain and functions as a protease co-factor (Erbel P., *et al*., 2006). Alanine scanning approaches of NS2B has revealed two sites critical for regulation of the proteolytic activity of NS2B-NS3 complex (Chappell KJ., *et al*., 2008). The interaction between NS2B and NS3 may also confer specificity for RNA unwinding of NS3 discriminating from DNA (Chernov AV., *et al*., 2008).

NS3: This is a highly conserved and multifunctional protein, consisting of the N-terminal serine protease domain localized to amino acids 1-169 and the C-terminal domain from residues 180-618, bearing helicase, nucleoside triphosphatase, and RNA triphosphatase activities, important for viral replication (Gorbalenya AE., *et al*., 1989; Wengler G. and Wengler G., 1991; Wengler G., *et al*., 1991a). The N- and C-terminal domains are linked via a flexible inter-domain, comprising residues 169-179 (Luo D., *et al*., 2008; Assenberg JM., *et al*., 2009). However, it is not active unless tethered to its cofactor, NS2B (Chappell KJ., *et al*., 2008a). This protease cleaves the viral polyprotein to release structural and non-structural proteins and, thus, disruption of its activity is lethal for virus replication. NS3 (and also its cofactor NS2B) has been localized within paracrystalline arrays (PC) or convoluted membranes (CM), suggesting that these membranes are the sites of proteolytic cleavage (Mackenzie JM., *et al*., 1998). Both the ATPase and helicase activity of NS3 have been shown to be regulated by NS4A (Shiryaev SA., *et al*., 2009), and the two activities can function independently of each other (Borowski P., *et al*., 2001). Within infected host cells, these functions appear to be regulated by their differential localization to separate virus-induced membranous compartments (Westaway EG., *et al*., 2001). All these properties of NS3 made of this protein and its active form, NS2B-NS3, a promising antiviral target (Martin-Acebes MA., *et al*., 2012)

NS4A: This is a small hydrophobic protein with several transmembrane domains that has been localized to the viral replication complex in virus induced membranes (VP, CM and PC) (Mackenzie JM., *et al*., 1998). The C-terminal region of NS4A can be cleaved by cell signalase generating the 2K fragment that may be responsible of membrane rearrangements in infected cells (Brinton MA., 2014). In addition, it has been reported that cleavage of NS4A C-terminal regional in DENV acts as a signal sequence for translocation into the ER of the adjacent NS4B protein (Miller S., *et al*., 2007). NS4A has been also related, together with NS2A, NS2B, and NS4B, to block type I interferon signalling in flavivirus infected cells (Martin-Acebes MA., *et al*., 2012; Brinton MA., 2014). Accumulation of NS4A (and also NS4B) into ER of infected cells seems to be involved in induction of the unfolded protein response upon WNV infection. Mutations in the 2K fragments have been related to

resistance against the antiviral action of the interferon-inducible 2', 5'-oligoadenylate synthetase 1b protein, and also to resistance against the flavivirus inhibitor lycorine thanks to the enhancement of RNA replication. NS4A has been proposed to also act as a cofactor regulating ATPase activity of the NS3 helicase (Martin-Acebes MA., *et al*., 2012).

NS4B: This is a small hydrophobic non-structural protein that is hypothesized to participate in viral replication and inhibition of host interferon signalling (Munoz-Jordan JL., *et al*., 2005). Mutations in NS4B affect viral RNA replication (Wicker JA., *et al*., 2006; Puig-Basagoiti F., *et al*., 2007; Welte JA., *et al*., 2006), possibly through its interaction with NS3 helicase (Xie X., et al., 2011) and can result in attenuation of WNV *in vivo* (Wicker JA *et al*., 2006; Welte T., *et al*., 2011). NS4B has been localized in perinuclear membranes and in the nucleus of WNV infected cells (Westaway EG., *et al*., 1997a; Pheng S. and Pei-Yong S., 2013) where it may be involved in the formation of viral replication complex.

NS5: This is located at the C-terminus of the viral polyprotein and is the largest and most conserved protein amongst members of the genus *Flavivirus*. NS5 contains two domains that have different enzymatic activities. The N-terminal region contains an S-adenosyl methionine methyltransferase (MTase) domain that has N7 and 2'-O MTase activities and also acts as guanylyltransferase (Brinton MA., 2014). This domain is necessary for capping the 5' end of the viral RNA that is performed by sequential methylation reactions. The C-terminal portion contains conserved sequence motifs characteristics of all RNA-dependent RNA polymerase for replication of viral genome (RdRp domain) (Martin-Acebes MA., *et al*., 2012). The methyltransferase activities together with the polymerase activities of NS5 are genetically validated to be essential for viral replication (Pheng S. and Pei-Yong S., 2013). This protein localizes to virus induced membranes in infected cells and colocalizes with dsRNA at viral replication complexes (Mackenzie JM., *et al*., 2007a). Due to the lack of proof-reading activity of NS5, WNV populations display a variable level of sequence diversity that favours selection of variants in response to selective pressures. NS5 is also a potent antagonist of interferon signalling to evade of host innate immune defences (Laurent-Rolle M., *et al*., 2010). Both the capping and RdRp activities made of NS5 also a promising antiviral target (Martin-Acebes MA., *et al*., 2012).

3.3 WNV REPLICATION CYCLE

The WNV life cycle consists of 4 principal stages: attachment/entry, translation, replication, and assembly/egress. WNV enters cells *via* receptor-mediated endocytosis after E protein interacts with one or more cell surface receptor(s). It is not completely clear which cellular receptors are involved in WNV binding, however DC-SIGN, several glycosaminoglycans, mannose receptor, c-type lectins and, although still controversial, integrin $\alpha_v\beta_3$ have been proposed as potential receptors (Tassaneetrithep B., *et al*., 2003; Lee E., *et al*., 2004; Davis CW., *et al*., 2006; De Filette M., *et al*., 2012; Martin-Acebes MA., *et al*., 2012). After binding to the cell, the viral particles are internalized into host cells *via* a clathrin dependent mechanism. Rab 5 was reported to be required for the cellular WNV entry (Krishnan MN., *et al*., 2007). The virus-containing endosome matures during internalization from the cell surface, with the pH dropping from neutral to slightly acidic in the early endosome and becoming more acidic during maturation to the late endosome. Acidification inside the late endosome triggers rapid conformational changes on the E protein resulting in fusion between the viral and endosomal membranes, and release of the virus nucleocapsid into the cytoplasm for genome uncoating (Gollins SW., *et al*., 1986; Modis Y., *et al*., 2004; Mukhopadhyay S. *et al*., 2005; Martin-Acebes MA., *et al*., 2012). The optimal pH for conformational rearrangements and viral fusion is 6.3-6.4, and this fusion process is dependent on the presence of cholesterol in the target membrane (Moesker B., *et al*., 2010; Martin-Acebes MA., and Saiz JC., 2011). Once viral RNA genome reaches the host cell cytoplasm it is translated into a single polyprotein, which is proteolytically processed by viral and host proteases to generates structural and non-structural proteins involved in viral replication and viron assembly. Whereas the cleavages at the junction C-prM, prM-E, E-NS1, NS4A-NS4B (Nowak T., *et al*., 1989), and likely also NS1-NS2A (Falgout B., *et al*., 1995), are performed by the host signal peptidase located within the lumen of ER, the remaining peptide bonds are cleaved by the virus encoded NS3 protease. The structural proteins form the virion that encapsidates the viral RNA, and the non-structural proteins form the replication complex that is required for synthesis of viral RNA (Suthar MS., *et al*., 2013). The original viral RNA is replicated by viral and cellular proteins into multiple copies to be used in the production of new virions. WNV replication requires the viral protein NS5, which is an RNA-dependent RNA polymerase (Rice CM., *et al*., 1986; Poch O., *et al*., 1989). An “antisense” negative strand RNA is produced by this enzyme, which then serves as a template for the synthesis of many new copies of the infectious positive strand RNA genome (De Filette M., *et al*., 2012). WNV induces changes in the cellular environment in

order to create conditions more appropriate for viral replication and undergo notable intracellular membrane remodelling. In particular, viral remodelling of ER (endoplasmic reticulum) membranes to form a network of replication complex provides a microenvironment required for productive viral replication (Bidet K., *et al*., 2014). These structures are important replication and virus protein processing and are termed vesicle packets (VP), paracrystalline arrays (PC) and convoluted membranes (CM) (Westaway EG., *et al*., 1997; Mackenzie JM., *et al*., 2001). Viral replication takes place at VPs, which are generated as invaginations of the membrane of ER and contact by pores with the cell cytoplasm. VPs contain dsRNA replication intermediates, and assembled virions bud into the ER (Gillespie LK., *et al*., 2010; Matin-Acebes MA., *et al*., 2012). A specific role of cholesterol and fatty acids in WNV-induced membrane structures has been proposed, and proteasome activity seems to be also important for viral replication (Mackenzie JM., *et al*., 2007; Gilfoy F., *et al*., 2009; Heaton NS., *et al*., 2010; Fernandez-Garcia MD., *et al*., 2011; Matin-Acebes MA., *et al*., 2012). Apart from providing the adequate platform for viral replication, these membrane rearrangements may also play a role for the evasion of innate immune response by interfering with the interferon signalling machinery (Hoenen A., *et al*., 2007; Mackenzie JM., *et al*., 2007). In addition, replication of WNV, accumulation of non-structural proteins at the ER induces ER stress activating the unfolded protein response and also induces apoptosis of infected cells (Parquet MC., *et al*., 2001; Medigeshi GR., *et al*., 2007; Ambrose RL., *et al*., 2011). Following replication and translation, genomes are packaged into virions, which travel to the cell surface in exocytic vesicles and mature through the ER-Golgi secretion pathway (Rice CM., 1996; Rossi SL., *et al*., 2010). This maturation process requires the cleavage of prM/M protein by a furin-like protease located at the acidic environment of the *trans*-Golgi network (Brinton MA., 2002). After maturation, viral particles are released by exocytosis from surface of infect cells (Rossi SL., *et al*., 2010).

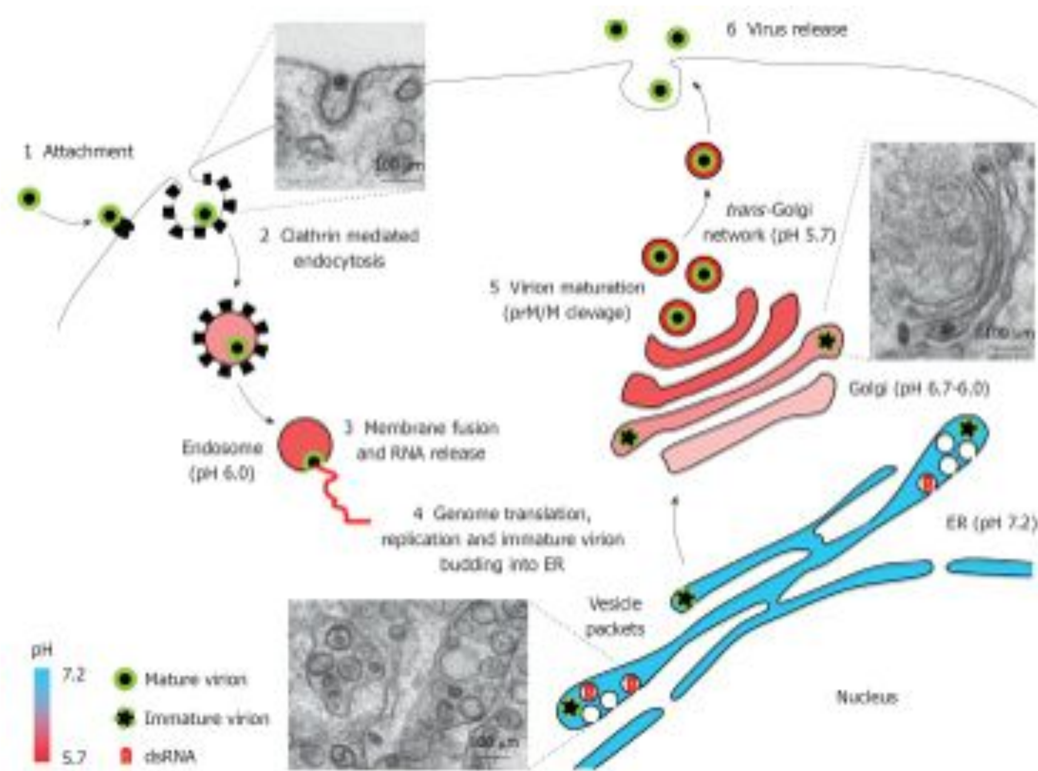


Figure 6: West Nile virus replication cycle. Schematic view of West Nile virus replication cycle in an infected cell. Electron micrograph of West Nile virus-infected Vero cells illustrate distinct snapshots from infectious cycle. WNV infects a wide range of target cells. Virion entry is initiated after the envelope protein, E, engages an unknown cellular receptor (or receptors) (step 1), followed by receptor-mediated endocytosis of the virus (step 2). The low-pH environment within the endosomal vesicle triggers viral fusion with the endosomal membrane, leading to virion uncoating and release of the viral positive-sense single-stranded RNA ((+)ssRNA) genome into the cytoplasm (step 3). The viral (+)ssRNA is translated into a single polyprotein at the ER and cleaved into mature proteins by the viral serine protease non-structural protein 2B–3B (NS2B–NS3) and cellular proteases (step 4). The NS proteins, including the viral RNA-dependent RNA polymerase NS5, form the replication complex for the synthesis of full-length negative-sense ssRNA ((–)ssRNA) intermediates. These serve as templates for the synthesis of full-length (+)ssRNAs. The viral capsid protein, C, is responsible for encapsidating viral genomic RNA, with assembly occurring on rough ER membranes (step 8). Immature virions are transported through the host secretory pathway, resulting in glycosylation of the viral E protein and host cell furin mediated-cleavage of the protein prM to the mature membrane protein, M (step 5). Mature virions are transported to the plasma membrane and released by exocytosis (step 6) (Martin-Acebes MA., *et al*., 2012; Suthar MS. *et al*., 2013).

3.4 MOLECULAR CLASSIFICATION

First classifications of WNV were based on cross-neutralization reactions and revealed that WNV is a member of the Japanese encephalitis virus serocomplex. This complex includes also other neurovirulent viruses such as Murray Valley encephalitis virus, St. Louis encephalitis virus, or Usutu virus (Poidinger M., *et al*., 1996; Beasley DW., 2005). Even though WNV has a single serotype, it nonetheless exhibits considerable genetic variation (Bondre VP., *et al*., 2007). Phylogenetic classification of WNV remains dynamic, with the large increase in genome sequence and surveillance data in recent years. Present analysis support that WNV aligns into at least seven different lineages (Fig. 7), on the basis of nucleic acid homology, with the major lineages diverging by 25%-30% nucleotide differences (Hubalek Z., *et al*., 1998; Lanciotti RS., *et al*., 1999; Lvov DK., *et al*., 2004; Mackenzie JS., *et al*., 2009; May FJ., *et al*., 2011; Papa A., *et al*., 2011). WNV strains that cause disease in humans and horses belong into the major lineages 1 and 2 (Marka A., *et al*., 2013; Di Sabatino D., *et al*., 2014), while other lineages have been sporadically detected in mosquitoes and birds but not associated with human disease (Vazquez A., *et al*., 2010). The phylogenic classification does not consistently correlate with the geographical distribution of WNV, which may be attributed to the broad dissemination of the virus by migrating bird species (Gray TJ. and Webb CE., 2014).

3.4.1 LINEAGE 1

Lineage 1, the largest and the most widespread, contains WNV strains isolates from Europe, Africa, Australia, Asia, North and Central America, as well as the Middle East (Hosseini NS., *et al*., 2014; Gray TJ. and Webb CE., 2014; Lanciotti RS., *et al*., 1999). Lineage 1 can be further subdivided into three different clades: 1a, 1b and 1c. Clade 1a is the most widely distributed and contains strains from the Americas (including the NY99 strain), Europe, Africa, the Middle East and Israel. Until recently, clade 1a comprises most of the isolates associated with outbreaks of human encephalitis, including the ongoing epidemic in North America (Lanciotti RS., *et al*., 1999). Interestingly, this clade displays close genetic relationship between geographically distant areas which are supposed to be the result of WNV introductions *via* migratory birds (Martin-Acebes MA., *et al*., 2012) This clade can further be divided in six clusters with distinct evolutionary histories (May FJ., *et al*., 2011). Sublineage 1b, contains the Australian Kunjin virus, that is an uncommon cause of human disease endemic to Australia and it is probably found in South East Asia and Papua New Guinea (Hall RA., *et al*., 2001; Gray TJ., *et al*., 2011; Rossi SL., *et al*., 2012; Hosseini NS.,

et al., 2014). While few human cases were reported, a major epidemic of illness in horses was reported in southeast Australia in 2011 (Frost MJ., *et al*., 2012). Clade 1c is only found in India. It has been proposed that isolates previously classified as sublineage 1c be reassigned to a new lineage 5 (Lanciotti RS., *et al*., 2002; Beasley DW., 2005; Bronde VP., *et al*., 2007). The fact that only one endemic genotype has been detected in India (1c) and one in Australia (1b), suggests that WNV was successfully introduced into these locations only once, as well as it was the casa in the American continent, where WNV was introduced in 1999 in the East Cost of the US (Lanciotti RS., *et al*., 1999; May FJ., *et al*., 2011). The first North American WNV isolate was most closely related to a strain isolated from a dead goose in Israel (lineage 1) during the 1998 outbreak, suggesting that North American WNV was derived from this epidemic (Lanciotti RS., *et al*., 1999). However, recent data suggest that the epidemic in Israel in 1998 was not the direct progenitor of North American epidemics, but rather that both epidemics originated from the same (unknown) location (May FJ., *et al*., 2011).

3.4.2 LINEAGE 2

Lineage 2 WNV, until the mid-2000s, was predominantly limited to sub-Saharan Africa and Madagascar, where it has been a cause of mild febrile illness in humans, rarely progressing to severe disease and typically not associated with outbreaks (Lanciotti RS., *et al*., 2002). However, in 2004 and 2005, WNV belonging to lineage 2 was first identified in wild birds in Hungary, with subsequent rapid spread to central Europe (Bakonyi T., *et al*., 2005; Bakonyi T., *et al*., 2006). Since 2004, lineage 2 has been observed in central and Eastern Europe. In 2010 it caused outbreaks in Romania and Greece and in 2011 it was detected for the first time in Italy (Bakonyi T., *et al*., 2006; Platonov AE., *et al*., 2008; Sirbu A., *et al*., 2010; Papa A., *et al*., 2010; Bagnarelli P., *et al*., 2011; Papa A., *et al*., 2011). These lineage 2 viruses have been implicated in avian, equine, and human cases of neuroinvasive disease with associated deaths, including cases reported in Russia, Hungary, Italy and Greece (May FJ., *et al*., 2011; Papa A., *et al*., 2011; Barzon L., *et al*., 2013; Magurano F., *et al*., 2012). The Greek and Italian strains showed the highest homology to Hungarian and South African strains, differing from the Russian lineage 2 strains. This means that at least two lineage 2 strains are circulating in Europe causing severe neuroinvasive infections in birds, horses and humans (Papa A., 2012; Papa A., *et al*., 2012). Although there are exceptions, in general, lineage 1 viruses are considered to be more virulent than the lineage 2 viruses (De Filette MD., *et al*., 2012): lineage 1 (clade 1a) viruses can cause severe human neurologic disease

whereas lineage 2 viruses generally cause a mild, self-limiting disease. Both, lineage 1 and 2, are now considered endemic in southeastern Europe, with an over 700% increase in cases reported in the region since 2009 (European Centre for Disease Prevention and Control, 2013).

3.4.3 LINEAGE 3

Lineage 3 WNV was first isolated near the Austrian and Czech Republic border in 1997. WNV belonging to lineage 3 has also referred as Rabensburg virus 97-103, named after the nearby Austrian town where the first infected *Culex pipiens* mosquitoes were isolated (Hubalek Z., *et al*., 1998; Bakonyi T., *et al*., 2005; Hosseini NS., *et al*., 2014). On the basis of genomic and antigenic diversity, it has been suggested that Rabensburg virus be assigned a new species within the Japanese encephalitis virus group (Bakonyi T., *et al*., 2005). Lineage 3 strain has not been isolated from humans, and the pathogenic potential remains uncertain, particularly as Rabensburg virus has been shown not to infect mammalian or avian cell cultures, nor infect experimentally exposed birds (Aliota MT., *et al*., 2012).

3.4.4 ADDITIONAL PROPOSED LINEAGES

Additional lineage subdivisions have been proposed for novel flavivirus isolates, including lineage 4 that contains a new variant of WNV (strain LEIVKrnd88-190), which was isolated in 1998 from *Dermacentor marginatus* ticks in a valley in the northwestern Caucasus Mountains of Russia (Bakonyi T., *et al*., 2005; Hosseini NS., *et al*., 2014). Lineage 5 WNV has been proposed for a group of human and mosquito isolate from India as early as the 1950s and cluster to form sublineage 1c (strain 804994) (Bondre VP., *et al*., 2007; Botha EM., *et al*., 2008). Lineage 6 WNV has been proposed for virus isolated from *C. pipiens* mosquitoes in southern Spain in 2006, strain HU2925/06, and forms a common evolutionary branch with lineage 4 (Vazquez A., *et al*., 2010). In addition to these minor lineages, the African virus Koutango (KOUV), first isolated in Senegal, is currently recognized as a separate species but could be considered as a seventh WNV lineage (De Filette MD., *et al*., 2012; Pesko KN. and Ebel GD., 2012). The human pathogenicity of lineages 4, 6 and 7 WNV is poorly understood, with human infection not reported (Gray TJ. and Webb CE., 2014).

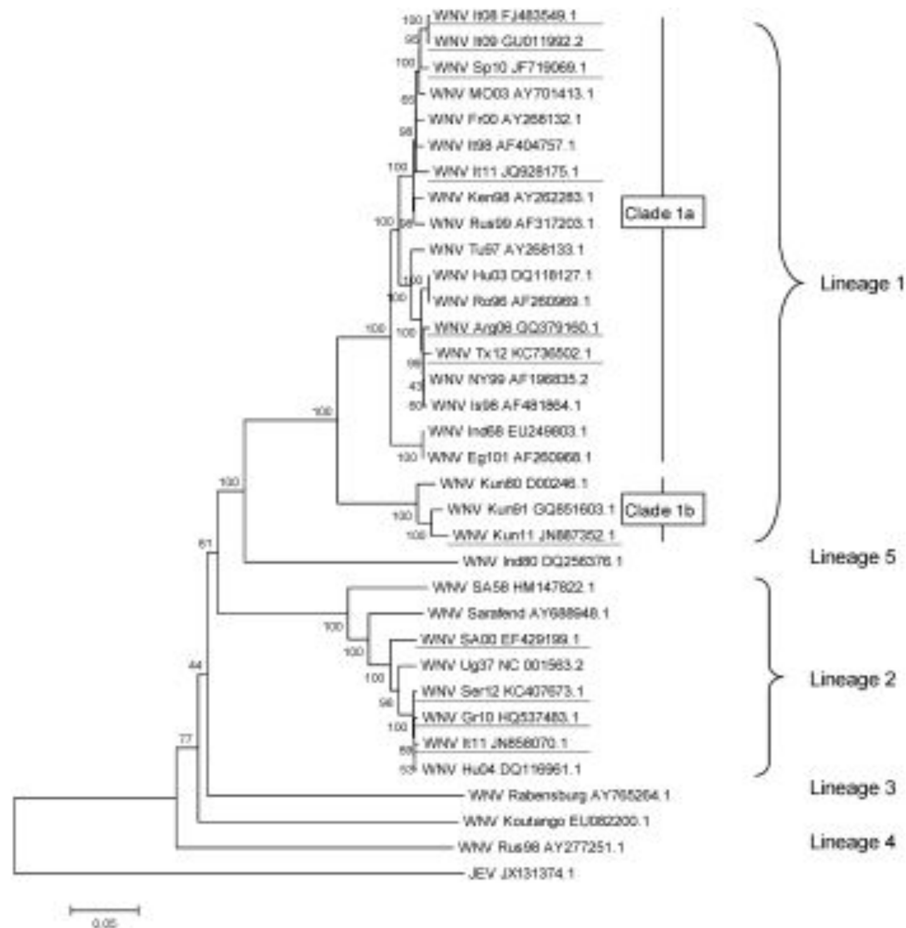


Figure 7. West Nile Virus (WNV) genetic diversity, evaluated using genetic alignment of complete genomic sequences. GenBank accession numbers are indicated on the tree branches of each virus; the first two or three letters stand for the country or the USA state reporting WNV (It = Italy, Sp = Spain, Mo = Morocco, Fr = France, Ken = Kenya, Rus = Russia, Tu = Tunisia, Hu = Hungary, Ro = Romania, Arg = Argentina, Tx = Texas, NY = New York, Is = Israel, Ind = India, Eg = Egypt, Kun = Kunjin Australia, SA = South Africa, Ug=Uganda, Ser = Serbia, and Gr = Greece) and the numbers indicate the year of isolation (96 = 1996, 10 = 2010). Japanese encephalitis virus (JEV), a closely related flavivirus, was used as an outgroup. The rooted phylogenetic tree was constructed using neighbor-joining with Jukes-Cantor parameter distances (scale bar) in MEGA (MEGA software, version 5.2) (Tamura K., et al., 2011). A bootstrapped confidence interval (1,000 replicates) and a confidence probability value based on the standard error test were also calculated using MEGA. The WNV strains responsible for recent human or equine outbreaks are underlined. The complete sequences of the most recent Romanian and Russian lineage 2 variants are not available, but at least two introduction events of lineage 2 strains have occurred in Europe: divergent lineage 2 strains have been observed in Romania/Russia and Hungary/Greece/Italy/Serbia/Austria (Ciccozzi M., *et al*., 2013; Donadieu E., *et al*., 2013).

