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REVIEW

West Nile virus: A re-emerging pathogen revisited

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Abstract

West Nile virus (WNV), a flavivirus of the *Flaviviridae* family, is maintained in nature in an enzootic transmission cycle between avian hosts and ornithophilic mosquito vectors, although the virus occasionally infects other vertebrates. WNV causes sporadic disease outbreaks in horses and humans, which may result in febrile illness, meningitis, encephalitis and flaccid paralysis. Until recently, its medical and veterinary health concern was relatively low; however, the number, frequency and severity of outbreaks with neurological consequences in humans and horses have lately increased in Europe and the Mediterranean basin. Since its introduction in the Americas, the virus spread across the continent with worrisome consequences in bird mortality and a considerable number of outbreaks among humans and horses, which have resulted in the largest epidemics of neuroinvasive WNV disease ever documented. Surprisingly, its incidence in human and animal health is very different in Central and South America, and the reasons for it are not yet understood. Even though great advances have been obtained lately regarding WNV infection, and although efficient equine vaccines are available, no specific treatments or vaccines for human use are on the market. This review updates the most recent investigations in different aspects of WNV life cycle: molecular virology, transmission dynamics, host range, clinical presentations, epidemiology, ecology, diagnosis, control, and prevention, and highlights some aspects that certainly require further research.

Key words: West Nile virus; Emerging pathogen; Molecular biology; Diagnosis; Pathology; Epidemiology; Vaccines; Antivirals

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VIRUS

West Nile virus (WNV) is a small enveloped virus about 50 nm in diameter. The genomic RNA is enclosed within a nucleocapsid formed by the capsid (C) protein that constitutes the core of the virion and is enveloped by a lipid bilayer derived from the host cell. Mature virions display a smooth outer surface with no projections or spikes. This outer shell is constituted by 180 copies of the small membrane (M) protein and an equal number of copies of the E glycoprotein disposed as 90 anti-parallel homodimers arranged in three distinct symmetry environments (Figure 1), thus resulting in a particle of icosahedral symmetry[1].

Genome

The WNV genome is constituted by a single-stranded RNA molecule of positive polarity (Figure 2). This RNA molecule of about 11 000 nucleotides in length encodes a

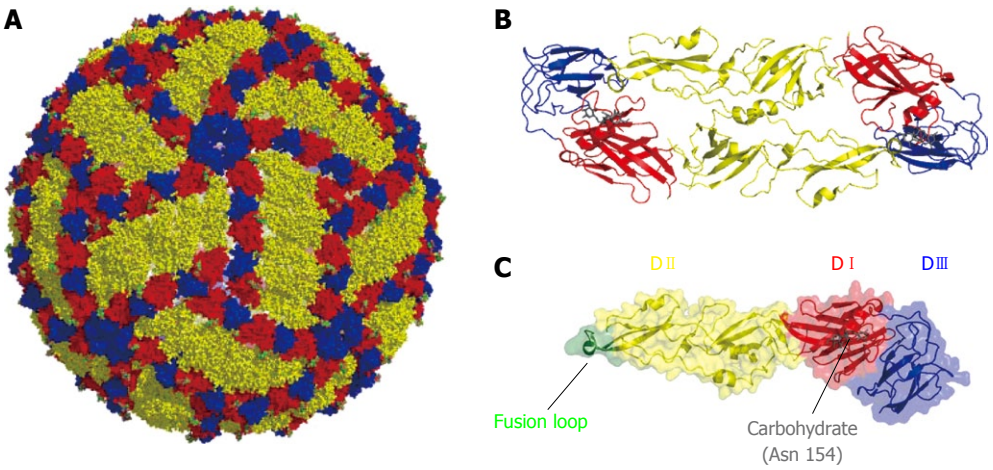

Figure 1 Organization of West Nile virus particle. A: Representation of West Nile virus particle based on cryoelectron microscopy data[230]. Glycoprotein E is shown on the surface of the particle. Color code: DⅠ (red), DⅡ (yellow) and DⅢ (blue); B: Ribbon diagram of a dimer of E glycoprotein; C: Structure of a monomer of the soluble ectodomain of E glycoprotein based on the atomic coordinates solved by X-ray crystallography[58]. Fusion loop is highlighted in green. The carbohydrate located at Asn 154 is shown in gray.