3.5 TRANSMISSION CYCLE

WNV is maintained in nature in an enzootic transmission cycle between avian hosts and ornithophilic mosquito vectors (Fig. 8). Mosquitoes become infected by feeding on birds that carry virus particles in sufficient concentrations in their blood (Marka A., *et al*., 2013). Apart birds, the virus can be transmitted to other animals including horses and humans as well (Hayes EB., *et al*., 2005). Humans and horses are considered incidental or "dead-end" hosts for WNV, as the low concentration of virus within the blood (viremia) in mammals is usually insufficient to infect a feeding naïve mosquito and maintaining the transmission cycle (Bowen RA., and Nemeth NM., 2007; Rossi SL., *et al*., 2010). Although human cases occur primary after mosquito inoculation, infection after blood transfusion, organ transplantation, and intrauterine transmission has reported (Hayes EB., *et al*., 2005).

3.5.1 VECTORS: MOSQUITOES AND OTHER ARTHROPODS

The ability of different mosquito species to acquire and transmit WNV is highly variable (Colpitts TM., *et al*., 2012). At least over 60 species of mosquitoes from 11 different genera have been described as competent vectors. Mosquitoes of the genus *Culex* are the predominant vectors in the enzootic cycle throughout the range of the virus distribution, although the particular species of *Culex* varies according to geographic locations (Martin-Acebes MA., *et al*., 2012). In North America *Cx. pipiens*, *Cx. restuans*, *Cx. quinquefasciatus*, *Cx. salinarus*, *Cx. tarsalis*, and *Cx. nigripalpus* have been described as the most efficient competent vectors; although other species such as *Aedes albopictus*, *Aedes vexans*, *Ochlerotatus japonicus* and *Ochlerotatus triseriatus* may also play role on viral transmission as bridging vectors that can transmit the virus to mammals (Brault AC., 2009). In Europe, the virus has been isolated from more than 40 different species, being again those of the *Culex* species the main vectors (Zeller HG. and Schuffenecker I., 2004). Several other species have been also implicated in the transmission cycle as competent vectors in other geographical areas, *Cx. univittatus* in Africa, *Cx. annulirostris* in Australia, and *Cx. vishnui* and *Cx. tritaeniorhynchus* in Asia (Hall RA., *et al*., 2002; Hayes EB., *et al*., 2005; Brault AC., 2009).

Vector competence varies between species and within populations of individual species. The *C. pipiens complex* contains two genetically distinct forms: *pipiens* and *molestus* that differ in physiology and behavior with obvious implications to their epidemiological importance. Form *pipiens* is thought to be exclusively ornithophilic, while the urban form *molestus* will feed on mammals. The two forms have been shown to not interbreed in the northern Europe,

in contrast to US and southern Europe population, which contain individuals with hybrid genetic signatures (*pipiens* x *molestus*) that may generate bridge vectors, disposed to feed on both birds and mammals. Indeed, US populations of *C. pipiens*, as well as *C. nigripalpus* and *C. tarsalis*, have been demonstrated to shift their feeding from birds to mammals in the late summer and early fall, and therefore may act as bridge vectors to infect equid and human hosts (Kramer LD., 2008).

Laboratory analyses have shown that *C. tarsalis* mosquitoes become infected after consumption of blood meals with viral concentrations over 10^7 PFU/mL, whilst only up to 30% do it if the concentration is in the 10^5 PFU/mL range (Goddard LB., *et al*., 2002). On the other hand, different species of mosquitoes inoculate quite variable doses of WNV ($10^{3.4}$ PFU to $10^{6.1}$ PFU) into vertebrate hosts during natural feeding, of which around 10^2 PFU are directly inoculated into the blood (Styer LM., *et al*., 2007).

The mechanism(s) of WNV perpetuation overwintering and years may vary by region and country, but possible mechanisms include continuous low-level virus transmission, reinitiation after reintroduction of virus by migratory birds from locations where virus is active year-round, vertical transmission to females about to enter reproductive diapause in winter, and recrudescence of low levels of virus in chronically infected birds when mosquitoes are active (Anderson JF. and Main AJ., 2006; Nasci RS., *et al*., 2001).

Beside from mosquitoes, WNV has been sporadically isolated in other arthropods: WNV has been isolated repeatedly in Russia from soft ticks (*Argasidae*). In addition, soft ticks have been demonstrated to transmit virus in the laboratory, and nonviremic transmission has been demonstrated. Hard ticks (*Ixodidae*) allow the virus to pass transstadially, but are incompetent vectors. Moreover, other arthropods have been suggested as alternative vectors, including dermanyssoid mites, swallow bugs, and hippoboscid flies, but their role in the transmission cycle is not clear (Martin-Acebes MA., *et al*., 2012).

3.5.2 BIRDS

Birds are the natural reservoir of WNV. More than 300 avian species representing over 200 birds families from North America have been reported as susceptible to WNV infection after its first introduction in 1999, confirming their role as primary vertebrate in the enzootic cycle (Martin-Acebes MA., *et al*., 2010; Kramer LD., 2008). Many studies have been conducted to determine the precise role of birds in the transmission of the virus and have demonstrated that birds vary significantly in susceptibility and response to infection, with a great diversity in the profile of viremia among the different avian species. Various experimental studies

have estimated that the limit, for mosquitoes to become infected after consumption of blood meals, is of 10^5 plaque forming units (PFU) viral concentration and different birds species can develop sufficient viremia titres before the birds become moribund and die a few days after being infected in order to allow the transmission of the virus to the feeding mosquitoes. These birds belong to the orders of *Passeriformes* (corvids, sparrows, finches, *etc*.), *Charadriiformes* (woodcocks, gulls, *etc*.) *Strigiformes* (owls, eagle owls, etc.) (Komar N., *et al*., 2003; Beasley DW., 2005). In contrast, species of the order of *Paciformes* (woodpeckers), *Columbiformes* (doves, pigeons, *etc*.) and *Anseriformes* (ducks, geese, *etc*.) develop lower viremia titres, in many cases insufficient to transmit the virus in mosquitoes and they do not contribute in the epizootic cycle (Marka A., *et al*., 2013).

Feeding by infected mosquitoes is the most common route of infection, but transmission to birds also has been demonstrated by direct contact via the fecal-oral route: many avian species shed large quantities of virus in their feces or oral secretions when infected (Komar N., *et al*., 2003), allowing direct transmission from bird-to-bird and even from bird-to-human. Experimental oral infection of birds has been demonstrated (McLean RG., *et al*., 2001) and prey-to-predator infection through ingestion of infected mosquitoes or of carrion by omnivorous birds such as corvids and raptors has been suggested (Garmendia AE., *et al*., 2000).

3.5.3 HUMANS, HORSES AND OTHER ANIMALS

Thirty species of mammals and occasionally other vertebrates including reptiles and amphibians have been found infected with WNV. Generally, humans, horses and other mammals infected in a spillover transmission are considered “dead end” hosts and their role in the transmission cycle is less significant than that of birds, because viral replication does not yield significant viremia to infect feeding mosquitoes. Enzootic in equines have occurred in the US, France, Italy, Marocco and in Israel. Unvaccinated equines develop infections ranging from asymptomatic to encephalitic disease, and demonstrate a case-fatality rate of about 25%. Because of their low viremias, they are considered incidental hosts in the transmission cycle. In experimental infections of horses with WNV, viremia levels are around 10^3 PFU/mL (Bunning ML., *et al*., 2002), thus being usually insufficient to sustain infectivity cycles (Martin-Acebes MA., *et al*., 2010). Several other animal species have been described as susceptible to WNV infection, with or without clear evidence of disease, including domestic and wild mammals such as: dogs, cats, pigs, cows, llamas, sheep, alpacas, deers, reinders, raccoons, bears, wolfs, squirrels, chipmunks, rabbits, and bats,

among others (Beasley DW., 2005; Blitvich BJ., 2008). As it has described for humans and horses, in most cases the viremia raised in these animals is low, only for rabbits and chipmunks, have been demonstrated in the laboratory to mount sufficiently high levels of virus in the blood to infect a small portion of feeding *Culex* spp. mosquitoes, but generally is probably not enough to initiate a new transmission cycle (Martin-Acebes., MA. *et al*., 2012). Apart from mammals, several reptiles and amphibians, such as snakes, crocodiles, alligators, iguanas and frogs (Kostiukov MA., *et al*., 1985; Steinman A., *et al*., 2003; Klenk K., *et al*., 2004; Steinman A., *et al*., 2006) have been also described as susceptible to WNV infection and some of them raise high viremia. In the US and Mexico, farmed alligators raised at high temperatures in crowded conditions demonstrated significant mortality and mount high viremia. Transmission appears to occur directly between alligators, as well as through ingestion of uncooked infected horse meat. However, the real contribution of animals other than birds and mosquitoes in maintaining WNV cycle in nature is still uncertain (Martin-Acebes MA., *et al*., 2010).

3.5.4 NON-VECTOR-BORNE TRANSMISSION

Even though the main mode of WNV transmission to vertebrate is *via* infected mosquito bite, it has been documented that alternative less common modes of non-vector-borne transmission in humans also exist: through solid organ transplantation from an infected donor to healthy recipient; the placenta from an infected mother to her fetus (vertical transmission), occupational infection concerning mainly laboratory professionals and through transfusion of infected blood and blood products (Martin-Acebes MA., *et al*., 2010; Marka A., *et al*., 2013). The first case of virus transmission through transfusion of red blood cells, platelets and fresh-frozen plasma has been reported in 2002 (Francis RO., *et al*., 2012) which drove, in 2003, to consequent screening of six million blood units with NAT test resulting in the removal of 818 positive for the virus units (Iwamoto M., *et al*., 2003; Pealer LN., *et al*., 2003; Hayes EB. and O'Leary DR., 2004; Paisley JE., *et al*., 2006). Routine testing of American Red Cross during 2003-2004 identified 540 donations that were WNV RNA positive but, although this technique is the one widely used for blood unit examination, a case of transmission followed by failure of NAT to detect units with a low viremia level was reported in Nebraska (De Oliveira AM., *et al*., 2004). In addition, in 2002 was reported the WNV transmission through solid organ transplantation from an organ donor, probably infected through blood transfusion, to four transplant recipients (CDC 2002; Iwamoto M., *et al*., 2003). Currently, there is not any national policy that requires organ donors screening,

but serious cases of neuroinvasive disease in recipients implies a need for ELISA and NAT testing of donors during transmission season (Inojosa WO., *et al*., 2012). In the same year, was reported the first case of transplacental WNV transmission in humans: a woman WNV infected, delivered at term a live infant that was positive for WNV-specific IgM and neutralizing antibodies with chorioretinits and severe cerebral abnormalities (white matter loss, focal cerebral destruction) (CDC 2002). In 2002, another case of probable non-vector-borne transmission of WNV through breast milk was reported but since there was no confirmed case reported from that time (Hayes EB., *et al*., 2004; Hayes EB., *et al*., 2005; Hinckley AF., *et al*., 2007). Two cases of laboratory-acquired infection were reported in USA. The most probable mode of transmission was through percutaneous inoculation (James FC., McCulloch CE., 2002; Sampathkumar P., 2003; Hayes EB., *et al*., 2005) or even through exposure to aerosol (Hayes EB., *et al*., 2005), as shown previously in mice (Nir Y., *et al*., 1965) or, as well as two turkeys breeders, they were handling were WNV infected. Nonetheless, the mode of transmission to these workers remains unknown (CDC 2003).

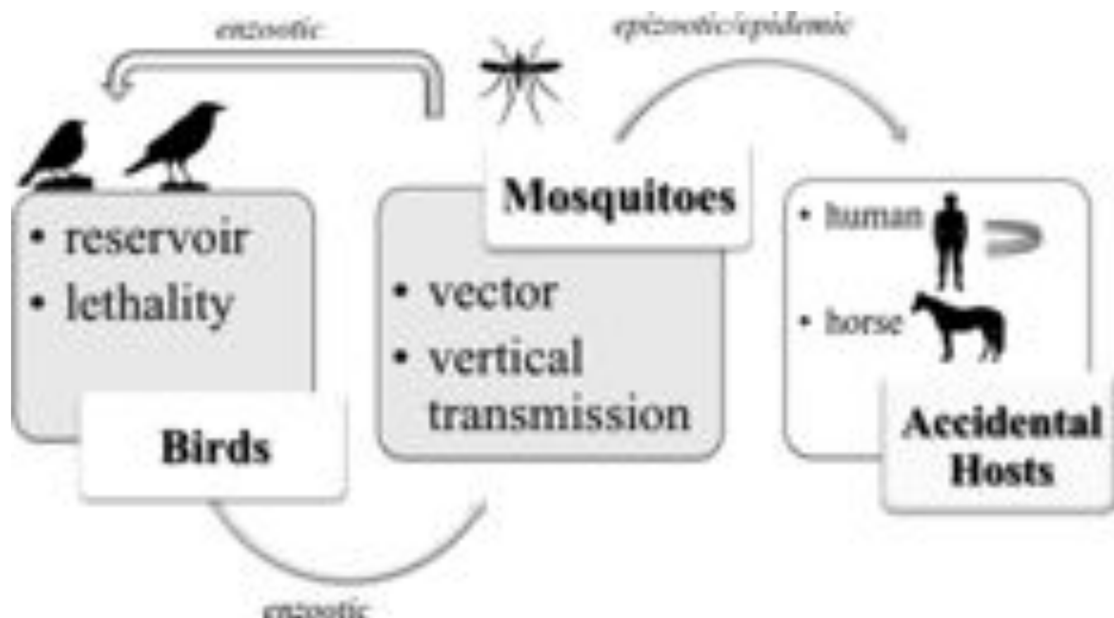


Figure 8. Diagram of the WNV transmission cycle. The maintenance of WNV in nature depends upon many avian and mosquito species. Humans and other incidental hosts, like horses, become infected when WNV-infected mosquito takes a bloodmeal from them (Rossi SL., *et al*., 2010).

3.6 EPIDEMIOLOGY OF WNV IN HUMANS

The epidemiology of WNV is continuously changing. WNV was originally isolated from the blood of a febrile woman in the West Nile province of Uganda in 1937 (Smithburn KC., *et al*., 1940). Subsequent isolations took place some years later in Egypt (Melnick JL., *et al*., 1951) Africa, Europe, Asia (Hubalek Z. and Halouzka J., 1999; Hayes CG., 2001) and then in Australia (Hubalek Z. and Halouzka J., 1999; Hall RA., *et al*., 2002; Zeller HG., *et al*., 2004; Hayes EB., *et al*., 2005), showed that the virus was widely distributed. Before 1994, outbreaks of WNV were sporadic with low clinical incidence that occurred primarily in the Mediterranean region, Africa and east Europe and WN disease was considered as minor risk for humans and horses. However, in the mid-1990s, WNV re-emerged in the Mediterranean region and in the eastern Europe, where it caused more severe symptoms, particularly affecting the nervous system, and higher mortality than had been observed in previous outbreaks (Anez G., *et al*., 2012). In 1999, WNV caused an outbreak in New York City marking its first appearance in the Americas. Subsequently, WNV has spread rapidly throughout the Western Hemisphere, including the USA, Caribbean, Mexico, Canada and as far south as Argentina and Brazil (Pepperell C., *et al*., 2003; Morales MA., *et al*., 2006; Adrian DL., *et al*., 2008; Pauvolid-Correa A., *et al*., 2011). In North America, the virus has caused meningitis, encephalitis, and poliomyelitis, resulting in significant morbidity and mortality. Including the Australian WNV subtype Kunjin virus, WNV is the most widely distributed arbovirus in the world (Fig. 9) and WNV infection is considered a serious animal or human health treat (Kramer LD., *et al*., 2007; Anez G., *et al*., 2012).

3.6.1 WORLDWIDE WNV EPIDEMIOLOGY

The virus was initially isolated in December 1937, from a 37-years-old, febrile woman in the West Nile district in the Northern Province of Uganda, currently the Arua district, during an epidemiological study defining the endemic zone of yellow fever (Smithburn KC., et al., 1940). Serum from the febrile case was inoculated intracerebrally in mice, with the subsequent viral particles shown to cause an encephalitic illness in selected vertebrate hosts. Since that time, the virus was not observed again until the 1950s when there were some sporadic reports of WNV circulation in Albania, Bulgaria, Belarus, Ukraine, and Moldavia (Hubalek Z. and Halouzka J., 1999) and caused the first WNV epidemics in Israel and Egypt (Melnick JL., *et al*., 1951; Bernkopf H., *et al*., 1953). In 1951 was reported the first outbreaks of human disease in Israel, with 123 cases of non-neuroinvasive disease. Since

were also reported the following year, while neuroinvasive cases (West Nile Neuroinvasive Disease, WNND) were recorded in 1957 and 1962 (Hayes CG., 2001). In Egypt, a large-scale epidemiological investigations and serosurveys brought up to light high WNV endemicity in southern parts of the Nile delta in contrast to a low one in the parts neighboring to the Mediterrean coast (Bernkopf H., *et al*., 1953). This report was the first to describe the seasonal pattern of WNV transmission, and to propose the natural enzootic cycle of WNV transmission between mosquitoes and birds (Hurlbut HS., *et al*., 1956). Other than sporadic epidemics was recorded in France in summer 1962, with several human cases of encephalitis, while two years later 13 human cases were reported and the virus was isolated from the blood of two entomologists as well (Murge B., *et al*., 2001). More recent outbreaks were reported in South Africa in 1974 where WNV disease caused approximately 10.000 human fever cases (Jupp PG., 2001; McIntosh BM., *et al*., 1976). Since that time, only sporadic outbreaks with low clinical incidence occurred and WNV was rarely seen and was considered of only minor importance to public health, but in the mid-1990s, the epidemiology of WNV apparently changed. Epizootics and epidemics of severe neurologic disease in horses, birds, and humans began to occur with increasing frequency (Tsai TF., *et al*., 1998; Hubalek Z. and Halouzka J., 1999; Bin H., *et al*., 2001; Giladi M., *et al*., 2001; Marfin AA. and Gubler DJ., 2001; Murgue B., *et al*., 2001; Murgue B., *et al*., 2001; Nash D., *et al*., 2001; Platonov AE., *et al*., 2001). The first cases of WNV in its lethal encephalitic form were reported in Algeria in 1994 with a total of 50 cases of WNV human infections including 20 WNND and one death (De Filette M., *et al*., 2012). Then, in 1996 the first large-scale epidemic took place in Bucarest, Romania, where WNV emerged as major cause of arboviral encephalitis. This outbreak was characterized by a high number of neuroinvasive cases with 393 recognized human cases of encephalitis and 17 deaths recorded in people over 50 years old (Tsai TF., *et al*., 1998; Campbell GL., *et al*., 2001). One year later, an epidemic took place during September-December with 173 WNND cases and eight deaths (Murgue B., *et al*., 2001). After 1996, outbreaks of West Nile viral encephalitis in people and horses were reported with increasing frequency in the Mediterranean basin (Hubalek and Halouzka., 1999; Triki H., *et al*., 2001), Russia (Platonov AE., *et al*., 2001) and Australia (Brown A., *et al*., 2002). In 1997, a new strain of WNV that kills young domestic geese (*Anser* spp.) was isolated in Israel (Malkinson M. and Banet C., 2002). WNV activity, with or without recorded human or horses clinical cases, have been lately reported in Algeria, Marocco, Egypt, Israel, Romania, Russia, Poland, Czech Republic, Hungary, Croatia, Serbia, France, Portugal, Spain, and Italy, which overall, have

accounted for hundreds of cases and dozen of deaths (Zeller HG. and Schuffenecker I., 2004; Calistri P., *et al*., 2010). In 1999, in the Volgograd region, Russia, 826 patients showed a clinical picture resembling that of WNND, but finally 183 was the number of confirmed WNV cases, 84 of which were diagnosed as encephalitis and 40 persons died (Platonov AE., 2001). In Europe, there is a history of recognized WNV outbreaks, characterized by human neuroinvasive disease, dating back to the mid-1990s. However, since 2008, there has been an unpredicted increased WNV activity in Hungary (2008–2009) (Bakonyi T., *et al*., 2013) and in Italy (2008–2009) (Rizzo C., *et al*., 2012), including the sustained emergence of lineage 2 WNV, with a rapid rise in the number of cases of neuroinvasive disease of animals and humans (Danis K., *et al*., 2011; CDC 2013). In 2012, there was a peak of 937 WNV cases in Europe and surrounding countries, with ongoing activity in 2013, with preliminary data reporting 783 WNV human cases, including 86 in Greece and 302 in Serbia (Gray TJ. and Webb CE., 2014).

Geographic expansion of WNV to the Western Hemisphere was detected in 1999. The epicenter of the outbreak took place in the Queens section of New York City and, by the end of the 1999 where the virus caused 62 human cases of severe and fatal neurologic disease including seven deaths. Numerous equine cases were documented, including an epizootic on Long Island, New York, with 25 clinical cases with nine deaths and an enormous mortality of birds, particularly among corvids (Nash D., *et al*., 2001; Ostlund EN., *et al*., 2001; Zeller HG. and Schuffenecker I., 2004; Hayes EB., *et al*., 2005; Murray KO., *et al*., 2010). Since then, WNV has spread quickly across the country, being, so far, responsible for over 1.100 fatalities, over 12.000 cases of meningitis/encephalitis, and more than 30.000 diagnosed human infections (http://www.cdc.gov). Genetic sequence studies have shown that the strain introduced in US, belonging to lineage 1, was identical to the WNV that caused the epizootic in domestic geese in Israel in 1998, but is still a mystery how the virus traversed the Atlantic Ocean (Beasley DW., *et al*., 2002; Brault AC., *et al*., 2004). The epizootic was soon spread across 48 contiguous states in the following years (Marfin AA. and Gubler DJ., 2001; Zeller HG. and Schuffenecker I., 2004): in 2000 to New Jersey and Connecticut, in 2001 to Florida, Louisiana, Maryland and Massachusetts and in 2002 to almost all states, and involved severe and fatal neurologic disease in humans, birds, horses and several other mammalian species reaching its peak of 9.862 cases in 2003 (CDC 2013). The largest state concerning population, California, had only sporadic cases in 2002–2003 but a large outbreak took place in 2004 with 778 cases and 28 deaths. Other large-scale epidemics took place in Illinois (884 cases, 2002), Colorado (2,947 cases, 2003), Nebraska (1,942 cases, 2003), South Dakota

(1,039 cases, 2003) and Texas (1,868 cases, 2012) (CDC 2013). Circulation of the virus to neighboring regions has been demonstrated through serological investigations and WNV activity has been reported in Canada, Central America, the Caribbean, and South America in mosquitoes, humans, horses, birds, and other animals, from some of which the virus has been occasionally isolated (Beasley DW., 2005; Blitvich BJ., 2008). In Canada, the first epidemic took place in 2002 with a total of 414 cases (Marka A., *et al*., 2013).

In Asia, outbreaks occasionally occurred in southern regions and especially in India, while sporadic cases were reported in Southeast Asia (Blitvich BJ., 2008). In Australia, Kunjin virus, considered to belong to a sublineage of WNV (Mackenzie JS., *et al.*, 2009), has caused a total of 13 human cases during the period from 1992 to 2010 (Gray TJ., *et al*., 2011).

The reason for this dramatic emergence of epizootic/epidemic disease caused by a virus that rarely gave rise to severe disease are not well understood (Marfin AA. and Gubler DL., 2001). Since the mid-1990s, three epidemiologic trends have emerged regarding WNV: 1. Increased frequency of outbreaks in humans and horses; 2. Increase in reported cases of neuroinvasive disease in humans; and 3. High case fatality rates in birds coinciding with human outbreaks, mainly in the USA and Israel (Petersen LR and Roehrig TJ., 2001). These more recent outbreaks have been attributed to evolution of a new, more pathogenic WNV variant belonging to lineage 1 (Murray KO., *et al*., 2010).

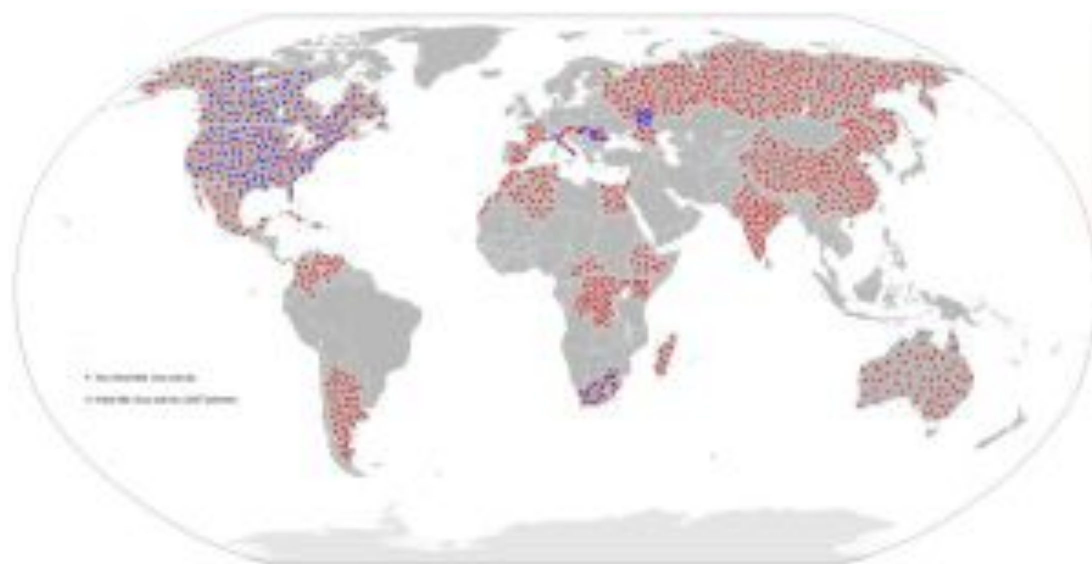


Figure 9. Distribution of WNV. Countries with historic or recent (2007-2010) WNV activity (isolations from mosquitoes, birds, horses or humans) are highlighted in red and blue, respectively (Rossi SL., *et al*., 2010).

3.6.2 EPIDEMIOLOGY OF WNV IN ITALY

The first evidence of the presence of WNV in Italy occurred during the late summer of 1998 in the Tuscany Region by the outbreak in horses, where 14 horses tested were positive for WNV with 6 fatal cases, while no infections in humans were recorded (Autotino GL., *et al*., 2002). The lineage that caused the equine outbreak in 1998 was related to WNV strains circulating at that time in the Mediterranean basin. This strain was no longer detected and was different from the strain, called Italy/2008-2009, that was responsible of the large outbreaks in humans and horses that occurred in the Po river area in 2008-2009 (Barzon L., *et al*., 2009; Rossini G., *et al*., 2011; Sotelo E., *et al*., 2011). In fact, notwithstanding the evidence of the presence of WNV in Italy al least since 1998, with the equine outbreak in horses and subsequent evidence of seroconversion in sentinel animals in different risk areas, human disease due to WNV infection was not documented for a decade, until the first human case of WNV neuroinvasive disease was diagnosed in 2008 (Rossini G., *et al*., 2008). The first human cases of WNND and WNF were detected in the Po river area in northeastern Italy in September-October 2008: these cases included three patients with WNND who were resident in Emilia-Romagna region (Rossini G., *et al*., 2008; Rizzo C., *et al*., 2012) and one patient with WNND and one with WNF who were in Veneto region (Rossini G., *et al*., 2008; Barzon L., *et al*., 2009; Gobbi F., *et al*., 2009), following the alert from the veterinary surveillance that reported equine cases of WNND in the same area (Macini P., *et al*., 2008). Retrospective analysis of CSF samples collected in the Summer 2008 in Veneto region from patients with aseptic encephalitis or meningitis led to the identification of further four human cases of WNND, with symptom onset in August-September and resident in the same area of WNV circulation (Barzon L., *et al*., 2009). A further five cases of asymptomatic WNV infection, including four resident in the affected area, were identified by active surveillance of farm workers (Barzon L., *et al*., 2009). On the basis of phylogenetic analyses, the WNV strains responsible for the Italian outbreaks in 2008-2009 belonged to lineage 1 and constituted a distinct monophyletic group within the WMed cluster (Rossini G., *et al*., 2011). In the decade 1998-2008, a possible explanation for the absence of human cases could be related to the underestimation of WNV activity and the under-diagnosis of WNV disease in Italy, especially in the years before the first human cases were identified. However, it cannot also be excluded that this was due to the lack of bridge transmission to humans during this decade or to the circulation of less pathogenic strains that did not cause symptomatic disease in humans, before the emergence of a new more virulent strain in 2008.

In 2009, WNV circulation was reported in larger area near the Po river that involved Veneto, Emilia-Romagna and Lombardy regions, with occurrence of several human cases (Rizzo C., *et al*., 2009; Calistri P., *et al*., 2010; Capobianchi MR., *et al*., 2010). In the period from the end of August to the end of September 2009, were diagnosed cases of WNV human infection: 18 confirmed cases of WNND identified and two positive organ and blood donors (Barzon L., *et al*., 2009; Rizzo C., *et al*., 2009; Angelini P., *et al*., 2010). These results of human surveillance were in agreement with those from veterinary and entomological surveillance that reported involvement of a territory surrounding the Po river larger than in the previous year, with evidence of WNV spread to western areas (Angelini P., *et al*., 2010; Busani L., *et al*., 2011). The year 2010 was characterized by a decrease of WNV activity, in parts as a result of effective vector control measures applied in the areas of WNV circulation surrounding the Po river (Calzolari M., *et al*., 2010). In fact, in 2010, human cases of infection (three cases of WNND, three of WNF, and two positive blood donors) were detected only in Veneto region, in areas located north of those affected in 2008 and 2009 (Barzon L., *et al*., 2011). An increasing WNV activity was observed in the following years in these new areas in Veneto region and in the nearby Friuli Venezia Giulia region: in 2011 was reported 10 cases of WNND, two of WNF, and six positive blood and organ (Rizzo C., *et al*., 2009; Rizzo C., *et al*., 2012) and in 2012 occurred the largest human outbreak ever recorded in Italy, with 25 confirmed cases of WNND, 17 of WNF, and 14 positive blood donors (Barzon L., *et al*., 2012; Barzon L., *et al*., 2013). In 2011 and 2012, human cases of WNV neuroinvasive disease were reported also in Sardinia island: five confirmed and one probable WNND cases recorded in 2011 (Magurano F., *et al*., 2012) and two confirmed WNND cases in 2012 (Barzon L., *et al*., 2013; EpiCentro; 2013). Surveillance in other Italian regions notified a sporadic case of WNF in the Marche Region, Central Italy, in 2011 (Bagnarelli P., *et al*., 2011) and a case of WNND in the South of Italy (Basilicata Region) in 2012 (Barzon L., *et al*., 2013). In 2011, human cases of WNV neuroinvasive infections registered in Veneto, Friuli Venezia Giulia and Sardinia were due by lineage 1 strains (Rizzo C., *et al*., 2012), while a case of WNV fever reported in Marche region was caused by lineage 2 strain (Fig. 10) (Bagnarelli *et al*., 2011).

In August of 2013, the epidemiology of WNV in northeastern Italy appears to be changing again. In fact, at least 12 human cases of WNV infection were reported in the Po area that was also affected in 2008–2009, while northern areas were less affected (Barzon L., *et al*., 2013).

These epidemiological data on human cases of WNV infection were in line with the results

from entomological and veterinary surveillance that reported WNV circulation and activity in the same areas where human cases were identified. In several cases, entomological and veterinary surveillance could predict the occurrence of human cases by reporting increased vector density and rate of infected mosquitoes and outbreaks in horses (Angelini P., *et al*., 2010; Gobbi F., 2012; Spissu N., *et al*., 2013; Mulatti P., *et al*., 2013).

In Italy, the onset of WNV disease in humans ranged from late July to late October, with peaks of cases reported in late August and early September. In patients with WNND, the overall percentage of death was approximately 10% and occurred generally in elderly and immunocompromised patients (Barzon L., *et al*., 2013).

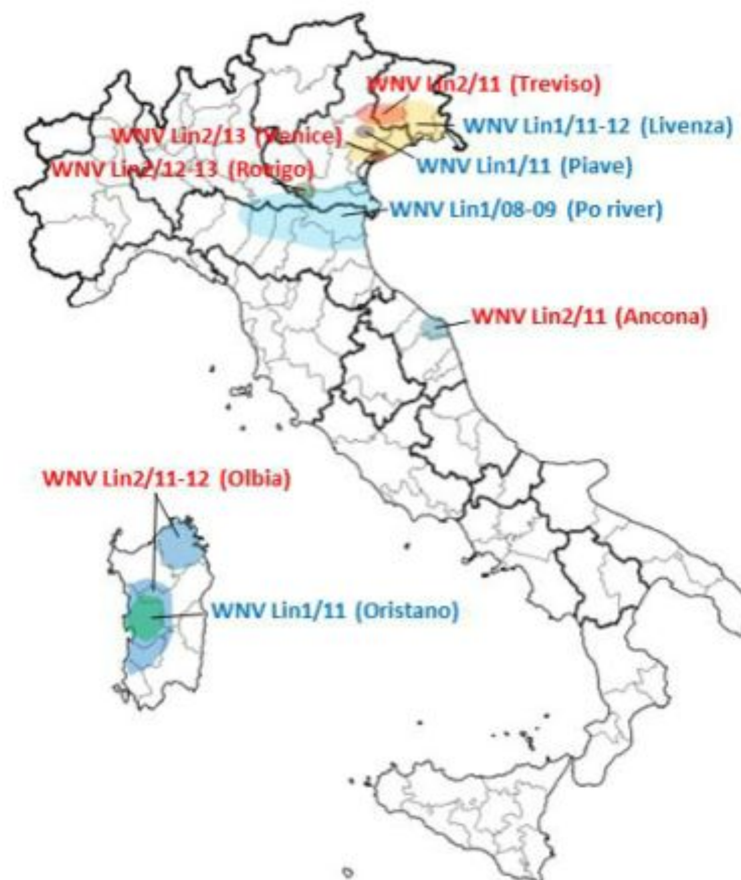


Figure 10: WNV epidemiology in Italy. Map of Italy showing the areas where different WNV strains were detected in the period from September 2008 to August 2013. WNV lineage 1 strains are indicated in blue; WNV lineage 2 strains are indicated in red (Barzon L., *et al*., 2013).

3.7 PATHOGENESIS

Understanding the full range of WNV pathogenesis in humans has been difficult, mainly due to the difference in virulence between WNV strains and the high prevalence of asymptomatic or sub-clinical infections. Little has been published about human infections with WNV of limited virulence. The vast majority of our current knowledge regarding WNV pathogenesis resulted from animal models (mostly rodent) infected under controlled conditions with a known amount of needle-inoculated virus. On the basis of these studies have been identified three distinct phases of WNV pathogenesis: 1. The early phase, with initial infection and spread; 2. The visceral-organ dissemination phase, with peripheral viral amplification; and 3. The central nervous system (CNS) phase, with WNV neuroinvasion. These phases may not accurately reflect the course of a natural infection in humans, but this sequence is thought to recapitulate the stages of pathogenesis in humans following infection by a mosquito (Samuel MA. and Diamond MS., 2006). Nevertheless, many descriptive accounts have been documented following the course of infection in humans suffering from West Nile fever (WNF) and West Nile neuroinvasive disease (WNND) resulting from a virulent lineage 1 WNV infection (Rossi SL., *et al*., 2010).

3.7.1 WNV PROPAGATION IN THE MOSQUITO HOST

Female *Culex* spp. mosquitoes acquire WNV after taking a blood meal from an infected viremic animal. The virus must then infect and replicate in cells of the mosquito midgut as the blood meal is being processed. After replication in the midgut epithelial cells, the virus spreads through the mosquito haemolymph to the salivary glands and other organs (Girard YA., *et al*., 2004). Accumulation of the virus in the salivary glands will eventually result in high viremia in the saliva, from where it can then be transmitted to its vertebrate hosts during the probing process of blood feeding (Colpitts TM., *et al*., 2012). A key step in WNV transmission and vector competence is the midgut barrier, which acts as a physical and immune barrier through the production of antimicrobial peptides and a peritrophic matrix (composed of chitin, proteins, glycoproteins and proteoglycans), which together limit viral replication and spread within the insect (Moskalyk LA., *et al*., 1996). A recent study suggests that C-type lectins facilitate WNV dissemination in mosquitoes (Cheng G., *et al*., 2010). A secreted C-type lectin protein, mosGCTL-1, binds to WNV and enhances viral attachment and infection through interaction with mosPTP-1, a mosquito surface protein that is a homologue of human CD45. WNV binds to secreted mosGCTL-1 in the haemolymph,

thus facilitating viral entry and invasion of different mosquito tissues. WNV infection triggers invertebrate innate immune programmes that can restrict infection. These include RNAi; innate immune signalling pathways mediated by Toll, immune deficiency (IMD) and JAK–STAT (Janus kinase–signal transducer and activator of transcription) proteins; and antimicrobial peptides (Arjona A., *et al*., 2011). Moreover, mosquitoes carry *Wolbachia* spp., which are symbiotic bacterial species that inhibit WNV replication in the insect (Glaser RL. and Meola MA., 2010). Mechanistically, *Wolbachia* spp. induce oxidative stress and reactive oxygen species in response to WNV infection, leading to activation of the Toll pathway and production of antimicrobial peptides, including defensins and cecropins, that inhibit flavivirus replication (Pan X., *et al*., 2012).

3.7.2 INITIAL INFECTION, VIRAL AMPLIFICATION AND SPREAD IN HUMANS

During feeding, infected mosquitoes probe host skin using their proboscid in order to inject mostly intradermally but also intravascularly pharmacologically active saliva proteins and to locate a blood source (Hudson A., *et al*., 1960; Ribeiro JM., *et al*., 1984; Ribeiro JM., *et al*., 1985). Dermal blood vessels are the targets for hematophagous insects. In order to locate these structures, the proboscis must navigate through a very elastic environment that has a high tensile strength. To efficiently move through this environment, mosquito saliva may contain components that liquefy the bite site. A salivary endonuclease with a proposed function to facilitate probing in host skin has been identified in *C. quinquefasciatus* (Calvo E. and Ribeiro JM., 2006). As part of the feeding process, a mosquito injects saliva and the viral particles that it contains. Depending on the mosquito species, up to 10^6 plaque-forming units (PFU) of infectious virus can be delivered into the host per bite (Styer LM., *et al*., 2007). Although many hematophagous insects can obtain a blood meal without functional salivary glands, the efficiency of blood feeding is severely limited (Hudson A., *et al*., 1960; Ribeiro JM., *et al*., 1984; Ribeiro JM., *et al*., 1985). In addition to viral factors that block the host immune response, saliva contains molecules that combat the host's hemostatic system, reduce inflammation and alter host immunity (Titus R., *et al*., 2006) that affect viral pathogenesis. All hematophagous insects inject at least one vasodilator, one coagulation inhibitor, and one platelet inhibitor, and often the saliva includes immunomodulatory, digestive, and antimicrobial proteins as well (Ribeiro JM., *et al*., 2001; Ribeiro JM. and Francischetti IM., 2001; Ribeiro JM., *et al*., 2007; Schneider BS. and Higgs S., 2008). Mice inoculated intradermally with WNV subsequent to feeding by *Culex* or *Aedes* spp. mosquitoes display more rapid infection kinetics, enhanced viraemia and accelerated

neuroinvasion compared with mice inoculated with WNV but not subjected to mosquito bites (Schneider BS., *et al*., 2006; Steyer LM., *et al*., 2011). While numerous proteins in the saliva of hematophagous insects have been described, many remain that have not been characterized, especially with respect to viral infection. In addition, mosquito saliva causes dysregulation of local immune responses, including alterations in cytokine levels, leading to local immunosuppression and reduced recruitment of neutrophils, dendritic cells (DCs) and T cells to the primary site of infection (Schneider BS. and Higgs., 2008; Schneider BS., *et al*., 2010). Host skin acts as an important barrier to many infections, though WNV antigen has been detected in skin at multiple phases of infection. WNV replication was observed in skin tissue at the inoculation site at 1 and 3 days post-infection (Schneider BS., *et al*., 2006), and WNV has also been shown to spread to areas of skin contralateral to the site of inoculation (Brown AN., *et al*., 2007). Infectious WNV has been shown to persist in skin at the inoculation site for at least 14 days post-infection (Appler KK., *et al*., 2010).

The early phase is defined by WNV replication in keratinocytes (Lim PY., *et al*., 2011) and skin-resident DCs, which can include dermal DCs and Langerhans cells (MHCCII+/NLDC1145+/E-cadherin+ cells) (Johnston LJ., *et al*., 2000) at the site of inoculation. Many reports document that both keratinocytes and fibroblasts are permissive to WNV infection *in vitro* and *in vivo* (Jarman RV., *et al*., 1968; Rezepova AI., *et al*., 1971; Kurane I. *et al*., 1992; Douglas MW., *et al*., 1994; Shen J. *et al*., 1995; Arnold SJ., *et al*., 2004, Cheng Y., *et al*., 2004; Cheng Y., *et al*., 2004; Fredericksen BL., *et al*., 2004; Fredericksen BL., *et al*., 2006; Kajaste-Rudnitski A., *et al*., 2006; Kajaste-Rudnitski A., *et al*., 2006; Scherbik SV., *et al*., 2007; Welte T., *et al*., 2009; Lazear HM., *et al*., 2011; Lim PY., *et al*., 2011). This is followed by traffic of activated dendritic cells to the draining lymph node (Johnston LJ., *et al*., 2000; Byrne S., *et al*., 2001) where the virus replicates further, antigen processing begins, and the early immune response may become evident (Kramer LD., 2008). Virus enters the blood stream by way of the efferent lymphatics and thoracic duct, which results in a viraemia that spreads the virus to the visceral organs of the body, including the spleen, heart, liver, kidneys and, possibly facilitates virus crossing the blood-brain barrier (BBB) resulting in CNS invasion and inflammation of the medulla, brain stem and spinal cord (Samuel MA. and Diamond MS., 2006). The specific target cells for WNV infection in the spleen and other peripheral tissues are not well defined, but are thought to be subsets of DCs, macrophages and possibly neutrophils (Ben-Nathan D., *et al*., 1996; Samuel MA., *et al*., 2006; Bai F., *et al*., 2010).

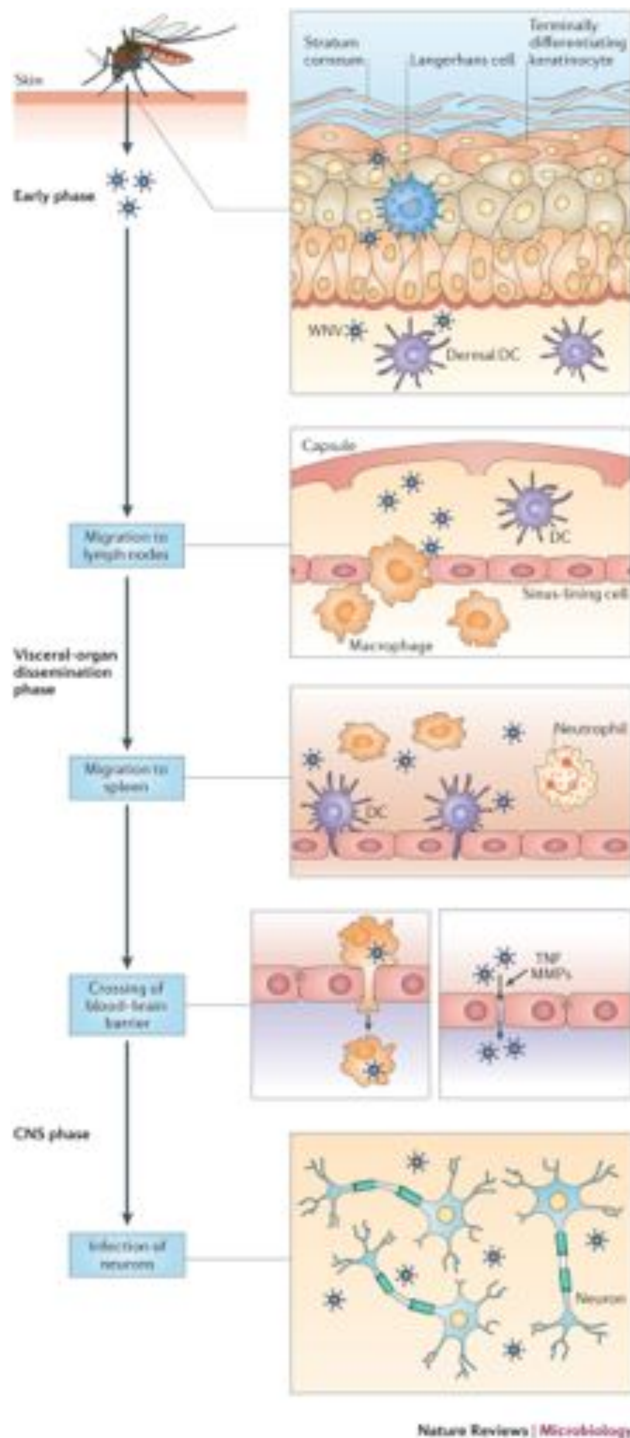


Figure 11. Pathogenesis of West Nile virus in humans. A schematic of West Nile virus (WNV) pathogenesis in humans is shown, created on the basis of mouse models. Following a subcutaneous bite of a mosquito, WNV is thought to replicate in keratinocytes (Lim PY., *et al*., 2011) and skin-resident dermal dendritic cells (DCs) and Langerhans cells. Infected DCs migrate to the regional draining lymph node and seed the virus within this node (Johnston LJ., *et al*., 2000). Replication within the draining lymph node leads to viraemia and subsequent infection of peripheral organs, including reasonably permissive tissues (such as the spleen) and non-permissive tissues (such as the kidney and liver). By day 4, viral replication peaks in the spleen and serum. Between day 6 and day 8 after infection, WNV is cleared from peripheral organs, and infectious virus is detected within the brain and spinal cord, in part owing to the virus crossing the blood–brain barrier. This is achieved by increasing endothelial cell permeability (through the secretion of tumour necrosis factor (TNF)), by the breakdown of endothelial cell junctions (through the action of matrix metalloproteinases (MMPs)) or through a ‘Trojan Horse’ mechanism, whereby the virus is transported to the central nervous system (CNS) by infected immune cells. In the CNS, WNV infects and causes injury to neurons within the brain stem, hippocampus, cortex, cerebellum and spinal cord (Suthar SM., *et al*., 2013).

3.7.3 NEUROINVASION

WNV is both neuroinvasive and neurotropic and invasion of the CNS tissues constitutes the third phase, where the virus targets and replicates in neuronal subsets. To establish infection in neurons of the brain, WNV first must cross the BBB. The BBB is highly regulated interface between the blood and the brain and is composed of four main cellular components: endothelial cells and their basement membrane (composed of collagen IV, laminin, proteoglycans, and glycoproteins); astrocyte and their food processes; microglial cells and pericytes (PCs) (Pardridge WM., 1983). The endothelium is the first line of defense against viral neuroinvasion: the tight junctions between endothelial cells form a diffusion barrier that restricts the entry of pathogens, immune cells, and immune mediators into the brain, thus preventing infection and limiting the potential side effects of immune system activation on generally non-renewable neurons (Ballabh P., *et al*., 2004; Muldoon LL., *et al*., 2013). Endothelial models have been developed to study the mechanism of WNV translocation across BBB *in vitro*, and mechanisms proposed include: transcellular transport of virions across the infected endothelial cells and an increased permeability of the BBB, which can then facilitate a paracellular entry of WNV into the CNS parenchyma (Suen WW., *et al*., 2014). In any case, the mechanisms by which the virus gains entry to the CNS remains poorly understood, an over-represention of *in vitro* studies without adequate *in vivo* validation continues to obscure our understanding of the mechanism(s). (Beasley DW., *et al*., 2002). The mechanism by which WNV and other encephalitic *flavivirus* cross the BBB may depend on the infection route and the pathogenicity of the WNV strain. Several models have been proposed for WNV entry into CNS (Fig. 12):

1. Crossing of the BBB likely occurs through a hematogenous route: as viremia develops following peripheral replication locally at the site of virus inoculation and/or in the draining lymph nodes, both resulting in systemic dissemination of the virus. According to current literature, increased viral burden in the serum correlates with greater and more rapid WNV entry into the CNS (Johnson RT., *et al*., 1968; Johnson RT., *et al*., 1968) and for this reason the hypothesis of hematogenous dissemination of WNV into the CNS has been a common focus of investigation;
2. Viral entry *via* passive diffusion as cell-free virions as result of blood-brain barrier (BBB) breakdown. The hypothesis of viral entry into CNS across by a more permeable BBB may due to intravascular levels of pro-inflammatory cytokine, which are produced during peripheral immune response that can mediate increased vascular permeability, also may

allow WNV to cross the BBB and infect neurons (Diamond MS., *et at*., 2003). WNV infection in peripheral tissue induces toll-like receptor (TLR)-3-mediated secretion of pro-inflammatory cytokines, including IL1β, IL-6, IL8 and TNF-α (Wang T., *et al*., 2004), which may disrupt the BBB. Secreted TNF-α can modulate BBB permeability by altering endothelial cell tight junctions which may allow WNV to cross the BBB and infect neurons (De Vries HE., *et al*., 1996; Fiala M., et al., 1997; Wang T., *et al*., 2004). Semaphorin 7A upregulation after WNV infection also is linked to increased TNF-α production. Mice lacking Semaphorin 7A showed reduced TNF-α levels in serum, less BBB permeability, and reduced viral entry into the brain (Sultana H., *et al*., 2012). The flux of WNV into CNS can be also enhanced through degrading the tight junction proteins of the BBB extracellular matrix by activation of matrix metalloproteinases (Wang P., *et al*., 2008). In BBB model studies *in vitro*, treatment with inhibitors of matrix metalloproteinases prevented the disruption of tight junction integrity associated with WNV infection (Verma S., *et al*., 2010).

3. The 'Trojan Horse' mechanism, *via* infected inflammatory cells: WNV is transported by infected immune cells (e.g., neutrophils or CD4+ or CD8+ T cells) across paracellular junction between endothelial cells into the brain parenchyma (Garcia-Tapia D., *et al*., 2006; Wang S., *et al*., 2008). The 'Trojan Horse' hypothesis, as proposed in many reviews (Lim SM., *et al*., 2011; Sips GJ., *et al*., 2012; Suthar MS., *et al*., 2013): Garcia-Tapia *et al.* (Garcia-Tapia D., *et al*., 2006) suggested that WNV infected Langerhan cells migrated from the site of inoculation to draining lymph node, where infection could then be relayed to mononuclear cells, such as monocytes and certain subsets of CD4+ lymphocytes. As hypothesized from a later study, Garcia-Tapia *et al.* (2007) suggested that the expression of lymphocyte and monocyte chemoattractants, such as IP-10 (CXCL10) and MCP-5 (CCL12), respectively, in WNV infected brains, post-footpad inoculation, could recruit peripheral mononuclear cells into the perivascular space in the CNS. Here, the recruited leukocytes could produce pro-inflammatory cytokines, such as TNF-α and interleukins, which as mentioned above, could compromise the BBB integrity (Wang T., *et al*., 2004; Garcia-Tapia D., *et al.,* 2007). Infected monocytes/macrophages and CD4+ lymphocytes could also facilitate productive viral replication in this region (Garcia-Tapia D., *et al.,* 2006; Rios M., *et al*., 2006), providing a source of infection for brain microvascular endothelial cells, which in turn may exacerbate the BBB permeabilization via the degradation of inter-endothelial tight junctions and upregulation of CAM expression (Dai J., *et al*., 2008; Verma S., *et al*., 2009). Increased expression of

ICAM-1 has been detected before WNV entry into brain and may plays an important role in virus neuroinvasion *in vivo* (King NJ., *et al*., 2003). Dai et al., (2008) showed that ICAM-1 knock-out (KO) mice had increased survival rates that were associated with significantly lower virus burdens and significantly fewer brain lesions, as well as decreased BBB leakage, following a lethal WNV challenge (Dai J., *et al*., 2008). These latter results, put in the context of other studies (Drevets, D.A.; Leenen, P.J., 2000; Hubbard AK., *et al*., 2000; Greenwood J., *et al*., 2002), suggest that ICAM-1 acts both as a ligand for leukocyte receptors at the surface of the BBB endothelium and as a signal transducer that influences BBB permeability and the neuroinflammation process, thus facilitating the transmigration of infected leukocytes (Donadieu E., *et al*., 2013). Further recruitment, margination and transmigration of infected leukocytes across the paracellular junction of the BBB could result in viral neuroinvasion and dissemination (Garcia-Tapia *et al.* 2008).

4. In some cases, WNV may penetrate into CNS through a transneural route and two neuroanatomical areas have been hypothesized to be involved in this mechanism: from the peripheral somatic nerves or from the olfactory nerves into the CNS (King NJ., *et al*., 2007; Murray KO., *et al*., 2010; Cho H. and Diamond MS., 2012). Peripheral neurons are susceptible to infection by WNV (Monath TP., *et al*., 1983; Hunsperger EA., *et al*., 2006) and investigators have shown that through direct injection of WNV into the sciatic nerve transneural spread of the virus from the peripheral nervous system (PNS) to the CNS could be a putative route for neuroinvasion (Samuel MA., *et al*., 2007; Wang H., *et al*., 2009). This study has specifically noted that WNV appeared to travel preferentially up motor nerves rather than sensor nerves. In addition, through compartmentalized neuron methods, has been demonstrated in hamsters that bidirectional axonal spread of WNV was possible (Samuel MA., *et al*., 2007). Retrograde axonal transport can bring WNV into CNS and accounts for acute limb paralysis, while anterograde transport would facilitate WNV spread in the CNS (Donadieu E., *et al*., 2013). Additional proposed mechanisms of CNS entry, as evident in a few *in vivo* challenge studies using neurotropic flaviviruses inoculated by either an intraperitoneal (Monath TP., *et al*., 1983), subcutaneous (foodpad) (Brown AN., *et al*., 2007) or intranasal route, include infection of olfactory neurons and rostral spread from the olfactory bulb (Brown AN., *et al*., 2007).
5. Other possible entry mechanisms for WNV include infection or passive transport through choroid plexus epithelial cells that has been documented in animal models (Kramer-Hämmerle, S., *et al*., 2005) or direct infection of brain microvascular endothelial cells

(Verma S., *et al*., 2009).

Drawing together the conclusions from the above *in vivo* and *in vitro* studies, the route of WNV neuroinvasion may be much more complex than one distinct path from the peripheral site of inoculation to the CNS and WNV may enter the brain though a combination of mechanisms. Although the precise mechanism(s) of WNV entry into the CNS in humans requires further study, it may differ depending on the route of infection and the pathogenicity of the WNV strain. (Beasley DWJ., 2002; Diamond MS., *et al*., 2009; Suen WW., *et al*., 2014).

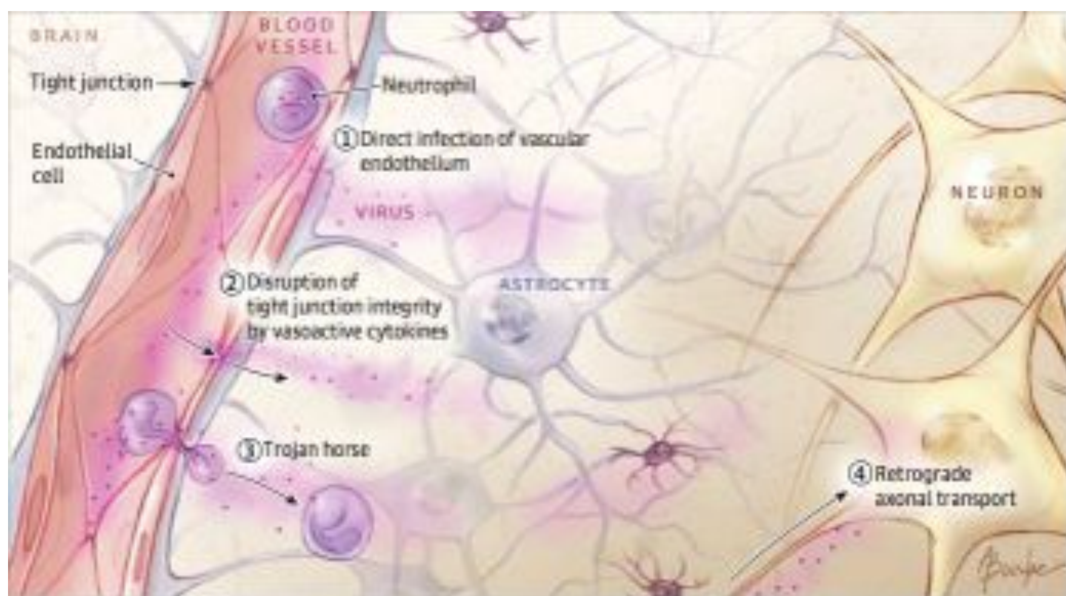


Figure 12. West Nile virus Neuroinvasive Mechanism. Potential mechanisms for neuroinvasion of West Nile virus include (1) direct infection of the vascular endothelium and subsequent entry to the central nervous system, (2) viral passage through the vascular endothelium due to disruption of the blood-brain barrier integrity by vasoactive cytokines, (3) a Trojan horse mechanism through which infected monocytes are trafficked into the central nervous system, or (4) retrograde axonal transport to the central nervous system following infection of peripheral neurons (Petersen LR., *et al*., 2013).

Upon CNS entry, WNV infects and injures several different neuronal cell populations, including those in the cerebral cortex, brain stem hippocampus, and spinal cord (Eldadah AH. and Nathanson N., 1967; Xiao SY., *et al*., 2001; Diamond MS., *et al*., 2003a; Omalu BI., *et al*., 2003; Shrestha B., *et al*., 2003; Fratkin JD., *et al*., 2004). Later in the course of infection, the virus induces inflammatory lesions and neuronal infection that comprises

degeneration, loss of cell architecture, and cell death, but the mechanism by which WNV induces neuronal injury is still unclear (Donadieu E., *et al*., 2013). Indeed, although neuronal injury may be directly caused by viral infection, it may also results from indirect mechanisms by leukocyte infiltration and the host inflammatory response (Chambers TJ., *et al*., 2003): mononuclear cells infiltrate appear diffusely throughout infected regions, but it is not clear whether these inflammatory cells eradicate infection or contribute to pathogenesis by destroying infected neurons and releasing pro-inflammatory cytokines (Lazear HM., *et al*., 2011). Elucidating WNV neuroinvasion in humans has proved to be difficult because WNV strains demonstrate variable virulence in mammals. However, animal models of WNV infection have provided insights into the pathogenesis of WNV in mammals to identify viral and host factors that control the viral dissemination and entry into the CNS (Donadieu E., *et al*., 2013).

3.8 TROPISM

WNV is transmitted to vertebrates by the bite of an infected mosquito, which deposits high doses of virus extravascularity in the skin (Styer LM., *et al*., 2007). The *in vivo* cell targets of WNV in the skin are unknown; however, it is believed that WNV infects Langerhan cells (LCs), the resident dendritic cells (DCs) of the skin and keratinocytes (Lim PY., et al., 2011). In fact, LCs are *in vivo* cell targets of another flavivirus, dengue virus (Wu SJ., *et al*., 2000), and DCs are susceptible to infection by WNV *in vitro* (Davis CW., *et al*., 2006; Silva MC., *et al*., 2007; Martina BE., *et al*., 2008; Lim PY., *et al*., 2010). Moreover, WNV RNA persists in the skin for up to 4 months post-inoculation (Appler KK., *et al*., 2010) and these results suggesting that WNV infects nonmigrating cells in the skin, likely keratinocytes, may contribute to WNV persistence in the skin (Lim PY., *et al*., 2011). Following WNV inoculation of mice, LCs migrate from the skin at the inoculation site to the draining lymph nodes (DLN) (Johnston LJ., *et al*., 2000). On the other hand, initial WNV replication after mosquito transmission or subcutaneous inoculation occurs in both the DLN and the skin (Brown AN., *et al*., 2007; Styer LM., *et al*., 2011), suggesting that, in addition to cells in the skin, lymph node are also productively infected, resulting in a primary viremia. WNV then spreads systemically to visceral organs, such as spleen, where takes place a second round of replication, presumably in epithelium cells and macrophages, respectively (Rios M., et al., 2006). Generally, WNV replication is typically restricted to the skin, draining lymph node, spleen, and CNS in humans and wild-type mice (Samuel MA. and Diamond MS., 2006; Lim PY., *et al*., 2011). Low levels of infectious virus can be recovered from the lung, kidney,

heart, pancreas and other peripheral tissues but not the liver, of wild-type infected mice (Brown AN., *et al*., 2007). Most peripheral organs, including the liver, are not typically associated with WNV replication in humans. However, reported cases of kidney, liver, and heart organ transplant-transmitted WNV infections have been described with outcomes ranging from asymptomatic infections to death in the recipients (Rhee C., *et al*., 2011). These clinical observations suggest that peripheral organs in humans are also capable of being infected by WNV but infection is restricted or controlled by immune defense programs. However, following WNV spreads to the spleen, where the virus is amplified, and a secondary viremia, WNV can cross the BBB and invade the central nervous system tissues. Regardless of how WNV enters the central nervous system, into the brain the virus must propagate efficiently within target cells to cause meningo-encephalitis. Studies in humans and mice have demonstrated that neurons are the primary cells targeted by WNV (Fredericksen BL., 2014). In humans, WNV is most often detected in neurons in the cerebral cortex, thalamus, brainstem, basal ganglia, cerebellar Purkinje cells, and spinal cord (mainly anterior horn), and in some cases, infection has been detected in the olfactory bulb and hippocampus (Fig. 13). WNV has been detected in the same regions of the brain of experimentally infected mice as in humans, indicating a similar tropism of WNV in humans and animal models (Xiao SY., *et al*., 2001; Omalu B., *et al*., 2003; Shrestha B., *et al*., 2003; Lim SM., *et al*., 2011). Moreover, WNV-positive brain microvascular endothelial cells and astrocytes have been detected in birds and humans, respectively, suggesting that these cells may serves as secondary targets *in vivo* (Lopes H., *et al*., 2007; Van Marle G., *et al*., 2007; Wunschmann A., *et al*., 2004). Astrocytes and endothelial cells form with neurons the neurovascular unit (NVU) and functions to regulate blood flow, the integrity of the BBB, and neuronal activity in response to environmental changes (Fredericksen BL., 2014). *In vitro* studies conducted at both low and high MOIs, demonstrated that pathogenic strains of WNV replicate within all NVU cell types, though replication in astrocytes was the most restricted (Cheeran MC., *et al*., 2005; Diniz JA., *et al*., 2006; Hussmann *et al*., 2013; van Marle G., *et al*., 2007; Verma S., *et al*., 2010). Moreover, although with several differences, neurons and astrocytes were found to support productive WNV infection, whereas viral growth was poorly permissive in microglial cells (Cheeran MCJ., *et al*., 2005). Mechanisms for this selective tropism among neuronal populations remain to be elucidated. Domain III of the envelope glycoprotein of WNV has been implicated in neuroinvasiveness, which constitutes the receptor-binding domain, and seems to be a primary virulence factor, but putative receptors on neuronal target cells have not yet been identified (Granwehr BP., *et al*.,

2004). Moreover, animal studies using intracranial inoculation indicate that most, if not all, strains of WNV can replicate within the CNS; nonetheless, the extent of neurovirulence is strain-dependent (Beasley DW., *et al*. 2002a; Shrestha *et al*. 2008). Recent studies have begun to define the host determinants for susceptibility to WNV in the various cell types comprising the neurons, endothelial and astrocytes cells and the viral factors responsible for the strain-dependent differences in neuropathogenicity (Beasley DW., *et al*. 2004; Beasley DW., *et al*. 2005; Cho et al. 2013; Hussmann et al. 2013; Shirato et al. 2004). Examination of WNV replication within the cells types comprising the neurons, endothelial and astrocytes cells infected at low MOIs demonstrated that high and low neuropathogenic strains of WNV replicate with similar kinetics and to equivalent levels in brain microvascular endothelial cells and neurons (Hussmann et al. 2013). However, astrocytes exhibited a reduced susceptibility to the low neuropathogenic strain compared to the high neuropathogenic strain, suggesting a possible role for this cell type in limiting WNV replication within the CNS.

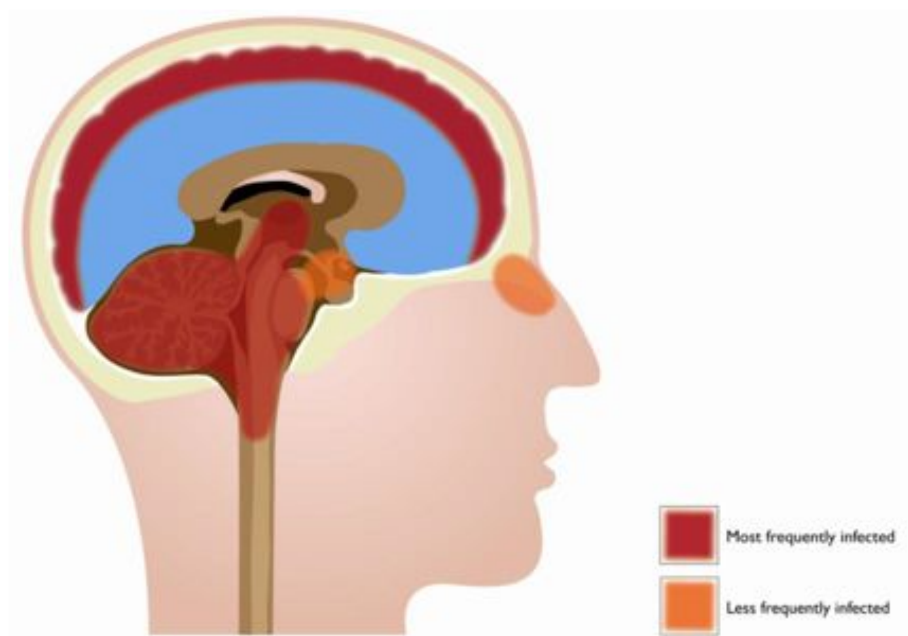


Figure 13. Frequency of infection of several regions of the human brain by West Nile virus. The areas most often infected by WNV include: the cerebral cortex, thalamus, basal ganglia, brainstem, cerebellum, and spinal cord (anterior horn) (indicated in dark red). Infection has less frequently been found in the olfactory bulb and hippocampus (indicated in orange) (Lim SM., et al., 2011).

3.9 CLINICAL MANIFESTATIONS IN HUMANS

WNV infection in humans causes a spectrum of manifestations from subclinical infection to death (Petersen and Marfin 2002). It is generally estimated that the majority (75 to 80%) of WNV infections in humans are asymptomatic. Of those who develop symptoms, approximately 20% of the infected people, develop an acute, systemic febrile illness, termed WN fever (WNF), and less than 1% of the symptomatic cases develop neurologic illness, which is primarily attributed to the neuroinvasive disease, where the virus breaches the intrathecal space and produces infection of CNS structures (Sejvar JJ., *et al*., 2003). West Nile Neuroinvasive disease (WNND) includes: aseptic meningitis (West Nile meningitis, WNM) that involves infection of the meninges (the outer covering of the brain and spinal cord) and makes up the largest percentage of the neuroinvasive disease in younger age groups; encephalitis (West Nile encephalitis, WNE) that involves viral infection of the brain parenchyma itself and is more typically manifested in older persons or immunocompromised individuals; acute poliomyelitis-like syndrome (West Nile poliomyelitis, WNP) that results from viral infection of the anterior horn cells of the spinal cord, leading to acute flaccid limb weakness (Campbell GL., *et al*., 2002; Granwehr BP., *et al*., 2004; Sejvar JJ., 2014). Overall, only 1 in 150 infections results in the most severe and potentially lethal form of the disease, although the relative risk is increased in the elderly or individuals with compromised immune systems (Sejvar JJ., 2007; De Filette M., *et al*., 2012). The incubation period for clinical illness generally ranges from 2 to 14 days after infection by mosquito bite, but prolonged incubation periods of up to 21 days have been observed among immunocompromised patients (Pealer LN., *et al*., 2003; Rhee C., *et al*., 2011).

3.9.1 WEST NILE FEVER (WNF)

WNF is the predominant clinical syndrome seen in most WNV infected persons that develop symptoms. All ages may be affected, but data suggest that the proportion of WNF may be higher among younger individuals (Pepperell C., *et al*., 2003, Brown J., 2004; O'Leary DR., *et al*., 2004; Hayes EB. and Gubler DJ., 2005). WNF can range from a mild infirmity lasting few days to a debilitating illness lasting weeks to months, and in some instances, they can result in hospitalization (Petersen LR., *et al*., 2013). Following an incubation period of approximately 2-14 days, infected persons typically begins with sudden onset of fever (usually >39°C), headache, fatigue, myalgia, often accompanied by gastrointestinal complaints, including nausea and vomiting that may lead to dehydration (Cambpell GL., *et*

al., 2002; Sejvar JJ., 2014). WNF may sometimes be associated with a rash that may be transient, lasting less than 24h in some persons, and tends to be morbilliform, maculopapular, and nonpruritic. The rash predominates over the torso and extremities, sparing the palms and soles and interestingly appears to be more frequently observed among younger persons than among older persons (Ferguson DD., *et al*., 2005). Although elderly persons with WNF may experience adverse outcomes and have a higher mortality rate than younger symptomatic persons, most patients experience complete recovery (Emig M. and Apple DJ., 2004; O'Leary DR., *et al*., 2004). However, some otherwise healthy persons may continue to experience a prolonged fatigue, headaches and difficulties concentrating for days or weeks following infection (Watson JT., *et al*., 2004). Deaths among persons with WNF occur primarily among older persons and among individuals with compromised immune systems and this is frequently attributable to cardiopulmonary complications (Sejvar JJ., *et al*., 2011).

3.9.2 WEST NILE NEUROINVASIVE DISEASE (WNND)

Approximately 5% of patients with symptomatic WNV infection develop neurologic disease. Severe WNND is associated with neurological involvement that varies from meningitis and/or encephalitis, to ocular manifestations, to poliomyelitis-like condition with acute flaccid paralysis that can result in respiratory failure (Campbell GL., *et al*., 2002).

West Nile meningitis (WNM), similar to that of other viral meningitides, is characterized by abrupt onset of fever and headache along with meningeal signs and photophobia. Headache may be severe, requiring hospitalization for pain control, and associated gastrointestinal disturbance such as nausea, vomiting and diarrhea, may result in dehydration (Sejvar JJ., *et al*., 2008). WNM, in cases that do not progress to meningoencephalitis, is generally associated with a favorable outcome and the fatality rate is low (Ceausu E., *et al*., 1997). Although, similar to WNF, some patients experience persistent headache, fatigue and myalgia (Sejvar JJ., *et al*., 2003; Sejvar JJ., *et al*., 2008).

Clinically, West Nile encephalitis (WNE) is generally typical of the arboviral encephalitides. WNE ranges in severity from a mild, self-limited confusional state to severe encephalopathy, coma and death: a prodrome of fever, headache, and other non-specific symptoms (i.e. typical WNF) lasting from 1 to few days occurs in some patients; while in others, a more abrupt onset of fever accompanied by symptoms and signs of encephalitis, especially mental status changes and vomiting, has been described and in about 15% of cases, cerebral dysfunction progress to coma. This manifestation is more commonly seen in older

individuals, particularly over the age of 55, as well as among organ transplant recipiens (Armali Z., *et al*., 2003; Kumar D., Prasad GV., *et al*., 2004; O'Leary DR., *et al*., 2004; Ravindra KV., *et al*., 2004). Whether other immunocompromised patients are at higher risk remains unclear, but severe WNV disease has been described in persons with malignancies (Guarner J., *et al*., 2004). Several neurological syndromes, primarily extrapyramidal (Pepperell C., *et al*., 2003; Sayao AL., *et al*., 2003; Sejvar JJ., *et al*., 2003; Burton JM., *et al*., 2004) and movement disorders, including severe tremors and parkinsonism, are frequently observed in patients with WNE (Hayes EB., *et al*., 2005; Robinson RL., *et al*., 2003; Sejvar JJ., *et al*., 2003). Indeed, patients with West Nile encephalitis frequently develop a coarse tremor, particularly in the upper extremities. The tremor tends to be postural and may have a kinetic component (Sejvar JJ., *et al*., 2003; Sayao AL., *et al*., 2003; Burton JM., *et al*., 2004). Myoclonus, predominantly of the upper extremities and facial muscles, may occur and may be present during sleep. Cerebellar ataxia, increased intracranial pressure, cerebral edema, and seizures have been described but are uncommon (Burton JM., et al., 2004; Sayao AL., *et al*., 2003; Kanagarajan K., *et al*., 2003). These movement disorders usually follow the onset of mental status changes and typically resolve over time. However, tremor and parkinsonism may persist in patients recovering from severe encephalitis (Sejvar JJ., *et al*., 2003; Pepperell C., *et al*., 2003). Up to 1 year may be necessary for convalescence following encephalitis. Analysis of the long-term outcomes of WNND has estimated that myalgia, confusion and lightheadedness may persist even beyond this period and prolonged depression persists in as many as 31% of patients (Sejvar JJ., 2007; Murray K., *et al*., 2010).
Clinical features in patients with WNM or WNE are usually familiar to many physicians and prompt them to search for a viral cause. However, acute flaccid paralysis may not be familiar to some clinicians, particularly when it occurs in the absence of meningitis or encephalitic signs and symptoms, resulting in difficulties for an accurate diagnosis (Li J., *et al*., 2003; Kramer LD., *et al*., 2007). In the 1999 New York City outbreak, about 10% of the hospitalised patients had acute flaccid paralysis (Li J., *et al*., 2003). However, the underlying cause (poliomyelitis) for this acute paralysis was not recognised until 2002 (CDC 2002; Glass JD., *et al*., 2002; Leis AA., *et al*., 2002). Acute and abrupt onset of limb weakness may be associated in WNV infection. In most cases, this limb paresis (partial weakness) or paralysis (complete loss of muscle power) is due to viral involvement of the anterior horn cells of the spinal cord, resulting in anterior (polio) myelitis (Glass JD., *et al*., 2002; Leis AA., *et al*., 2002; Jeha LE., *et al*., 2003; Sejvar JJ., Leis AA**;** *et al*., 2003; Sejvar JJ., *et al*., 2005). The clinical features of West Nile Poliomyelitis (WNP) are characteristic and

generally dramatic, allowing for differentiation from the characteristic diffuse "muscle weakness" described by many persons with severe fatigue associated with WNV infection (Sejvar JJ., *et al*., 2014). WNP generally develops soon after illness onset: asymmetric weakness usually develops rapidly within the first 48 hours after symptom onset, although patients with extensive spinal cord involvement develop a more symmetric dense quadriplegia. Central facial weakness, frequently bilateral, can also occur (Jeha LE., *et al*., 2003). Sensory loss or numbness is generally absent, though some patients experience intense pain in the affected limbs just before or during the onset of weakness (Sejvar JJ., *et al*., 2005). The most severe manifestation of WNP is the involvement of respiratory muscle innervation that leads to diaphragmatic and intercostal muscle paralysis and resulting in respiratory failure and requiring emergent endotracheal intubation. Respiratory involvement in WNP is associated with high morbidity and mortality, and among survivors, prolonged ventilatory support lasting months may be required (Sejvar JJ., *et al*., 2005). However, in some cases, patients are unable to be weaned from mechanical ventilation, and the withdrawal of ventilator support leads to death (Sejvar JJ., *et al*., 2014). Other forms of Acute flaccid paralysis (AFP) associated with West Nile virus infection include Guillain-Barré syndrome (GBS) and other demyelinating neuropathies, motor axonopathy, axonal polyneuropathy, involvement of ventral spinal roots, myasthenia gravis, and brachial plexopathies (Leis AA. and Stokic DS., 2012). The weakness associated with GBS is usually symmetric, ascending (e. g. beginning in the legs and subsequently involving arms and cranial nerve innervates muscles, and is associated with sensory and autonomic dysfunction.

Other manifestations have been described in the setting of West Nile virus infection include multifocal choroiditis, vitritis, myocarditis, pancreatitis, fulminant hepatitis, rhabdomyolysis, stiff-person syndrome, and autonomic instability (Southam CM. and Moore AE., 1952; Parelman A. and Stern J., 1974; McIntosh BM., *et al*., 1976; Petersen LR., *et al*., 2012). However, after fever and neuroinvasive disease, chorioretinitis and vitris are the most commonly reported clinical manifestation of WNV infection (Adelman RA., *et al*., 2003; Bains HS., *et al*., 2003; Kuchtey RW., *et al*., 2003; Hershberger VS., *et al*., 2003; Vandenbelt S., *et al*., 2003; Shaikh S., *et al*., 2004). Chorioretinal lesions have been described as multifocal and with a "target-like" appearance and have also been noted retinal hemorrhages. Lesions tends to be clustered primarily in the temporal and nasal regions of the periphery of the fundus and this distribution and appearance of the chorioretinal lesions have been proposed that may be distinctive for WNV infection (Hershberger VS., *et al*., 2003). An inflammatory vitritis has occurred concomitantly with the chorioretinitis and may be

significant enough to obscure the optic disc. Symptomatic persons describe gradual visual blurring and loss, floaters and flashes (Sejvar JJ., 2014).
Numerous other clinical manifestations have been described in association with WNV infection, but these are rare manifestations and a definitive association with WNV infection is difficult to substantiate. These rare extraneurological manifestations include: 1. Rhabdomyolysis, that has been temporally associated with WNV infection suggesting a viral myositis, although the presence of virus in muscle tissue has not been observed (Kulstad EB., *et al*., 2003; Jeha LE., *et al*., 2003); 2. Pancreatitis and fulminant hepatitis that have been reported in case of severe WNV infection (Perelman A., *et al*., 1974; Sampson BA,. *et al*., 2000). In this case, WNV has been identified in hepatic and pancreatic specimens at pathology, suggesting that viscerotropic WNV disease may be an infrequent manifestation of infection; 3. Myocarditis and cardiac arrhythmias: the first has been seen pathologically in WNV infection and the second has occurred in individuals with WNP, suspected to be due to autonomic dysfunction (Fratkin JD., *et al*., 2004); 4. Moreover, in some cases convalescent patients may have persistent or chronic infection detected through PCR of the urine, which suggested ongoing viral replication in renal tissue (Murray K., *et al*., 2010; Murray KO., *et al*., 2011). Although persistence of WNV has also been noted in several animals models (Pogodina VV., *et al*., 1983; Tesh RB., *et al*., 2005; Siddharthan V., *et al*., 2009), it has not been uniformly evident in assays of urine (Gibney KB., *et al*., 2011).

3.9.3 HOST RISK FACTORS

Overall, among all individuals who become infected, approximately 25% develop WNF (Zou S., *et al*., 2010) and only 1 in 150 to 250 develops WNV neuroinvasive disease WNND (Mostashari F., *et al*., 2001; Petersen LR., *et al*., 2012). Infections in humans are predominantly subclinical, but reported infection manifestations may range from fever and myalgias to meningoencephalitis and death (Petersen LR. and Marfin AA., 2002). Several factors influence the outcome of WNV infection in the human and animal hosts including virus strain, age, immune status, and genetic susceptibility. The most important risk factor for acquiring WNV infection is exposure to infected mosquitoes. An analysis of the locations of WNV disease cases during the 1999 outbreak in New York found that cases were clustered in an area with higher vegetation cover, indicating favorable mosquito habitat (Brownstein JS., *et al*., 2002). Risk factors for developing WNF following infection are poorly defined. A follow-up study of asymptomatic, viremic blood donors indicated that increasing viral load and female sex, but not age, subsequently increased the risk of

developing WNF (Zou S., *et al*., 2010). A smaller follow-up study of viremic blood donors suggested that younger persons were more likely to develop WNF (Brown JA., *et al*., 2007).

In contrast, elderly and immunocompromised individuals are more susceptible to develop a neuroinvasive disease (WNND), particularly encephalitis (Lindsey NP., *et al*., 2010; Carson PJ., *et al*., 2012) that may result in death. Surveillance data from the US indicate that age is the most important host risk factor for the development of WNND after infection. Indeed, although neuroinvasive disease of WNV infection has been reported among all ages, the proportion of individuals who progress to WNND is greater among older compared to younger persons. It has been estimated that the incidence of neuroinvasive disease increases approximately 1.5-fold for each decade of life, resulting in a 20-fold increased risk of neuroinvasive disease and death among individuals over 50 years of age and 30 times greater for persons 80-90 years old compared to children younger than 10 years old, the case-fatality rate ranges from 15% to 29% (Chowers MY., *et al*., 2001; Nash D., *et al*., 2001; Petersen LR. and Marfin AA., 2002; O'Leary DR., *et al*., 2004). Among those older adults who survive, as many as 50% may have significant postillness morbidity for at least a year following infection (Campbell GL., *et al*., 2002) and may have an increased risk of death for up to 3 years after acute illness (Lindsey NP., *et al*., 2012). Some possible explanations for the higher incidence of WN meningoencephalitis in the elderly include factors that enhance viral entry into the CNS by disruption of the cerebral endothelium (e.g. hypertension, cerebrovascular disease) or an increase in the magnitude and duration of viremia (e.g. immunosuppression, immune senescence). Higher fatality is also seen in infected infants and immunocompromised patients (Granwehr BP., *et al*., 2004). Based upon a limited number of cases, persons infected through transplant of infected organs are likely at higher risk of developing neuroinvasive disease and death compared with patients infected through the natural route of mosquito bite inoculation (Rhee C., *et al*., 2011; Nett RJ., *et al*., 2012). However, conflicting data exist regarding risk of severe neurologic disease among other organ transplant recipients and may be related to the interval between infection *via* mosquito bite and transplantation or the type of post-transplant immunosuppresive therapy (Kumar D., *et al*., 2004; Freifeld AG., *et al*., 2010; Sejvar JJ., 2014). In addition to old age, immunosuppression, such as that of transplanted people or human immunodeficiency virus infected patients, a history of cancer, diabetes, alcohol abuse, a history of cardiovascular disease or chronic renal disease, hepatitis C infection and as well as male sex may increase the risk of neuroinvasive disease (Campbell GL., *et al*., 2002; Bonde AV., *et al*., 2003; Murray K., *et al*., 2006; Lindsey NP., *et al*., 2010; Sejvar JJ., *et al*., 2011; Carson PJ., *et al*.,

2012; Cho H. and Diamond MS., 2012; Lindsey NP., *et al*., 2012).

Given the fact that only a small minority of those infected develop severe disease and the fact that risk factors, apart from older age and immunosuppression, are not well defined, there is a strong rationale to suspect a genetic predisposition to WNV neurological complications: specific host genetic factors that influence the severity of infection with WNV and antiviral innate immune response have been identified (Table 2) (Loeb M., *et al*., 2011; Colpitts TM., *et al*., 2012). Certain HLA types appear to be associated with risk of a more severe outcome (HLA-A*68 and C*08) or better resistance to infection (B*40 and C*03) (Lantieri MC., *et al*., 2011). Single nucleotide polymorphism (SNP) studies have detected SNPs in key regulators of immune function, including interferon pathway elements. In particular, an association of SNP between symptomatic and asymptomatic WNV infections and IRF3 and MX-1 innate immune response and effector genes has been reported (Bigham AW., *et al*., 2011). IRF3 encodes a member of the interferon regulatory transcription factor family involved in the upregulation of type 1 IFN genes as well as other pathway genes. However, IRF3 has been reported to protect mice from WNV-induced disease by both IFN-dependent and independent mechanisms (Daffis S., *et al*., 2001; Fredericksen BL., *et al*., 2004). After a peripheral WNV infection, *irf3*$^{-/-}$ mice exhibited increased mortality, early viral entry into CNS, and increased virus levels in the brain and spinal cord compared to wild-type mice (Daffis S., *et al*., 2001). For this reason IRF3 may a candidate for influencing the risk of symptomatic WNV infection in humans. Also MX1, that is a GTPase with antiviral functions and belongs to MX (myxovirus resistance) family of IFN-induced proteins, may have an effect on flavivirus infections in humans. Indeed, upon viral infection, a host cell secretes type 1 IFN that, in turn, induce the production of MX proteins that diminish viral replication. In mice, Mx1 confers resistance to orthomyxoviruses including influenza viruses, but has not been demonstrated to confer resistance to flaviviruses (Staeheli P., *et al*., 1988). Another study examined CCR5Δ32, a relatively common 32-bp deletion in the coding region of the chemokine receptor 5 (CCR5), which is known to be protective in infection with HIV, that was initially reported to be associated with both increased susceptibility to WNV infection and death. A greater incidence (4.2%) of loss-of-function CCR5Δ32 homozygotes was observed in symptomatic and lethal WNV cases compared to that in the general population (1.0%), suggesting that CCR5 may mediate resistance to WNV infection in humans (Glass WG., *et al*., 2006; Lim JK., *et al*., 2008). More recently, this association was not replicated, but results suggestive of a link to clinical manifestations of infection with CCR5Δ32 mutation were reported (Lim

JK., *et al*., 2010). In certain mouse strains, susceptibility to flavivirus, including WNV, maps to a truncated isoform of the 2’-5’ oligoadenylate syntetase (OAS1b) gene: a member of an IFN-regulated gene family involved in degradation of viral RNA. Compared with the resistant mice, susceptible mice produce an OAS1b protein lacking 30% of the C-terminal sequence, resulting in the inactivation of the OAS/RNaseL pathway. Consequently, a large amount of virus is produced in the susceptible mice. A recent study suggests that allelic variants in two human ortholog genes, OAS1 and OASL, are associated with increased risk of WNV susceptibility or WNND, although subsequent attempts to replicate the association with OASL were unsuccessful (Yakub I., *et al*., 2005; Lim JK., *et al*., 2009). The products of OAS1, OAS2, OAS3, OASL, and their downstream effector RNaseL each influence host defense by blocking viral replication (Samuel CE., 2001). Thus, in humans, variation in OAS1 is a genetic risk factor for initial WNV infection although not for disease severity (Diamond MS., *et al*., 2009). Moreover, a dominant negative splice variant of RNase L, which functions in the antiproliferative roles of interferon, was detected more often in WNV patients than in control patients (Yakub I., *et al*., 2005). Thus genetic variation in the IFN response pathway appears to correlate with the risk of symptomatic WNV infection in humans. Another genomic study investigated >1.500 symptomatic subjects, with severe versus mild disease, and showed that SNPs in RFC1 (a replication factor), SCN1a (a sodium channel), and ANPEP (an aminopeptidase) genes have been associated with a more severe neurological disease, although even more differences might have been revealed when comparing asymptomatic and symptomatic cases (Loeb M., et al., 2011).

Gene(s)	SNP(s)	Comparison groups (*n*)	Study results
OASL	rs3213545	WNV^{+} cases (33) vs healthy controls (16)	Associated with increased susceptibility to WNV infection
CCR5	Δ32 deletion	WNV^{+} cases (395) vs WNV^{-} (1,463)	Increased risk of symptomatic WNV infection
		WNV^{+} cases (224) vs healthy controls (1,318)	Increased risk of symptomatic WNV infection
		WNV^{+} cases (634) vs WNV^{-} (422)	Not a risk factor for WNV initial infection; associated with symptomatic WNV infection
OAS1	rs10774671	WNV^{+} cases (501) vs healthy controls (552)	A risk factor for initial infection with WNV
IRF3, MX1, OAS1	rs2304207, rs7280422, rs34137742	Symptomatic cases (422) vs asymptomatic cases (331)	Associated with symptomatic WNV infection
RFC1, SCN1A, ANPEP	rs2066786, rs2298771, rs25651	Severe WNV cases (560) vs mild WNV cases (950)	Associated with neuroinvasive disease in patients infected with WNV

Table 2. Genes and corresponding SNPs important in human WNV infection (Colpitts TM., *et al*., 2012).

3.9.4 VIRAL RISK FACTORS

Several factors influence the outcome of WNV infection in the human, including viral factors. For example the capacity of WNV to infect several target cells and evade immune response allows WNV to survive and cause disease within the host (Table 3). Certain aspects of the biology of WNV facilitate its ability to cause severe disease. WNV productively infects diverse cell populations from many animal species, suggesting usage of multiple and/or well-conserved receptors (Xiao SY., *et al*., 2001; Banet-Noach CL., *et al*., 2003; Farajollanhi A., *et al*., 2003; Jacobson ER., *et al*., 2005; Root JJ., *et al*., 2005; Tesh RB., *et al*., 2005; Garcia-Tapia D., *et al*., 2006). The relatively diverse tropism of WNV allows viral replication in several tissues in animal and human hosts and may contribute to the wide spectrum of clinical manifestations (Sejvar JJ., *et al*., 2003; Yim R., *et al*., 2004; Hayes EB., Komar RS., *et al*., 2005; Paddock CD., *et al*., 2006). Moreover, WNV is cytolytic and induces apoptosis in a variety of cell types, including neurons (Parquet MC., *et al*., 2001; Shrestha B., *et al*., 2003). Although few studies have investigated the mechanisms of WNV-induced cell death *in vivo*, individual WNV proteins may contribute to virus-mediated cytotoxicity. *In vitro*, expression of either NS3 or capsid protein induced rapid, caspase-dependent apoptosis, and capsid protein expression *in vivo* resulted in cell death (Yang JS., *et al*., 2002; Ramanathan MP., *et al*., 2006).

In addiction, genetic variation may affect WNV virulence (Samuel MA. and Diamond MS, 2006). Sequence-based phylogenic analyses of global WNV strains have revealed two major lineages: lineage 1 and 2. Lineage 1 strains are detected worldwide and are commonly involved in human and equine outbreaks, including in Romania (1996), Russia (1999), Israel (1998 to 2000), and the Americas (1999 to 2005) (Dauphin G., *et al*., 2004; Mackenzie JS., *et al*., 2004). In contrast, lineage 2 strains appears to be localized to central and southern Africa and have caused only occasional human infections (Jupp PG., 2001; Lanciotti RS., *et al*., 2002). Generally, lineage 1 strains induce significant encephalitis and mortality in birds and mammals, although isolates from both lineages can be neuroinvasive (Samuel MA. and Diamond MS., 2006). Thus, while lineage 1 WNV isolates appear to be linked to the recent increase in severe infection of humans, pathogenic lineage 2 isolates have been identified and have the potential to induce significant human disease. The specific sequence determinants of virulence are an area of intensive study. N-linked glycosylation of the E protein appears important for neuroinvasion as mutations of E-protein glycosylation sites attenuated viral replication and pathogenesis (Beasley DW., *et al*., 2002; Shirato K., *et al*.,

2004; Beasley DW., Whiteman MC., *et al*., 2005). Moreover, E-protein glycosylation modulates WNV virulence by altering virion stability, viral replication, and particle assembly (Beasley DW., Whiteman MC., *et al*., 2005; Hanna SL., *et al*., 2005; Li J., *et al*., 2006). Glycosylation of the NS1 protein has also been linked to WNV pathogenesis: WNV NS1 contains three N-linked glycosylation sites (residues 130, 175, and 203) and mutants lacking glycosylation at either two or three sites induced lower viremia and decreased lethality *in vivo* (Samuel MA. and Diamond MS., 2006).

WNV has also evolved specific strategies to avoid and/or attenuate innate and adaptive immune responses. Flaviviruses, including WNV, are largely resistant to the antiviral effects of IFN once cellular infection is established. Through studies with WNV, and others flavivirus, this phenotype has been largely ascribed to the actions of non-structural proteins NS2A, NS2B, NS3, NS4A, NS4B, and NS5. These non-structural proteins suppress host IFN-induced responses at multiple stages within the cell by delaying IRF-3 activation and IFN-α gene transcription and by preventing the phosphorylation and activation of JAK1 and Tyk2 (Muñoz-Jordan JL., *et al*., 2003; Lin RJ., *et al*., 2004; Best SM., 2005; Guo JT., *et al*., 2005; Liu WJ., *et al*., 2005; Lin RJ., *et al.*, 2006; Murray K., *et al*., 2006). The replication fitness and virulence of lineage 1 and lineage 2 strains has been linked to control of host IFN responses (Keller BC., *et al*., 2006): while a pathogenic lineage 1 Texas isolate actively antagonized IFN signalling, an attenuated lineage 2 strain from Madagascar lacked this activity. The replication and virulence of the lineage 2 isolate were restored in cells and mice that lacked the IFN-α/βR. These data suggest that inhibition of type I IFN responses may be a key feature in the evolution of pathogenic WNV strains. Consistent with this, an aminoacid substitution (Ala30Pro) in NS2A protein of Kunjin virus could reduce NS2A-mediated inhibition of the IFN response resulted in an attenuated neurovirulence (Liu WJ., *et al*., 2006). Escape from the humoral immune response may also contribute to WNV pathogenesis. Flaviviruses have a low-fidelity RNA-dependent RNA polymerase that generates quasispecies *in vivo* (Jerzak G., et al., 2005). This antigenic variation may allow viral quasispecies to escape antibody-mediated neutralization (Beasley DW. and Barrett AD., 2002), as strains with mutations at the dominant neutralizing epitope in DIII of the E protein can emerge (Li L., *et al*., 2005).

Trait	Source	Effect on pathogenesis
Diverse cellular and species tropism	Use of multiple and/or well-conserved receptors	Replication in multiple tissues and diverse clinical manifestations
Induction of rapid cell death	NS3, capsid	May contribute to neuropathology
N-linked glycosylation of E protein	Genetic variation	Alters virion stability, replication, particle assembly, pathogenesis
N-linked glycosylation of NS1 protein	Genetic variation	Lack of glycosylation reduces pathogenesis in vivo
IFN resistance	NS2A, NS2B, NS3, NS4A, NS4B, genetic variation	May suppress IFN production in vivo and allow increased replication or pathogenesis
Quasispecies generation	Low-fidelity RNA-dependent RNA polymerase	Potential for escape from antibody neutralization and T-cell lysis
Upregulation of MHC class I expression	Dependent on NF-κB activation	May inhibit NK cell responses

TABLE 3. WNV virulence factors (Samuel MA. and Diamond MS., 2006).

3.10 DIAGNOSIS

Laboratory diagnosis relies on isolation of virus, detection of viral antigens or RNA in blood or tissues, or detection of virus-specific IgM antibody that should be further confirmed by detection of IgG antibody in the same or a subsequent sample (Fig. 14).

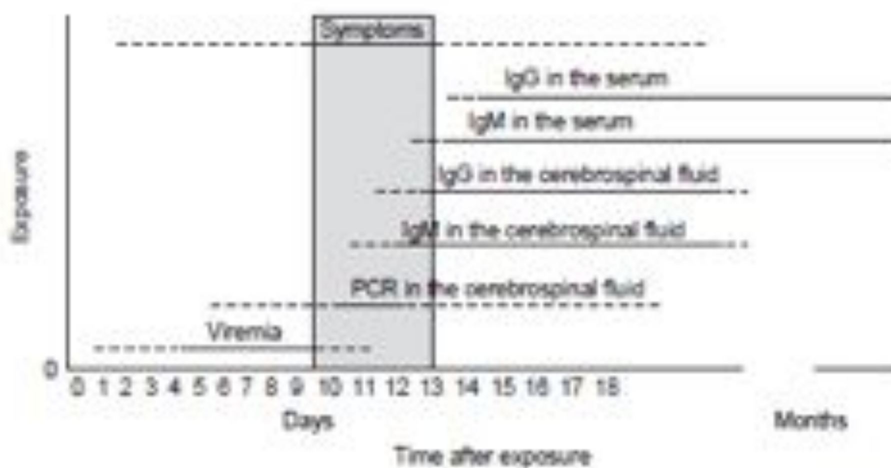


Figure 14. Schematic of virologic and serologic tests in West Nile virus encephalitis. Solid lines represent the more common results; broken lines represent reported ranges. The shaded box is an example of a typical patient (Kramer LD., 2008).

3.10.1 NUCLEIC ACID BASED TESTS FOR WNV

The use of nucleic acid detection techniques has provided an opportunity to diagnose WNV in patients prior the production of specific IgM antibody, with the circulation of detectible levels of WNV RNA in blood, an average, 4 days prior to the first detection of IgM antibodies (Busch MP., *et al*., 2008). WNV nucleic acid detection has become a routine test for screening blood products in endemic areas, and the introduction of such measures in these areas has essentially eliminated WNV acquisition through the donated blood or organ supply (Busch MP., *et al*., 2005). Several methods for detection of viral RNA have been applied for WNV surveillance and diagnosis, mainly reverse transcription polymerase chain reaction (RT-PCR) assays, quantitative real-time RT-PCR and nucleic acid sequenced-based amplification (Lanciotti RS., 2003). All these assays have been extensively used in mosquito pools, and animal and human samples (blood and/or CFS), although the latter are usually collected after the onset of clinical signs, when virus is unlikely to be present on them (Martin-Acebes MA., *et al*., 2012).

3.10.2 SEROLOGIC DIAGNOSIS OF WNV INFECTIONS

Serological testing remains the most widely used method for detection of anti-WNV antibodies in human and animal samples (Beasley DW., 2005a). Following exposure to WNV, both IgM and IgG antibodies are produced. In most cases, IgM antibodies can be detected within 4 to 7 days after the initial exposure and may persist in the serum for more than one year in some patients (Roehrig JT., *et al*., 2003; Colpitts TM., *et al*., 2012). In comparison, anti-WNV IgG are reliably detected approximately 8 days after the onset of symptoms and they have a limited use in the initial diagnosis of WNV infection (Tardei G., et al., 2000). The commercial IgM antibody capture enzyme-linked immunosorbent assay (MAC-ELISA) can be applied to both CSF and serum: the detection of IgM antibody in the CSF is indicative of infection of the CNS and in conjunction with evidence of neurological has been accepted as diagnostic of WNV disease; whereas the presence of IgM antibody in the serum alone is strongly suggestive of recent infection but not definitive due to persistence for al least 16 months (199 days in the CSF) in patients with WNND and to some cross-reactivity with antibody to other flaviviruses. Indeed, the main weakness that limits the clinical relevance of serological methods is the broad antigenic cross-reactivity that exists between related flavivirus (Japanese encephalitis virus, St. Louis encephalitis virus, Yellow fever virus, and Dengue virus), and thus, if suspected, sera have to be tested against different related viruses and results have to be subsequently confirmed by different assays. The

diagnosis methods can be subdivided into two main groups: the first includes the enzyme-linked immunosorbent assays (ELISAs) and immunoflourescence (IF) based tests; the second includes the Plaque Reduction Neutralization Test (PRNT), considered as the gold-standard (Dauphin G. Zientara S., 2007) which an be carried out using a highly sensitive 50% or less-sensitive 90% endpoint ($PRNT_{50}$, and $PRNT_{90}$ respectively), both of which require the constant availability of standardised-validated infectious viruses and appropriate cell cultures (Sambri V., *et al.*, 2013). The hemagglutination-inhibition test (HIA) is still used to detect pan-flavivirus immune response whereas the complement fixation test (CFT) is rarely used in today's laboratories. Thus, initially serological testing was based on the techniques included in the first group are widely used due to their relative applicability in routine laboratory and the ability to automate a part of the workflow (IgM antibody capture assays (MAC-ELISA) and in indirect IgG ELISAs), but they are less specific as a consequence of their inability to distinguish between WNV-specific and cross-reactive antibody responses (Dauphin G., Zientara S., 2007) and any positive result identified, using these methods, must be confirmed by the more specific tests, i.e., those that constitute the second group. The $PRNT_{50}$ method is considered the gold-standard for detecting immune responses which is able to detect, specifically, low titre, low avidity immune responses (Sambri V., *et al.*, 2013).

3.11 VACCINES

Even though notable progress for WNV vaccine development has been made, no FDA approved vaccines exist for human use (Dauphin G. and Zientara S., 2007; Rossi SL., *et al.*, 2010; Beasley DW., 2011), and their cost-effectiveness for human treatment is still uncertain. On the other hand, there are effective, licensed vaccines for the treatment of horses that had greatly contributed to the decrease incidence of equine cases in the US, whilst the number of human cases still remains growing (Ward MP., *et al.*, 2006; Dauphin G. and Zientara S., 2007). There are several strategies being pursued for WNV vaccine development (Table 5). The first strategy is based on the use of live attenuated or chemically inactivated virus obtained from infected cell cultures or from inoculated suckling mouse brains (Ng T., *et al.*, 2003; Samina I., *et al.*, 2005). Fort Dodge Animal Health developed this strategy by formalin inactivating whole virus (Innovator®, FortDodge, Princeton, NJ, US) and this formulation has been approved for horses (Rossi SL., *et al.*, 2010). Additionally, a commercially available formaldehyde inactivated vaccine derived from infected suckling mice brains and live attenuated vaccines have been administered to

domestic geese in Israel (Malkinson M., *et al*., 2001; Samina I., *et al*., 2005). The second strategy involves the production of WNV antigens from a heterologous virus backbone. The vectors being used are: recombinant live canarypox vaccine that express prM and E genes formulated with Carbopol adjuvant (Recombitek^{TM}), Yellow fever virus (Chimerivax^{TM}), and Dengue 4 (WNV-DENV4) (Pletnev AG., *et al*., 2003; Arroyo J., *et al*., 2004; Minke JM., *et al*., 2004; Monath TP., *et al*., 2006). The Recombitek^{TM} (MerialLtd., Athens, GA, US) vaccine has been licensed for use in horses. The third approach is through use of recombinant DNA technology that has been applied for engineering DNA and recombinant vaccines based on the use of viral proteins (or fragments of them) synthesized in diverse systems (from bacteria to insect cells and larvae) (Dauphin G. and Zientara S., 2007; Rossi SL., *et al*., 2010; Beasley DW., 2011). Vertical transfer of acquired maternal immunity to the offspring has been demonstrated in mice immunized with recombinant proteins (Alonso-Padilla J., *et al*., 2011). For horses a plasmid DNA vaccine, pCBWN, that encodes WNV structural antigens (prM-E) (Fort Dodge and Center for Disease Control and Prevention) commercialized in the US. The success of veterinary vaccines has encouraged others to develop these and other strategies for human vaccines that should induce a good response on higher risk groups and achieve an affordable cost/benefit ratio (Rossi SL., *et al*., 2010; Martin-Acebes MA., *et al*., 2012).

Type	Antigen	Sponsor	Stage of Development
Chimeric (vector)			
Recombitek ™ (canarypox)	WNV-prM-E	Merial	Licensed for horses
Chimerivax ™ (yellow fever virus)	WNV-prM-E	Acambis	Phase II
WNV-DENV4 (dengue virus 4)	WNV-prM-E	NIAID/NIH	Phase II
DNA			
WNV-DIII	WNV-DIII	Multiple labs	Preclinical
WNV-E	WNV-E	Multiple labs	Preclinical
WNV-prM-E	WNV-prM-E	Multiple labs	Preclinical
Inactivated/Killed			
Innovator ™	Whole virus	Fort Dodge Animal Health	Licensed for horses
Subvirion Particles/Virus-like Particles			
WNV-prM-E	WNV-prM-E	Multiple labs	preclinical

Table 5. WNV vaccines. Partial list of licensed and preclinical vaccines against WNV (Rossi SL., *et al*., 2010).

4. INNATE IMMUNITY

4.1 INNATE IMMUNITY TO VIRUS INFECTION

The innate immune system acts as the first line of defense against invading viral pathogens and it is critically important for controlling infection. It consists of multiple antiviral programs that work in concert to control viral replication and spread as well as promoting the cell-mediated innate and adaptive immune responses. The proper and controlled induction of this aim of the immune response is mediated by specialized cellular proteins termed pattern-recognition receptors (PRRs). These are proteins expressed by a variety of cells, which are responsible for sensing the presence of pathogens invasion through evolutionary conserved viral components, known as pathogen-associated molecular patterns (PAMPs), such as glycoprotein, double-stranded RNA (dsRNA), single-stranded RNA (ssRNA), and DNA that are broadly shared by different microorganisms and essential to the survival or infectivity of the pathogen (Takeuchi O. and Akira S., 2009; Rossi SL., *et al*., 2010; Jensen S., *et al*., 2012; Ye J., *et al*., 2012; Fredericksen BL., 2014). Currently, three classes of PPRs have been shown to be involved in the recognition of PAMPs in non-immune cells or cells of the innate immune system, namely: retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs); Toll-like receptors (TLRs); and nucleotide oligomerization domain (NOD)-like receptors (NLRs). Among these receptors types, RLRs and TLRs detect pathogen structures in immune cells and active intracellular signalling cascades that leads to production of type I interferons (IFNs) and proinflammatory cytokines whereas NLRs are known to play a role in the production of mature interleukin-1-beta through activation of caspase-1 (Fig. 15) (Kanneganti TD., *et al*., 2007; Petrilli V., *et al*., 2007).

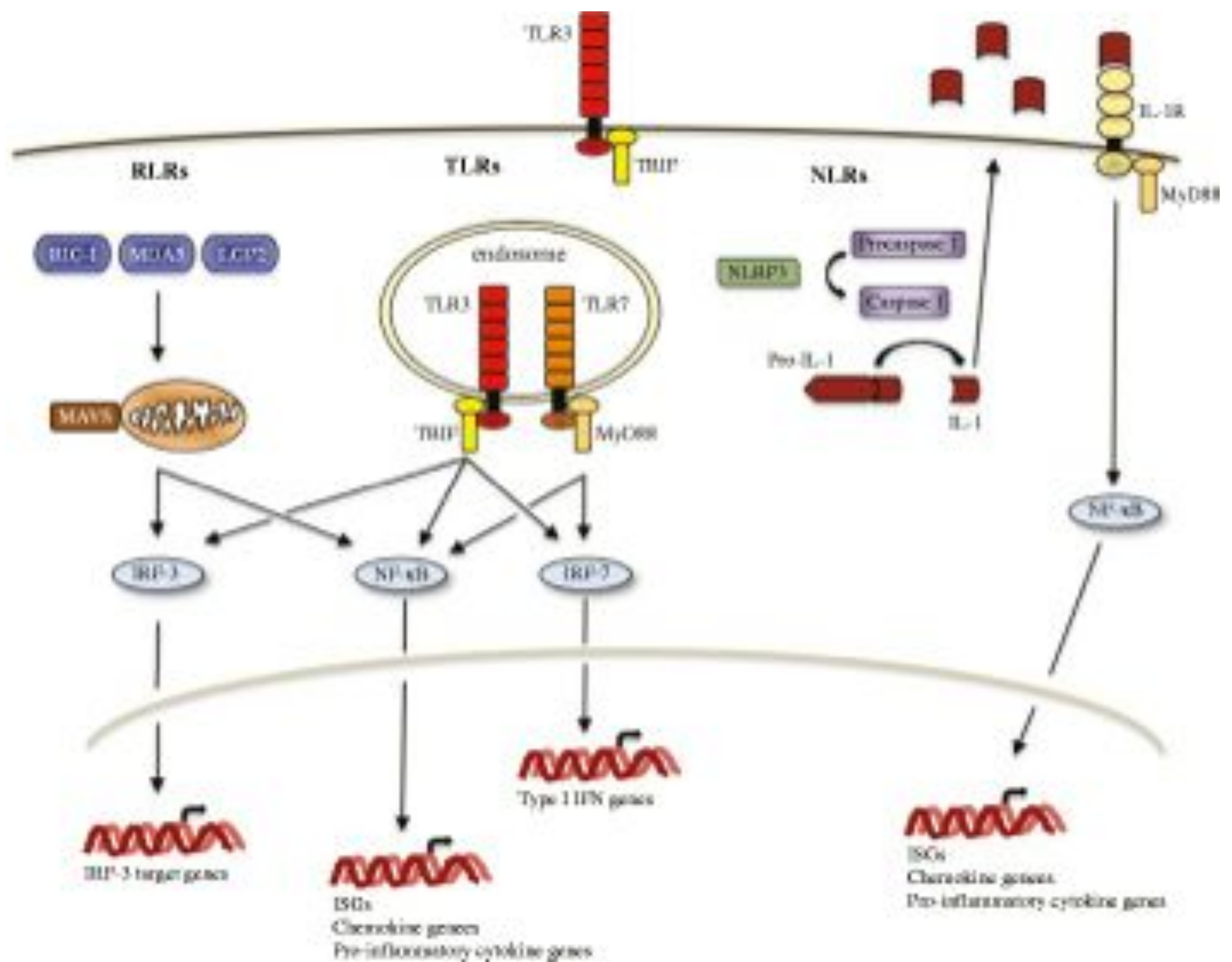


Figure 15. Cell-intrinsic innate antiviral response. Schematic of the key signalling pathways contributing to the innate antiviral response to WNV. Abbreviations: retinoic acid-inducible (RIG-I)-like receptors (RLRs); Toll-like receptor (TLRs); melanoma differentiation antigen 5 (MDA5); laboratory of genetics and physiology 2 (LGP2); nucleotidebinding oligomerization domain (NOD)-like receptors (NLRs); NLR family PYD-containing 3 (NLPR3); TIR-domain-containing adapterinducing interferon-β (TRIF); myeloid differentiation primary response 88 (MyD88); interferon stimulated genes (ISGs) (Fredericksen BL., 2014).

4.1.1 RIG-I-LIKE RECEPTORS SIGNALLING

The RIG-I-like receptors (RLRs) are cytosolic proteins consisting of three members of relevance: RIG-I (also known as DDX58), melanoma differentiation-associated antigen 5 (MDA5), and laboratory of genetics and physiology-2 (LGP2) (Kang DC., *et al.*, 2002; Yoneyama M. *et al.*, 2004; Yoneyama M., *et al.*, 2005; Yoneyama M. and Fujita T., 2007; Jensen S. and Thomsen R., 2012). RLRs are critical sensors of viral RNAs in the cytoplasm

and are expressed basally in most cells of human organism, except plasmacytoid dendritic cells (pDCs) which preferentially employ TLRs for detection of RNA virus infection, and are induced to high levels by type I interferons (IFNs) (Kang DC., *et al*., 2004; Yoneyama M., *et al*., 2004; Kato H., *et al.*, 2005; Yoneyama M., *et al.,* 2005). However, in response to viruses that circumvent the endosomal TLRs of the pDCs by direct membrane fusion, cytosolic recognition by RLRs is assumed to be of great importance also in pDCs. RIG-I and MDA5 consist of two N-terminal tandem caspase-activation and recruitment domains (CARDs), an RNA helicase domain, and a C-terminal repressor domain (RD). In contrast, LGP2 lacks the tandem N-terminal CARDs but contains an RNA helicase domain and a C-terminal repressor domain. Whereas the helicase domain and RD are important for the recognition of viral RNA, the CARDs are essential for triggering intracellular signalling cascades (Yoneyama M., *et al*., 2004; Saito T., *et al*., 2007). LGP2, that lacks the N-terminal CARD, has been suggested to function as a negative regulator, rather than an initiator, of RLR signalling (Komuro A. and Horvath CM., 2006; Venkataraman T., *et al*., 2007; Suthar MS., *et al*., 2012). RNA virus infection leads to the generation of dsRNA and RNAs with 5'-triphosphate ands in infected cells. Long dsRNA is not normally present in cells, and 5' ends of host RNAs are typically capped. In response to detection of viral RNAs in the cytoplasm, both RIG-I and MDA5 are post-translationally modified and translocate to mitochondria and mitochondrial-associated membranes (Gack MU., *et al*., 2010; Horner SM., *et al*., 2011; Liu HM., *et al*., 2012; Wies E., *et al*., 2013). Here, RIG-I and MDA5 interact with an adapter protein designated IFN-β promoter stimulator-1 (IPS-1), also know as mitochondrial antiviral signalling (MAVS), through CARD repeats that leads to the formation of the IPS-1/MAVS-signalosome, comprised of RLR signalling adaptors, protein kinases, and transcription factors (interferon regulatory factors (IRF)-1, -3, -5, -7, and NF-κB) (Daffis S., *et al*., 2007; Daffis S., *et al*., 2008; Daffis S., *et al*., 2009; Lazear HM., *et al*., 2013). IPS-1-deficient mice are impaired in the production of proinflammatory cytokines and type I in response to all RNA viruses recognized by RIG-I and MDA5 (Kumar H., *et al*., 2006; Sun Q., *et al*., 2006). These findings indicate that IPS-1plays essential roles in RIG-I/MDA5 signalling (Takeuchi and Akira., 2009). IPS-1 itself is probably not directly involved in the signalling process, but serves as a platform to coordinate the activation of two of the signalling pathway also utilized by the TLRs (Jensen S. and Thomsen R., 2012). Recently, another adaptor, stimulator of IFN genes (STING, also called MITA) was described (Ishikawa H. and Barber GN., 2008; Zhong B., *et al*., 2008). STING is also found in the mitochondrial membrane but resides predominantly in the endoplasmic reticulum.

Considering that STING interacts with RIG-I and IPS-1 in the mitochondrial membrane, this potentially opens the possibility for cross talk between the two organelles in viral sensing (Arnoult D., *et al*., 2009). However, the precise importance of such interactions is not clear at this moment (Jensen S. and Thomsen R., 2012). IPS-1 coordinates the activation, through TNF receptor-associated death domain (TRADD), of two of the same pathways as those activated by TRIF during downstream signalling from TLR3: the activation of RIP1 for NF-kB nuclear translocation, and the activation of IKKs, for IRF3 phosphorylation and translocation (Hemmi H., *et al*., 2004). Activated transcription factors translocate to the nucleus and drive transcription of IFN-β, IFN-α4, proinflammatory cytokines, and interferon-stimulated genes (ISGs) that aid in cellular defense against viral infection (Quicke KM. and Suthar MS., 2013). In addition, research also suggests that RIG-I activation can trigger inflammasome formation and cysteine-aspartic protease 1 (caspase-1) activity, leading to the maturation of proinflammatory cytokines such as interleukin-1β (IL-1β) (Poeck H., *et al*., 2010). This IPS-1-indipendent pathway is also used by NLRP3 (Jensen S. and Thomsen R., 2012).

4.1.2 TOLL-LIKE RECEPTORS SIGNALLING

In addition to RLRs, TLRs are important for recognizing virus infection. TLRs are transmembrane glycoprotein receptors with an N-terminal extracellular PAMP-binding region, a transmembrane domain (LRRs) and a C-terminal cytoplasmic domain designed the Toll/IL-1 receptor (IL-1R) homology (TIR) domain (Akira S., *et al*., 2006) which mediates downstream signalling events upon activation of the receptor (Bowie A. and O'Neill LAJ., 2000; Akira S. and Takeda K., 2004). TLRs, unlike the RLRs, are transmembrane proteins suitable for detecting distinct viral and bacterial PAMPs outside of the cells as well as in cytoplasmic vacuoles after phagocytosis or endocytosis (Takeuchi O. and Akira S., 2009). Upon extracellular ligand recognition, TLR dimerization is thought to be induced, bringing together the cytoplasmic TIR domains and subsequently recruting adaptor molecules to initiate the signalling process (Akira S., *et al*., 2006; O'Neill LAJ., 2006). Among more than 10 TLRs present in mammals, TLR2, TLR3, TLR4, TLR7, TLR8 and TLR9 are thought to be importance in the recognition of structural components of RNA viruses. Among these receptors, TLR2 and TLR4, present on the plasma membrane, are involved in the recognition of viral envelope proteins on the cell surface. By contrast, the TLRs 3, 7, 8 and 9, reside on cytoplasmic vesicles, such as endosomes and ER, and recognize microbial nucleotides (Takeuchi O. and Akira S., 2009; Quicke KM. and Suthar MS., 2013). TLR3

recognizes dsRNA, whereas TLR7/8 and TLR9 recognize GU-rich ssRNAs and DNA with CpG motifs, respectively (Alexopoulou L., *et al*., 2001; Diebold SS., et al., 2004; Heil F., *et al*., 2004). TLR3 and TLR7/8 are important in regulating immunity to WNV but, unlike the RLRs, function in a cell- and tissue-specific manner: while TLR3 signalling in cortical neurons, but not in macrophages or DCs, promotes type I IFN production and is required for controlling virus replication (Daffis S., *et al*., 2008a); TLR7/8 signalling is important for triggering type I IFN and proinflammatory cytokine production within neurons, macrophages, and keratinocytes, but not DCs (Welte T., *et al*., 2009; Szretter KJ., *et al*., 2010). Upon binding PAMP RNA, all TLRs, except TLR3, activate three major signalling pathways: mitogen-actived protein kinases (MAPKs), one or more interferon regulatory factors (IRFs), and a nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), that lead to the production of type I IFN and proinflammatory cytokines *via* myeloid differentiation factor 88 (MyD88) (Adachi O., *et al*., 1998; Yamamoto M., *et al*., 2003). MyD88 is a protein comprised of a C-terminal TLR-binding TIR domain and an N-terminal death domain (DD), and through the latter it forms a complex with two interleukin-1 receptor-associated kinases (IRAKs). Mammals have four IRAK family members, called IRAK-1, IRAK-2, IRAK-M and IRAK-4 that are characterized by an N-terminal DD and a C-terminal serine/threonine kinase domain. Upon activation, IRAK-4 phosphorylates IRAK-1 that, activated, binds the C-terminal domain of TNF receptor-associated factor 6 (TRAF6). This IRAK-1/ TRAF6 complex then dissociates from the TLR. Downstream of IRAKs, TRAF6 is activated and catalyzes the formation of a K63-linked polyubiquitin chain on IKK-γ/NF-kB essential modulator (NEMO) (Chen ZJ., 2005) and on tumor growth factor-β (TGF-β)-activated kinase 1 (TAK1). IKKγ subsequently associates with IKKα and IKKβ. IKKβ is phosphorylated by the activated TAK1 associated with the TAK1 binding protein 1 (TAB1), TAB2, and TAB3. This leads to the IKK-mediated phosphorylation and subsequent degradation of IkB, which in the unphosphorylated state is coupled to NF-kB. NF-kB, formerly sequestered in the cytosol, is now free to enter the nucleus to induce gene expression. TAK1 in association with TAB1, TAB2, and TAB3 also triggers a MAPK pathway leading to the formation of AP-1. Similar to NF-kB, AP-1 enters the nucleus, and together, NF-kB and AP-1 induce the expression of genes involved in inflammatory responses (Akira S. and Takeda K., 2004; Takeuchi O. and Akira S., 2009). IRF5 and IRF7 also interact with the complex of IRAKs and TRAF6. This leads to IRAK1-dependent phosphorylation and subsequent nuclear translocation of both molecules (Takaoka A., *et al*., 2005; Uematsu S., *et al*., 2005). While IRF5 is involved primarily in regulating the induction

of proinflammatory cytokines (e.g., IL-6 and IL-12p40) (Takaoka A., et al., 2005), IRF7 is a key mediator in TLR7/TLR8-dependent type I IFN production (Honda K., et al., 2005; Honda K., *et al*., 2006).

TLR3 is localized to the intracellular compartment in macrophages, B lymphocytes, and cDCs and is found both intracellulary and on the surface of NK cells, epithelial cells and fibroblasts (Hornung V., *et al*., 2002; Matsumoto M., *et al*., 2003; Kawai T. and Akira S., 2008; Paludan SR., *et al*., 2011). In response to stimulation with dsRNA, TLR3 dimerizes and recruits another adapter protein, shared only with TLR4 among TLRs: TIR domain-containing adapter inducing IFN-β (TRIF) (Yamamoto M., *et al*., 2002; Oshiumi H., et al., 2003; Yamamoto M., et al., 2003). TRIF associates with TRAF6 through TRAF-binding motifs presents in its N-terminal portion for the activation and translocation of NF-kB and AP-1 as that seen for TLRs signalling described above. In addition to this pathway, recent studies showed that TAK-1-indipendent pathway of NF-kB activation is also triggered. This pathway is initiated when TRIF interacts with RIP1 and RIP3 *via* the RHIM (receptor-interacting (RIP) homotypic interaction motif) presents in its C-terminal portion (Sato S., *et al*., 2003; Meylan E., *et al*., 2004) and subsequently converges on IKKβ, which is also used by the TAK1-dependent route (Meylan E., *et al*., 2004). TRIF associates also with TRAF3 for association with TRAF family member-associated NF-kB activator (TANK)-binding kinase 1 (TBK1) and IKKε. TBK1/IKKε subsequently phosphorylates IRF3 (Sharma S., *et al*., 2003; Fitzgerald KA., 2003). The downstream signalling molecules for the expression of IFN-inducible genes are shared between the TLR3 and RLR signalling pathways. IRF3, upon phosphorylation, dimerizes and translocates to the nucleus to initiate transcription of type I IFNs (IFN-β and IFN-α4). In a positive feedback system, these type I IFNs, among many other effects, upregulate the level of IRF7 expression in responding cells. IRF7, when upregulated, is phosphorylated by TBK1/IKKε, as is IRF3. Dimerized IRF7 then stimulates further type I IFN release (entire range of IFN-α species) (Kawai T. and Akira S., 2006). Furthermore, as type I IFN release stimulates the expression of TLR3 in cells that were initially TLR3 negative, this adds to the positive feedback loop, enhancing the capacity to provide the antiviral response. In addition to the TLR3-mediated expression of type I IFNs and inflammatory cytokines, TLR3 activation also provides a link between the innate immune system and the adaptive immune system (Jensen S. and Thomsen R., 2012).

4.1.3 NOD-LIKE RECEPTORS SIGNALLING

Nucleotide-binding oligomerization domain-containing (NOD)-like receptors (NLR), are cytosolic proteins regulating inflammatory and apoptotic responses, and recent studies point to the importance of these receptors in the antiviral defense (Jensen S. and Thomsen R., 2012). These proteins contain: an LRR domain at the C-terminal, that is considered to be the sensor region of the NLRs; a NACHT (NAIP, CIITA, HET-E, TP-1) domain located centrally that mediates oligomerization and activation; and at the N-terminal an effector-binding domain, most often a CARD or a pyrin domain (PYD), that signals downstream following induced proximity upon activation and oligomerization of the NLRs (Jensen S. and Thomsen R., 2012). NLRP3 (NOD-, LRR-, and pyrin domain-containing 3) oligomerizes upon activation and recruits an adapter, ASC (apoptosis-associated speck-like protein containing a CARD), and procaspase-1 to form an inflammasome complex. The inflammasome is an innate immune signalling complex comprised of cytosolic PRRs (34 NLR genes in mice and 22 NLR genes in humans) that regulates immune programmes and promotes viral clearance through the secretion of pro-inflammatory cytokines of the IL-1β family, including IL-1β, IL-18, and IL-33. Inflammasome activation is regulated by two signals: 1. A priming signal to induce the secretion of pro-inflammatory cytokines of the IL-1β family, including IL-1β, IL-18, and IL-33 which can be mediated by the TLR, RLR, or NLR signalling pathways; and 2. A maturation signal wherein the inflammasome complex, comprised of an activated NLR, ASC adaptor protein and caspase-1, that processes pro-IL-1β and pro-IL-18 into their mature forms which are subsequently secreted from the cell. These cytokines can have opposing effects: can elicit protective immunity by promoting immune cell trafficking to and activation at sites of infection, and can trigger pathological responses by driving a programmed cell death response known as pyropoptosis (Suthar MS., *et al*., 2013; Quicke KM. and Suthar MS., 2013).

4.1.4 TYPE I INTERFERON SIGNALLING

RLR and TLR signalling both activate IRF transcription factors, in particular IRF3 and IRF7, which are essential for regulating the type I IFN response following viral infections (Sato M., *et al*., 2000; Honda K., *et al*., 2005). These IRFs induce the production of type I IFNs (IFN-α and IFN-β) that, together with type II (IFN-γ) and type III (IFN-λ) IFNs, act as important innate immune system controls of viral infections (Samuel MA and Diamond MS., 2006). IFN-α and IFN-β is produced by most cell types following virus infection and induces an antiviral state by upregulating genes with direct and indirect antiviral functions.

IFN α/β are secreted from the cell and can bind to the IFN-α and IFN-β receptor complex (IFNAR) in an autocrine or paracrine manner, leading to activation of the receptor-associated kinases tyrosine kinase 2 (TYK2) and JAK1. These events are followed by phosphorylation of STAT1 (signal transducer and activator of trascription 1), STAT2 and assembly of the ISG factor 3 (ISGF3) complex, consisting of a STAT1– STAT2 heterodimer and IRF9 (Quicke KM. and Suthar MS., 2013). This complex translocates to the nucleus and binds specific DNA sequences known as interferon stimulated response elements (ISRE) to initiate transcription of IFN-stimulated genes (ISGs) (Horvath CM., 2004). In addition, inhibitor of NF-κB kinase-ε (IKKε) phosphorylates STAT1 at serine 708 to alter the specificity of the ISGF3 complex, thus enhancing the expression of IKKε-dependent antiviral genes, including IFN-induced protein with tetratricopeptide repeats 2 (IFIT2) and ADAR1 (also known as DRADA) (Tenoever BR., *et al*., 2007; Perwitasari O., *et al*., 2011).

4.2 INNATE IMMUNE EVASION STRATEGIES OF WNV

In order to replicate and spread, WNV has evolved mechanisms to counteract the innate immune pathways of the host cells (Katze MG., *et al*., 2002) (Fig. 16). With respect to RLR signalling, initial observations evaluating the kinetics of IRF3 activation following WNV infection revealed a disparity between early viral protein accumulation and IRF3 activation, suggesting that WNV either actively antagonizes or evades detection by RLRs. Subsequent studies indicated that WNV uses a passive evasion strategy, possibly by masking or sequestering viral RNA from recognition by RLRs (Fredericksen BL., *et al*., 2004). In support of this finding, WNV replication complexes, which are a source of viral dsRNA, accumulate in ER membrane vesicles (Gillespie LK., et al., 2010), thus providing a possible mechanism for WNV to avoid sensing by RLRs. The exact mechanism by which WNV evades PRR detection is not understood yet. Recent studies revealed that WNV does not actively inhibit the RIG-I pathway leading to the production of IFN α/β. Rather, WNV strains appear to delay activation of PRR signalling long enough to give the virus a replicative advantage within the cells during the early stages of infection (Fredericksen BL., *et al*., 2006). Virus replication during this window period supports an accumulation of viral proteins that exert effects on IFN α/β actions (Keller BC., *et al*., 2007). This evasion strategy allows WNV to establish infection and synthesize viral factors that subsequently block other downstream innate immune signalling pathways, namely TLR3 and type I IFN signalling (Keller BC., *et al*., 2006; Wilson JR., *et al*., 2008).

WNV has also been shown to interfere with the type I IFN response. Several groups have

recently reported that Lineage 1 WNV strains, but not lineage 2 WNV-MAD (strain Madagascar-AgMg798), is capable of blocking the phosphorylation of TYK2 to inhibit type I IFN-induced phosphorylation and activation of STAT1 and STAT2. This inhibition correlates with the onset of viral protein synthesis within infected cells. The exact viral protein that inhibits TYK2 has not yet been identified, but it has been proposed that the NS2A, NS2B3, NS4A and NS4B viral proteins each have inhibitory activity against IFN signalling (Keller BC., *et al*., 2007). In support of this study, the WNV non-structural proteins mediate the degradation of IFNAR1 through both lysosome- and proteasome-dependent pathways (Evans JD., *et al*., 2011). It is unclear whether the inhibition of TYK2 and the degradation of IFNAR1 are two separate events or are directly linked, and this warrants further investigation into the mechanism involving inhibition of type I IFN signalling by WNV. Other strategies by which WNV evades IFN signalling include redistribution of cellular cholesterol to sites of viral replication complexes, thus altering membrane-associated signalling in favour of viral replication (Mackenzie JM., *et al*., 2007), and synthesis of the viral non-coding subgenomic RNA, which has been identified as an IFN antagonist (Schuessler A., *et al*., 2012). The viral proteins that are responsible for inhibiting these IFN signalling components have not been identified. However, several WNV proteins have been implicated in the antagonism of the type I IFN signalling cascade. Both structural and non-structural proteins of lineage 1 WNV suppress type I IFN signalling (Evans JD., 2007; Suthar MS., *et al*., 2012a): NS2A (Liu WJ., *et al*., 2005; Liu WJ., *et al*., 2006), NS2B (Liu WJ., *et al*., 2005), NS3 (Liu WJ., *et al*., 2005), NS4A (Liu WJ., *et al*., 2005), NS4B (Liu WJ., *et al*., 2005; Munoz-Jordan JL., *et al*., 2005; Evans JD., 2007) and NS5 (Laurent-Rolle M., *et al*., 2010) all suppress type I IFN production and/or signalling, albeit through varied mechanisms.

WNV actively evades the antiviral effects of IFIT1, an ISG protein that is highly induced following viral infection. IFIT1 exerts its antiviral function through multiple mechanisms, including interacting with eukaryotic translation initiation factor 3 (eIF3) to inhibit translation, inhibiting translation of viral RNAs lacking 2′-*O*-methylation, sequestering viral RNAs that contain a 5′ triphosphate and directly interacting with viral proteins to inhibit their function (Fensterl V. and Sen GC., 2011). A WNV mutant lacking 2′-*O*-methyltransferase activity (WNV-NS5-E218A) is inhibited in cells expressing IFIT genes, revealing that WNV uses 2′-*O*-methylation as a mechanism to evade the antiviral effects of IFIT1; indeed, wild-type WNV strains show no growth advantage in *Ifit1*−/− cells or mice compared with in wild-type cells or mice (Szretter KJ., *et al*., 2012).

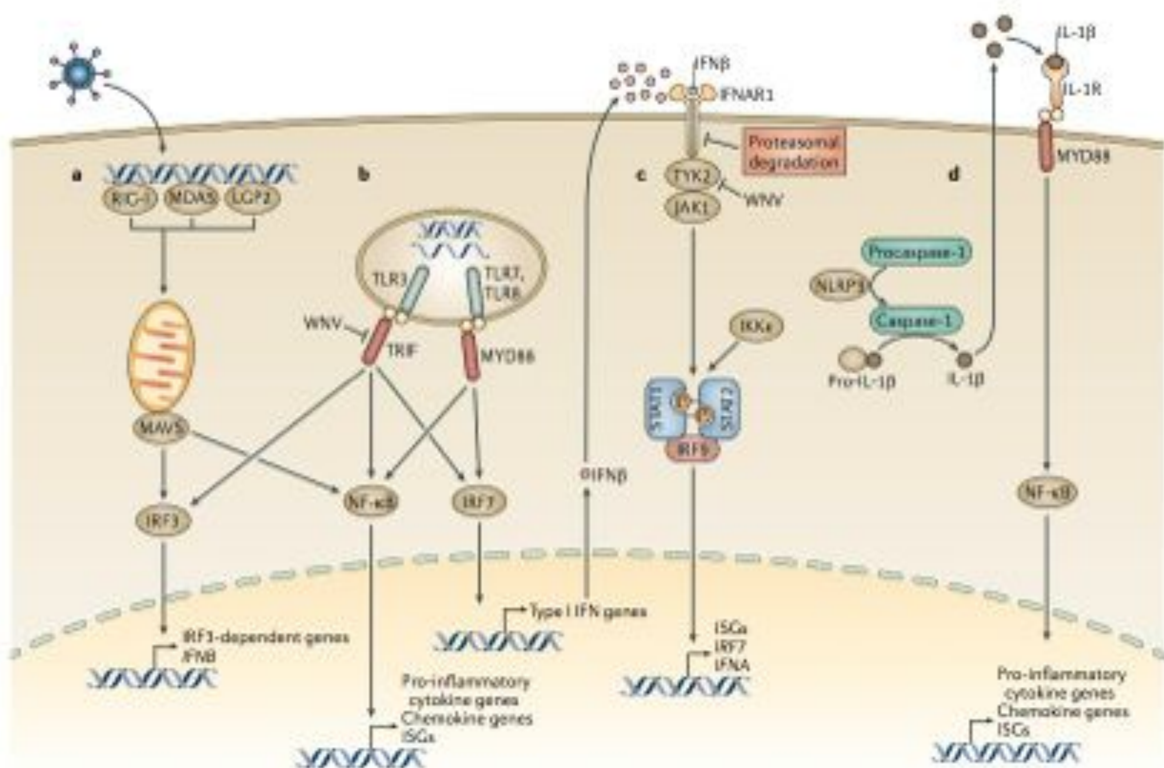


Figure 16. Cell-intrinsic innate immune response to West Nile virus infection. Innate immune responses to, and immune evasion by, West Nile virus (WNV). **a** | The recognition and binding of non-self RNA ligands by the RIG-I-like receptors (RLRs) retinoic acid-inducible gene I protein (RIG-I), melanoma differentiation antigen 5 (MDA5) and LGP2 induces the activation of these receptors through ATP hydrolysis, followed by a conformational change in the RLR, oligomerization of the RLR and the subsequent RLR subcellular redistribution to membranes. Following this redistribution, RIG-I and MDA5 bind to the adaptor protein MAVS (mitochondrial antiviral signalling). Formation of the MAVS signalling synapse drives activation and nuclear translocation of the latent transcription factors IFN regulatory factor 3 (IRF3) and nuclear factor-κB (NF-κB), which induce expression of their target genes, including interferon β (IFNβ). WNV is thought to use a passive evasion strategy, possibly by masking or sequestering viral RNA (not shown). WNV proteins might also inhibit type I IFN production. **b** | Following binding of non-self RNA ligands within the endosomal compartment, Toll-like receptor 3 (TLR3), TLR7 and TLR8 signal through their adaptor proteins — TRIF (TIR domain-containing adaptor inducing IFNβ) and MYD88 (myeloid differentiation 88) — to promote NF-κB-, IRF3- and IRF7-dependent gene expression. WNV inhibits TLR3 signalling. WNV proteins might also inhibit type I IFN production. **c** | IFNβ is secreted from the infected cell and binds in an autocrine and paracrine manner to IFNα and IFNβ receptor complex 1 (IFNAR1), leading to phosphorylation of the associated tyrosine kinase 2 (TYK2) and Janus kinase 1 (JAK1) and assembly of the trimeric IFN-stimulated gene factor 3 (ISGF3) complex containing signal transducer and activator of transcription 1 (STAT1)–STAT2 heterodimers and IRF9. This complex translocates to the nucleus and amplifies cell-intrinsic immunity by promoting the expression of IFN-stimulated genes (ISGs) and various IFNα subtypes. The IFN-induced phosphorylation of STAT1 by inhibitor of NF-κB kinase (IKKε) further enhances expression of a subset of ISGs. WNV blocks the activity of TYK2 and induces the degradation of IFNAR1. **d** | The NLRP3 (NOD-, LRR- and pyrin domain-containing 3) inflammasome is activated in response to WNV infection, resulting in cleavage of pro-interleukin-1β (pro-IL-1β) into the mature IL-1β form. IL-1β is secreted from the cell and binds to IL-1 receptor (IL-1R) to trigger activation of NF-κB and transcription of NF-κB-dependent genes, as well as to regulate ISG expression (Suthar MS., *et al*., 2013).

AIM

West Nile virus (WNV) is an emerging neurotropic, arthropod-borne flavivirus that is maintained in nature in an enzootic transmission cycle between mosquitoes and birds, although the virus also infects and causes disease in other vertebrates. Indeed, WNV can occasionally infect humans through mosquito bites, and human-to-human transmission *via* infected blood or organ donation has also reported. In 70%-80% of human cases, WNV infection remains asymptomatic. 20-40% of human cases may develop a mild flu-like illness, and less than 1% of clinical cases progress to severe neuroinvasive disease such as encephalitis, meningitis or acute flaccid paralysis, all of which may be fatal or accompanied by long term neurological sequelae.

Based on nucleotide sequence data, WNV strains are phylogenetically classified into at least five genetic lineages, but only lineages 1 and 2, which have a nucleotide sequence identity of approximately 75%, have been associated with major epidemics. WNV strains belonging to lineage 1 distributed throughout the world and have been associated with outbreaks of encephalitis and meningitis in Africa, Europe, the Middle East, India, and North America. WNV strains belonging to lineage 2 were initially confined to the Africa subcontinent and the island countries of Madagascar and Cyprus and were considered to be less neurovirulent than lineage 1 strains up to their association to meningo-encephalitis outbreaks in Greece in 2010. In Italy, the first human cases of WNV-associated fever and/or neurological disorders were reported in 2008 in Emilia Romagna and, since then, WNV circulation was thereafter reported in other Italian regions (Veneto, Lombardia), with occurence of several human cases. On basis of phylogenetic analysis, the WNV strains that caused Italian outbreaks in 2008-2009, belonged to lineage 1 strains. Although the WNV strains are highly genetically conserved, stochastic mutations in their genome may lead to the emergence of new strains, as was observed in Italy in 2011 with the identification of new lineage 1 strains that caused human cases of WNV neuroinvasive infections. Furthermore, the first human cases of WNV fever caused by a lineage 2 strain, related to Hungarian-Greek strains, were also reported in the Marche and Sardinia regions.

A large variety of WNV strains from America have been described in terms of genetic, phenotypic and pathogenic properties, but there are few experimental studies on biological properties of WNV strains that are circulating in Europe/Italy and on WNV lineage 2 pathogenic strains.

The aim of this study is investigate the growth kinetics of five strains of WNV: four WNV strains belonging to lineage 1, including one from North America and three isolated in Italy and one WNV strain belonging to lineage 2 from Italy. The phenotypic properties of these WNV strains were examined to observe if there were differences in replication in two cell types: Vero and human astrocytes cells. Moreover, in order to understand if IFN may influence the growth kinetics of divergent lineage 1 and 2 viruses, we compared the influence of IFN-α action on their capacity of replication in Vero and human astrocytes cells.

MATERIALS AND METHODS

1. CELLS AND VIRUSES

Vero and 1321N1 cell lines were maintained at 37°C in a humidified atmosphere containing 5% CO_2. Vero cells (African green monkey kidney epithelial cells) were grown in Minimal Essential Medium (MEM) supplemented with 10% Fetal Bovine Serum (FBS), L-glutamine and penicilline-streptomycin (MEM complete). 1321N1 (human astrocytoma) cell line was propagated in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), L-glutamine and penicilline-streptomycin (DMEM complete).
Five strains of WNV were used for this study:

- Four strains belong to lineage 1, clade 1a: WNV-NY99 (GeneBank accesion no. DQ211652), WNV-TOS (GeneBank accesion no. HM991273), WNV-AN 1 (GeneBank accesion no. JN858069), and WNV-DON B;
- One strain belongs to lineage 2: WNV-CIP.

All virus strains were isolated from human plasma inoculated on Vero cells. Virus isolates were propagated for one passage on Vero or 1321N1 cells. Viral stocks were aliquoted and stored at -80C°.

2. VIRUS TITRATIONS

Viral titres were quantified as $TCID_{50}$ endpoint titers ($TCID_{50}$/mL) calculated by the method of Spearman-Karber. Briefly, the samples were titrated on 96-well flat-bottom tissue culture plate where Vero cells were seeded into all wells. Each sample was serially diluited in a 10-fold series and it was titrated in triplicate. The 96-well titration plates were incubated at 37°C in a humidified 5% CO_2 incubator and presence of cytopathic effect was evaluated after 3-5 days.

3. GROWTH CURVES

For analysis of virus growth kinetics, 1 day after seeding 1 x 10^6 per well of Vero or 1321N1 cells in a 6-well plates, cells were infected with WNV-NY99, WNV-TOS, WNV-AN 1, WNV-DON B or WNV-CIP at MOI of 1 and 0,01 in a volume of 4 ml of MEM or DMEM, respectively. Cells were infected for 1.5 hours, inoculum was removed and cells were washed two times with fresh medium and 4 ml of appropriate medium, MEM or DMEM, was added. At 2, 4, 6, 8, 10, 12, 18, 24, 48, 72 and 80 hours after infection, cell culture supernatants were harvested and stored at -80°C. The supernatants were titrated in 96-well

plates by $TCID_{50}$ method on Vero cells as described above. Each growth curve experiment was performed in triplicate, and each $TCID_{50}$ assay was undertaken in duplicate.

4. VIRUS STRAINS SUSCEPTIBILITY TO INTERFERON-α (IFN-α) ACTION

24 hours after seeding 1 x 10^6 per well of Vero or 1321N1 cells in a 6-well plates, cells were incubated for 24 hours in presence (pre-treatment) and in absence of 100 U/ml of IFN-α-2b human (H6166-10UG SIGMA). After 24 hours, in presence and in absence of IFN-α-2b, Vero and 1321N1 cells were infected with WNV-NY99, WNV-TOS, WNV-AN 1, WNV-DON B or WNV-CIP at MOI of 1 in a volume of 4 ml of MEM or DMEM. Cells were infected for 1.5 hours, inoculum was removed and cells were washed two times with fresh medium and 4 ml of appropriate medium, MEM or DMEM, with and without 100U/ml of IFN-α-2b was added. At 0, 24 and 48 hours post-infection, the cell culture supernatants were harvested and stored at -80°C. The supernatants were titrated in 96-well plates by $TCID_{50}$ method on Vero cells. Each experiment was performed in triplicate, and viral titre, at each time point, was evaluated in duplicate.

RESULTS

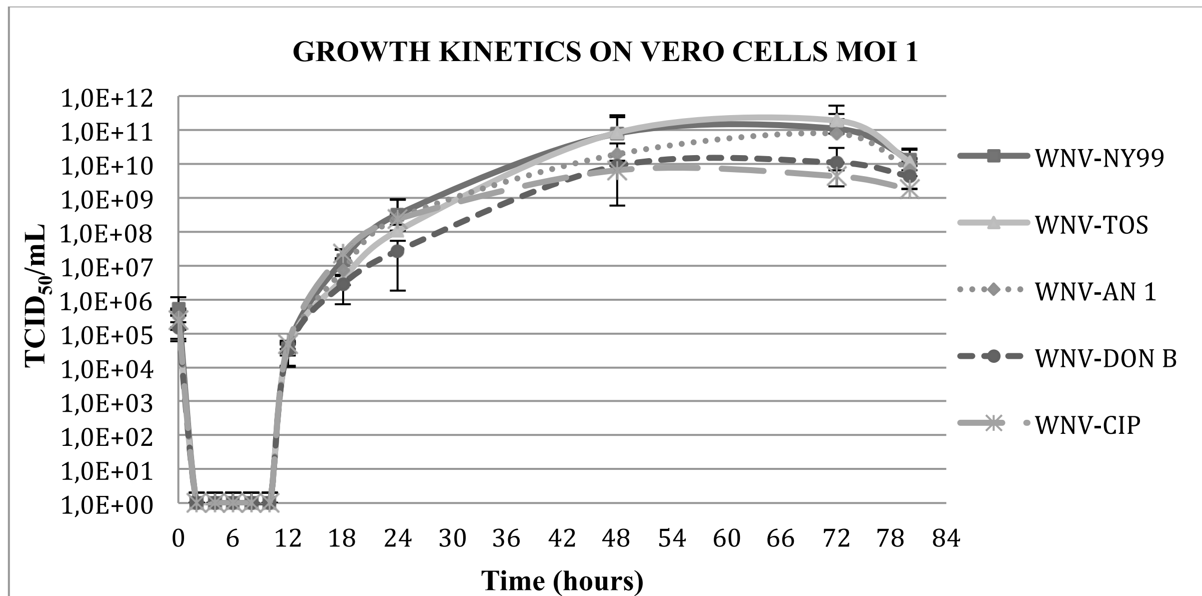
In the present study, we compared the growth properties of five WNV strains in two cell lines: Vero (African green monkey kidney epithelial cells) and 1321N1 (human astrocytoma). Four WNV strains belong to lineage 1: WNV-NY99 from North America, WNV-TOS, WNV-AN 1 and WNV-DON B from Italy. One WNV strain isolated in Italy, WNV-CIP, belongs to lineage 2. In order to understand the influence of IFN-α on viral growth, we compared the influence of IFN-α on growth kinetics of different lineage 1 and 2 WNV strains in Vero and 1321N1 cells.

1. GROWTH PROPERTIES OF WNV STRAINS ON VERO CELLS

To investigate possible differences on the kinetics of replication of both lineages 1 and 2 WNV strains circulating in Italy, we compared growth kinetics of viruses on Vero cells. Cells were infected at a multiplicity of infection (MOI) of 1 and 0.01 with WNV-NY99, WNV-TOS, WNV-AN-1, WNV-DON B or WNV-CIP. At 2, 4, 6, 8, 10, 12, 18, 24, 48, 72 and 80 hours after infection, the cell culture supernatants were harvested and titrated by $TCID_{50}$ method on Vero cells. For each MOI and viral strain tested, growth curve experiments were performed in triplicate. Viral titre, at each time point, was evaluated in duplicate and the results are summarized in Fig. 17.

Viral growth analyses in Vero cells revealed that all WNV strains examined display a similar growth kinetic. Extracellular infectious particles were detectable starting from 12 hours post-infection for all WNV strains for each MOIs tested. At 18 hours post-infection the titre reached was approximately 10^6 $TCID_{50}$/ml and 10^4 $TCID_{50}$/ml for all WNV strains at MOI of 1 and 0.01, respectively. At 24 hours post-infection the titres were in the range 10^7-10^8 $TCID_{50}$/ml at MOI of 1 and 10^5-10^6 $TCID_{50}$/ml at MOI of 0.01. When cells were infected at MOI of 1, at 48 hours post-infection the peak of infectious virus production was reached with a titre of approximately 10^{11} $TCID_{50}$/ml for WNV-NY99, WNV-TOS and WNV-AN 1, and about 10^{10} $TCID_{50}$/ml for WNV-DON B and WNV-CIP and at 72 and 80 hours post-infection viral titres slowly decreased and the *plateau* was reached with titres ranging from 10^{10}-10^{11} $TCID_{50}$/ml. When cells were infected at MOI of 0.01, the peak of infectious virus production was reached at 72 hours post-infection with a titre of about 10^9 $TCID_{50}$/ml for all WNV strains tested and the *plateau* was reached at 80 hours post-infection with viral titles of $10^9$$TCID_{50}$/ml, the same title reached at 72 hours post-infection.

A



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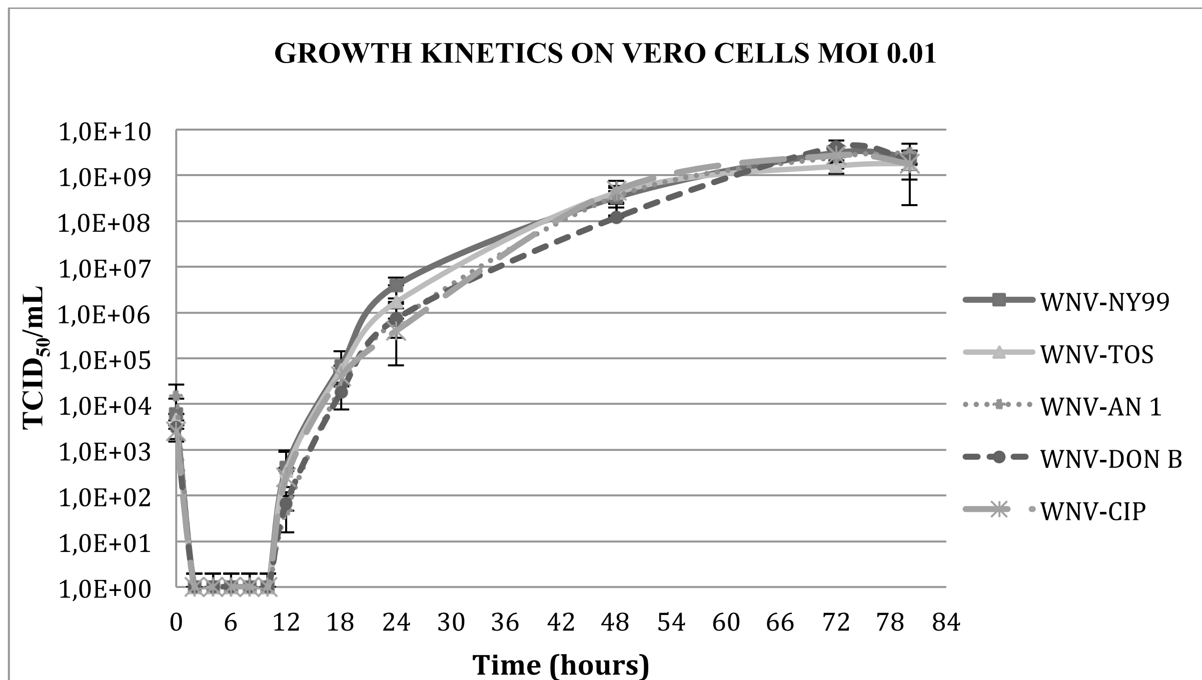


Figure 17. Growth kinetics of WNV strains on Vero cells. Vero cells were seeded in six-well dishes and infected with WNV-NY99, WNV-TOS, WNV-AN 1, WNV-DON B, or WNV-CIP at multiplicity of infection (MOI) of 1 (A) and 0.01 (B). At indicated times 2, 4, 6, 8, 10, 12, 18, 24, 48, 72 and 80 hours post-infection, cell culture supernatants were harvested and the titres of the viruses were determined by $TCID_{50}$/ml assay on Vero cells. Each growth curve experiment was performed in triplicate, and each $TCID_{50}$ assay was undertaken in duplicate. Results are expressed as the mean $TCID_{50}$/ml ± standard deviation.

2. GROWTH PROPERTIES OF WNV STRAINS ON 1321N1 CELLS

Astrocytes are star-shaped and constitute the most abundant cell type found in the CNS. They have a number of important functions in the brain homeostasis, including maintenance of functional integrity in the BBB, regulation of neuronal blood flow, modulation of neuronal health and activity through the uptake of excess neurotransmitters and secretion of nutrients. Moreover, astrocytes can produce acute-phase proteins and some pro-inflammatory cytokines and play a crucial role in controlling leukocyte influx. Here, we compared the ability of four WNV strains belonging to lineage 1 (WNV-NY99, WNV-TOS, WNV-AN 1 and WNV-DON B) and one WNV strain belonging to lineage 2 (WNV-CIP) to replicate in 1321N1 cells (human astrocytoma cell line).

1321N1 cells were infected at a multiplicity of infection (MOI) of 1 and 0.01 with WNV-NY99, WNV-TOS, WNV-AN-1, WNV-DON B or WNV-CIP. At 2, 4, 6, 8, 10, 12, 18, 24, 48, 72 and 80 hours after infection, the cell culture supernatants were harvested and titrated by $TCID_{50}$ method on Vero cells. For each MOI and viral strain tested, growth curve experiments were performed in triplicate. Viral titre, at each time point, was evaluated in duplicate and results are summarized in Fig. 18.

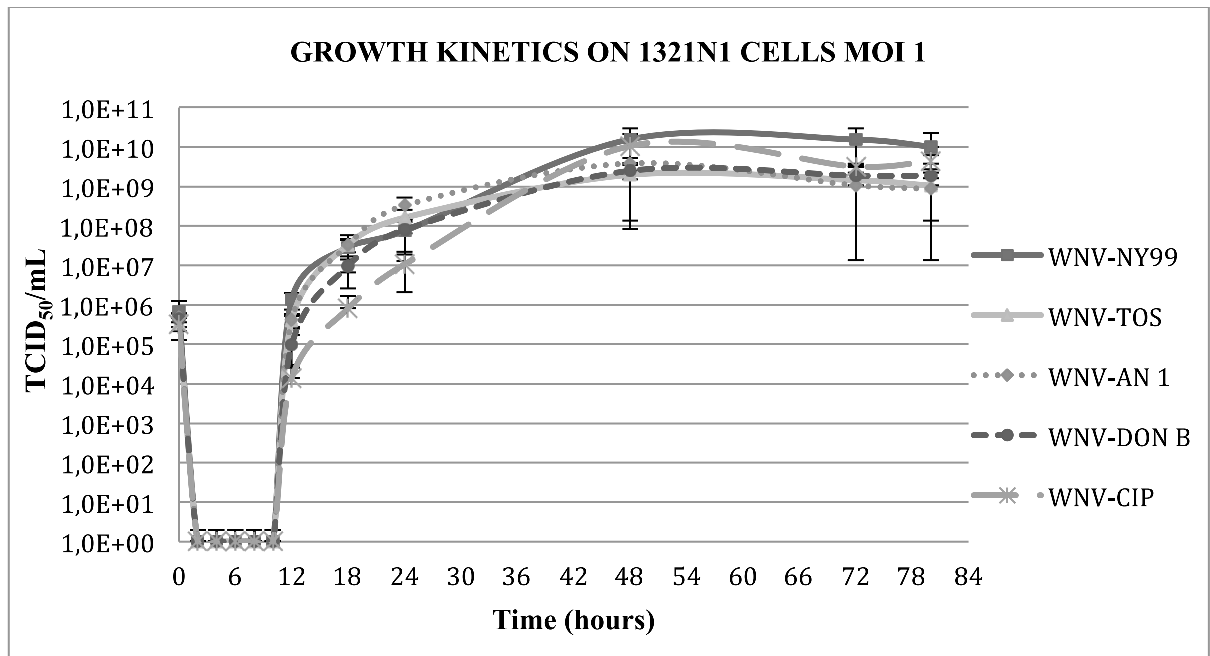
Viral growth analyses in 1321N1 cells revealed that all WNV strains display a similar growth kinetic although the kinetic of replication of WNV-CIP, lineage 2 WNV strain, was slightly delayed at early times post-infection compared to those lineage 1 WNV strains. When cells were infected at MOI of 1, extracellular infectious particles were detectable starting from 12 hours post-infection for all WNV strains, but the titres were approximately 10^6 $TCID_{50}$/ml for lineage 1 WNV strains and 10^4 $TCID_{50}$/ml for lineage 2 WNV strain. By comparison, at 12, 18 and 24 hours post-infection, growth kinetic of WNV-CIP was delayed and the titers were decreased of approximately 10-100 fold, compared to those of lineage 1 WNV strains at the same time points. However, for all WNV strains tested, the peak of infectious virus production was reached at 48 hours post-infection and the titres were in the range 10^9-10^{10} $TCID_{50}$/ml. Then at 72 and 80 hours post-infection the *plateau* was reached with titres ranging from 10^9-10^{10} $TCID_{50}$/ml.

When cells were infected at MOI of 0.01, extracellular infectious particles were detectable starting from 12 hours post-infection for WNV lineage 1 strains and from 18 hours post-infection for WNV lineage 2 strain, WNV-CIP. In fact, at early times post-infection, growth kinetic of WNV-CIP was delayed and the titers were decreased of approximately 10-100 fold, compared to those of lineage 1 WNV strains. However, for all WNV strains tested, at 48 hours post-infection the titre reached was approximately 10^7 $TCID_{50}$/ml and the peak of

infectious virus production was reached at 72 hours post-infection with ranging from 10^8-10^9 $TCID_{50}$/ml. Then at 80 hours post-infection the *plateau* was reached, the viral titres slowly decrease with titres ranging from 10^7-10^9 $TCID_{50}$/ml.

Despite the slight growth delay of WNV-CIP at early times post-infection, 1321N1 cells were highly permissive for all WNV strains examined.

A



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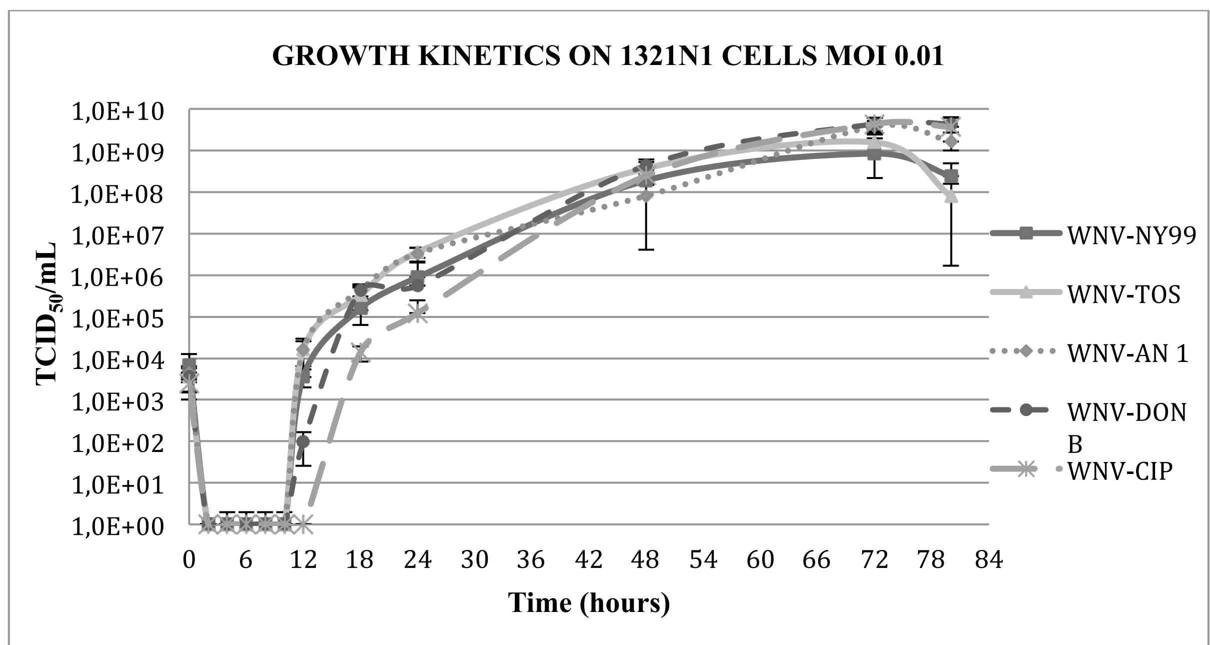

Figure 18. Growth kinetics of WNV strains on 1321N1 cells. 1321N1 cells were seeded in six-well dishes and infected with WNV-NY99, WNV-TOS, WNV-AN 1, WNV-DON B, or WNV-CIP at multiplicity of infection (MOI) of 1 (A) and 0.01 (B). At indicated times 2, 4, 6, 8, 10, 12, 18, 24, 48, 72 and 80 hours post-infection, cell culture supernatants were harvested and the titres of the viruses were determined by $TCID_{50}$/ml assay on Vero cells. Each growth curve experiment was performed in triplicate, and each $TCID_{50}$ assay was undertaken in duplicate. Results are expressed as the mean $TCID_{50}$/ml ± standard deviation.

3. WNV STRAINS SUSCEPTIBILITY TO INTERFERON-α (IFN-α) ACTION ON VERO CELLS

After comparing growth properties of WNV strains on Vero cells, we evaluated if different strains belonging to lineage 1 and 2 exhibited differential susceptibility to the antiviral effects of interferon-α (IFN-α). Vero cells were left treated or untreated with 100 U/mL of IFN-α for 24 hours to induce an intracellular antiviral state (IFN-pre-treatment) before infection. Then cells were infected at a multiplicity of infection (MOI) of 1 with WNV-NY99, WNV-TOS, WNV-AN-1, WNV-DON B or WNV-CIP in the presence or absence of IFN-α and maintained under these conditions for the duration of the experiment. At 0, 24 and 48 hours after infection, the supernatants were harvested and titrated by $TCID_{50}$ method on Vero cells. For each viral strain tested, growth curve experiments were performed in triplicate, and viral titre at each time point was evaluated in duplicate. The results are summarized in Fig. 19.

Viral growth analyses in Vero cells treated or untreated with 100 U/mL of IFN-α revealed that all WNV strains have similar susceptibility to IFN-α action. When cells were infected at MOI of 1 in absence of IFN-α, the titres for all WNV strains tested were in the range 10^7-10^8 $TCID_{50}$/ml and 10^9-10^{10} $TCID_{50}$/ml at 24 and 48 hours post-infection, respectively. When cells were infected at MOI of 1, in presence of 100 U/ml of IFN-α, at 24 and 48 hours post-infection the tires were in the range 10^4-10^5 $TCID_{50}$/ml and 10^5-10^6 $TCID_{50}$/ml, respectively. Thus, treatment with 100 U/ml of IFN-α2b greatly reduced approximately of 1.000-fold and 10.000-fold infectious particle production in all WNV strains examined at 24 and 48 hours post-infection, respectively. These results demonstrate that all WNV strains tested are highly susceptible to antiviral process induced by IFN-α action and there are no differences in susceptibility among different WNV strains.

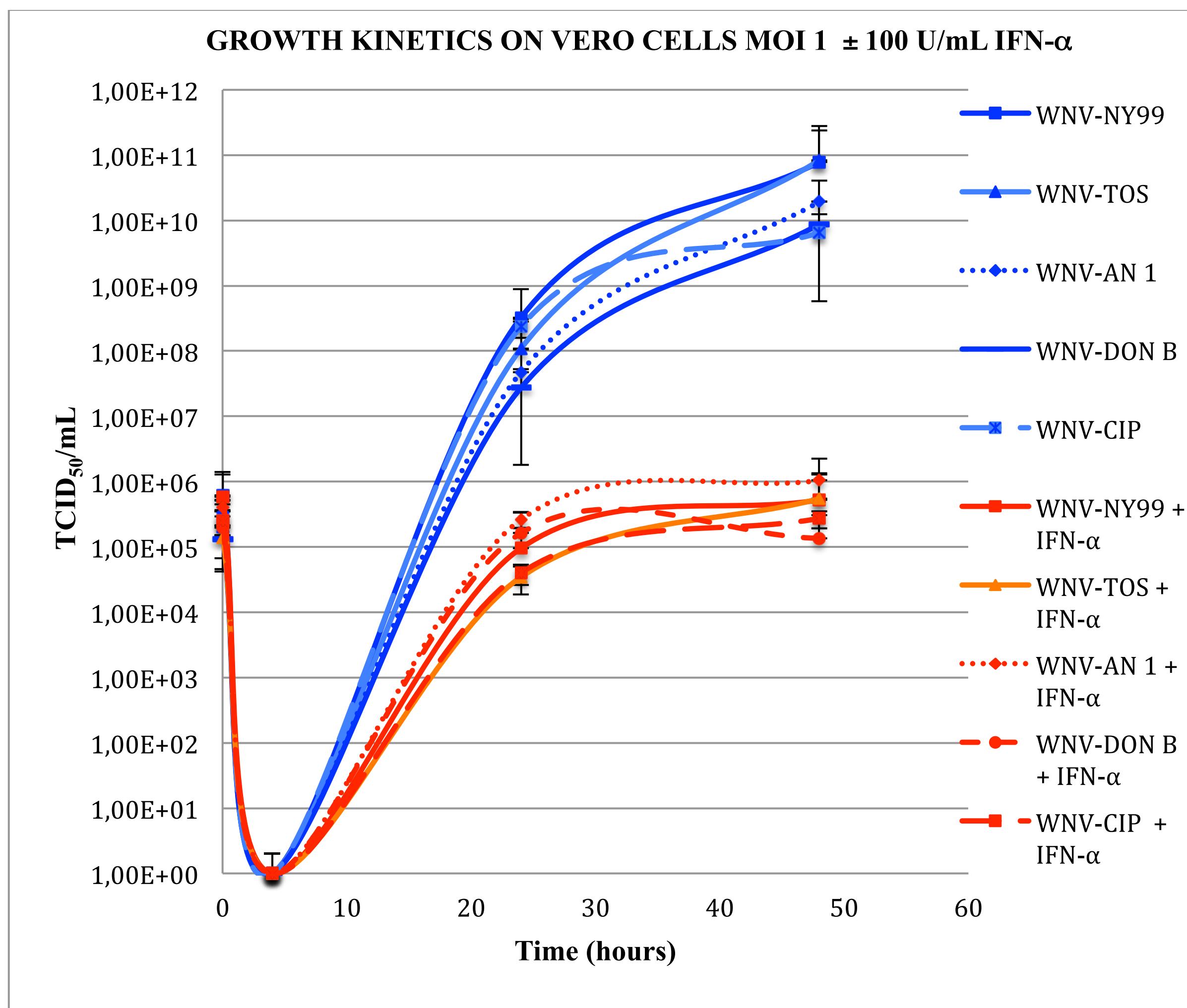

Figure 19. Growth kinetics of WNV strains on Vero cells and susceptibility to IFN-α action. Vero cells were pre-treated with 0 (bleu lines) or 100 U/mL IFN-α (red lines) for 24h prior to WNV infection. Cells were then infected (1 MOI) with WNV-NY99, WNV-TOS, WNV-AN 1, WNV-DON B, or WNV-CIP for 1,5 hours. Following infection, medium containing 0 (blue lines) or 100 U/mL IFN-α (red lines) was added to cells. At indicated times post-infection, cell culture supernatants were harvested and the titres of the viruses were determined by $TCID_{50}$/ml assay on Vero cells. Each growth curve experiment was performed in triplicate, and each $TCID_{50}$ assay was undertaken in duplicate. Results are expressed as the mean $TCID_{50}$/ml ± standard deviation.

4. WNV STRAINS SUSCEPTIBILITY TO INTERFERON-α (IFN-α) ACTION ON 1321N1 CELLS

After comparing growth properties of WNV strains on 1321N1 cells and their susceptibility to interferon-α action on Vero cells, we evaluated if different strains belonging to lineage 1 and 2 WNV exhibited differential susceptibility to the antiviral effects of interferon-α (IFN-α) on 1321N1 cells. Cells were treated or untreated with 100 U/mL of IFN-α for 24 hours to induce an intracellular antiviral state (IFN-pre-treatment) before infection and then were infected at a multiplicity of infection (MOI) of 1 with WNV-NY99, WNV-TOS, WNV-AN-1, WNV-DON B or WNV-CIP in the presence or absence of IFN-α. At 0, 24 and 48 hours after infection, the supernatants were harvested and titrated by $TCID_{50}$ method on Vero cells. For each viral strain tested, growth curve experiments were performed in triplicate, and viral titre at each time point was evaluated in duplicate. The results are summarized in Fig. 20.

Viral growth analyses in 1321N1 cells treated or untreated with 100 U/mL of IFN-α revealed that: when cells were infected at MOI of 1 in absence of IFN-α, the growth kinetics were similar for all WNV strains tested, with a slight delay in infectious particle production at 24 hours post-infection for WNV-CIP compared to that WNV lineage 1 strains and the titres for all WNV strains tested were in the range 10^6-10^8 $TCID_{50}$/ml and 10^9-10^{10} $TCID_{50}$/ml at 24 and 48 hours post-infection, respectively. When cells were infected at MOI of 1, in presence of 100 U/ml of IFN-α, at 24 hours post-infection the tires for WNV-NY99, WNV-TOS and WNV-AN-1 were in the range 10^1-10^2 $TCID_{50}$/ml and for WNV-DON B and WNV-CIP were in the range 10^3-10^4 $TCID_{50}$/ml. At 48 hours post-infection the titres reached were approximately of 10^1 $TCID_{50}$/ml for WNV-NY99, WNV-TOS and WNV-AN-1, and in the range 10^3-10^4 $TCID_{50}$/ml for WNV-DON B and WNV-CIP. Thus, treatment with 100 U/ml of IFN-α resulting in an approximate 6-log and 8-log decrease in infectious particle production in WNV-NY99, WNV-TOS and WNV-AN 1 at 24 and 48 hours post-infection, respectively. For WNV-DON B and WNV-CIP titres were reduced of approximately 4-log and 6-log at 24 and 48 hours post-infection, respectively. These results demonstrate that, although WNV-DON B and WNV-CIP seem more resistances than others WNV strains examined, all WNV strains are highly susceptible to antiviral process induced by IFN-α action in 1321N1 cells, resulting a more reduction in infectious particle production than that observed in Vero cells. The different response of WNV-DON B and WNV-CIP to IFN-α action may due to major resistance to the antiviral effects of IFN-α or develop different mechanism(s) to counteract the antiviral process induced by IFN-α action.

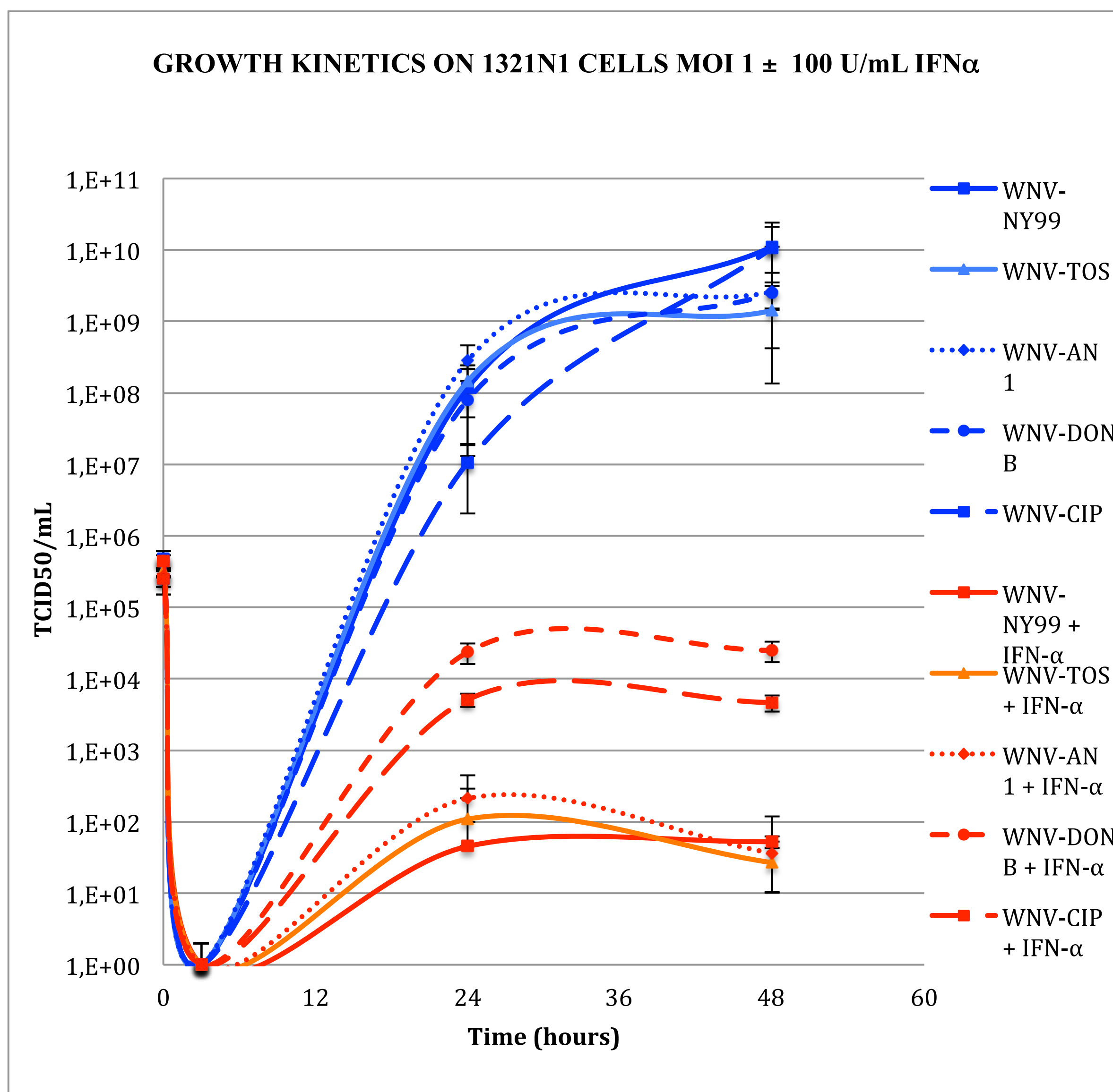

Figure 20. Growth kinetics of WNV strains on 1321N1 cells and susceptibility to IFN-α action. 1321N1 cells were pre-treated with 0 (bleu lines) or 100 U/mL IFN-α (red lines) for 24h prior to WNV infection. Cells were then infected (1 MOI) with WNV-NY99, WNV-TOS, WNV-AN 1, WNV-DON B, or WNV-CIP for 1,5 hours. Following infection, medium containing 0 (bleu lines) or 100 U/mL IFN-α (red lines) was added to cells. At indicated times 0, 24, and 48 hours post-infection, cell culture supernatants were harvested and the titres of the viruses were determined by $TCID_{50}$/ml assay on Vero cells. Each growth curve experiment was performed in triplicate, and each $TCID_{50}$ assay was undertaken in duplicate. Results are expressed as the mean $TCID_{50}$/ml ± standard deviation.

DISCUSSION

This study examined the phenotypic and interferon-α (IFN-α)-regulatory properties of different WNV lineage 1 and 2 strains that are circulating in Europe/Italy in two cell lines: Vero and 1321N1.

West Nile virus (WNV) is a neurotropic, arthropod-borne flavivirus that is maintained in nature in a mosquito-bird cycle, whereas humans and horses are considered incidental or "dead-end" hosts. In humans, infection with WNV remains asymptomatic in most of cases, about 80%. However, in 20% of cases, WNV causes a mild flu-like illness (West Nile Fever, WNF) and less than 1% of clinical cases progress to severe neuroinvasive disease (West Nile Neuroinvasive Disease, WNND). Phylogenetic analyses have described two major distinct lineages of WNV strains: lineage 1, the largest and most widespread, is found in Africa, Asia, the Middle East, Europe, Australia and Americas; and lineage 2 isolates are found primarily in sub-Saharan Africa and Madagascar with recent introductions into Europe (Greece, Hungary and Italy) and Russia (McMullen AR., et al., 2013). The first human cases of WNV infection in Italy were reported in 2008 in Emilia-Romagna region. In the following years, WNV circulation was reported in other Italian regions associated with outbreaks of meningo-encephalitis caused by different viral strains belonging to distinct lineages. The rapid spread of highly pathogenic strains of WNV into naïve populations in Europe, Israel, and the USA has resulted in both increased number of human cases and severity of disease compared to previous outbreaks. This may suggest that epidemic forms of virus may have undergone genetic variations that may affect WNV virulence and/or may encode mechanisms to counteract the host immunity. The innate immune system acts as the first line of defense against invading viral pathogens and it is critically important for controlling infection. WNV infection triggers a delayed host response including the interference with type I interferon (IFN) response. IFNs are a family of immuno-modulatory cytokines that are produced in response to virus infection and serve as integral signal initiators of host intracellular defenses.

While a large variety of WNV strains from America have been described in terms of genetic, phenotypic and pathogenic properties, there are few data about biological characteristics of European/Italian WNV isolates highlight the importance to achieve a better knowledge on this emerging viral infection. Initially, we examined the biological properties of four WNV lineage 1 strains (WNV-NY99, WNV-TOS, WNV-AN 1 and WNV-DON B) and one WNV lineage 2 strain (WNV-CIP) circulating in Europe/Italy in Vero cells (African green monkey kidney epithelial cells). We found that growth analyses of WNV lineage 1 strains replication

in Vero cells display growth kinetics and peak infectious virus production nearly identical to WNV strains well-characterized lineage 1 strains isolated in America. These finding are in accord with Keller BC., *et al.*, 2006, which found that TX02, a WNV lineage 1 strain isolated from the brain of an infected grackle in Texas in 2002 and sharing genotypic traits with other American lineage 1 WNV strains, exhibited similar phenotypic growth to that of other WNV lineage 1 strain: WNV-NY3356, a strain 99.9% identical to the WNV-NY99. However, our data are in contrast with Keller BC., *et al.*, 2006 about results with WNV lineage 2 strain. In fact, while Keller BC., *et al.*, 2006 found that growth kinetics of WNV-MAD78, a nonpathogenic lineage 2 strain, was delayed and peak infectious virus production was decreased 10-fold relative to lineage 1 strains; we found that growth analyses of WNV-CIP replication in Vero cells exhibits a similar replication fitness and biological properties nearly identical to those of WNV lineage 1 strains. We hypothesized that the different phenotypic properties of WNV-CIP, compared to WNV-MAD78, may be due to their different pathogenicity: whereas WNV-MAD78 is an avirulent lineage 2 African isolate with no association with human disease, WNV-CIP is a pathogenic lineage 2 strain isolated from a patient with neuroinvasive disease.

Moreover, we investigated if there were differences in the virulence and neuroinvasion properties or specific-strain restriction at the blood-brain barrier (BBB) between WNV lineage 1 and 2 strains. Several studies have proposed that the severity of WNV infection in immunocompetent animals is unrelated to the virus lineage but is highly strain-specific and that a determining factor for neuropathogenicity dependents on the capacity of the strain to invade the CNS though breakdown of the BBB. Astrocytes and microglia are the principal cells within the CNS responsible for initiating, regulating, and maintaining neuroimmune response to viral infections. Although WNV is a neurotropic virus, its relative ability to replicate in astrocytes is unknown. Thus, we compared the ability of four WNV strains belonging to lineage 1 (WNV-NY99, WNV-TOS, WNV-AN 1 and WNV-DON B) and one WNV strain belonging to lineage 2 (WNV-CIP) to replicate in human astrocytoma 1321N1 cells. The results of the present study demonstrated that all WNV strains tested exhibited similar growth kinetics and peak infectious virus production in 1321N1 cells, although WNV-CIP had a slight delay at early times post-infection compared to those lineage 1 WNV strains. In addition, astrocytes supported productive WNV replication *in vitro*. Our results of growth properties with WNV lineage 1 strains are in accord with previous *in vitro* Cheeran MCJ., *et al.*, 2005 and Hussmann KL., *et al.*, 2013 studies which tested the growth properties of WNV lineage 1 strains, WNV-NY99 and WNV-NY3356 respectively, in

astrocytes where they found that astrocytes supported productive WNV lineage 1 strains infection. However, our findings are in contrast with Hussmann KL., *et al*., 2013 studies about growth properties of WNV lineage 2 strains. In fact, whereas we found that WNV-CIP shown similar growth kinetics in astrocytes compared to those of WNV lineage 1 strains, Hussmann KL., *et al*., 2013 observed that replication of WNV-MAD78, a WNV lineage 2 strain, in astrocytes was both delayed and reduced compared to that of WNV lineage 1 strain, WNV-NY3356. The hypothesis for these discordant results about WNV lineage 2 strains is due to different pathogenicity of lineage 2 strains used, probably due to evolution of this lineage. In fact, WNV-MAD78 used by Hussmann KL., *et al*., 2013 is an avirulent WNV lineage 2 strain isolated from an infected parrot in Madagascar in 1978, whereas in this study we used WNV-CIP, a WNV lineage 2 strain isolated from an infected patient with neuroinvasive disease in Italy in 2014. Until recently, viruses in lineage 2 were not thought to be of public health importance due to few outbreaks of diseases being associated with viruses in this lineage. However, recent epidemics of lineage 2 in Europe (Greece and Italy) and Russia have shown the increasing importance of this lineage. The McMuller AR., *et al*., 2013 study shows that lineage 2 has evolved over the past 300-400 years and appears to correlate with a change from mouse attenuated to virulent phenotype based on previous studies by their group. Moreover, this evolution mirrors that which is seen in lineage 1 isolates, which have also evolved to a virulent phenotype over the same period of time (McMuller AR., *et al*., 2013). Thus, WNV-CIP, associates with a recent outbreak in Italy, is a WNV lineage 2 strain with a virulent phenotype and it is possible that it has a transmission behavior characteristic more similar to pathogenic WNV lineage 1 strains than to that seen in avirulent WNV lineage 2 strains.

IFN-α plays an integral role in intracellular innate immunity as well as in the linkage of the innate immune response to cell-mediate defenses against virus infection. In order to replicate and spread, viruses direct processes to attenuate the initiation of IFN production and/or to antagonize the antiviral actions of IFN inside the host cell. The process by which members of the family *Flaviviridae* regulate host defence and IFN actions vary widely. In order, to understand if IFN may influence the growth properties of WNV lineage 1 and 2 strains, we compared the susceptibility to interferon-α (IFN-α) action of four WNV strains belonging to lineage 1 (WNV-NY99, WNV-TOS, WNV-AN 1 and WNV-DON B) and one WNV strain belonging to lineage 2 (WNV-CIP) in Vero and 1321N1 cells. Our results show that both WNV lineages 1 and 2 examined exhibit a similar susceptibility to IFN-α action in Vero cells resulting in a similar reduction of infectious particle production, suggesting that WNV

lineage 2 strain has the same susceptibility to IFN action compared to WNV lineage 1 strains. Our results are in accord with previous study of Keller BC., *et al*., 2006, in the same experimental conditions. In fact, Keller BC., *et al*., 2006, to analyze the differential responses WNV-MAD78, WNV lineage 2 strain, and WNV-TX02, WNV lineage 1 strain, to antiviral effect of IFN, treated cells with IFN founding a similar reduction in infectious particle production in both WNV-MAD78 and WNV-TX02. We then evaluated the influence of IFN on viral growth in 1321N1 cells. Our results shown that treatment with IFN in 1321N1 cells results in an greatly decrease in infectious particle production in both WNV lineages 1 and 2, however there is a more reduction in infectious particle production in WNV-NY99, WNV-TOS and WNV-AN 1 than in WNV-DON B and WNV-CIP. The hypotheses for this different response of WNV-DON B and WNV-CIP to IFN action may due to major resistance to the antiviral effects of IFN for some other undefinited mechanism(s) that may contribute to control and counteract the antiviral process induced by IFN action in 1321N1 cells. However, these results demonstrate that, although WNV-DON B and WNV-CIP seem more resistances to IFN action on 1321N1 cells than others WNV strains examined, all WNV strains are highly susceptible to antiviral process induced by antiviral IFN action in 1321N1 cells, resulting in a more reduction in infectious particle production than to that observed in Vero cells.

While recent works with WNV has focused primarily on lineage 1 and 2 isolates, particularly in America (Cheeran MCJ., *et al*., 2005; Keller BC., *et al*., 2006; Hussmann KL., *et al*., 2013), there are few experimental studies on biological properties of WNV strains isolated in Europe/Italy and on recent WNV lineage 2 pathogenic strains. This is the first study that examined the phenotypic properties of WNV strains circulating in Europe/Italy including a WNV lineage 2 strain and their susceptibility to IFN action. This study contributes to augment our knowledge about WNV strains that are circulating in Europe/Italy on their pathogenetic potential in terms of tropism for different cell types and susceptibility to antiviral action of IFN. In addition, it provides us new knowledge about phenotypic properties of WNV lineage 2 associated with outbreaks of meningoencephalithis disease. A better understanding of how European/Italian WNV isolates are evolving can provide insights into the future evolution of WNV strains, and allow us to better predict how these isolates will lead to future outbreaks and epidemics. Moreover, an augmented knowledge on the pathogenesis of this neuroinvasive infection and on virus-host interactions, including how WNV control the IFN system, can facilitate the development of novel therapeutic approaches.

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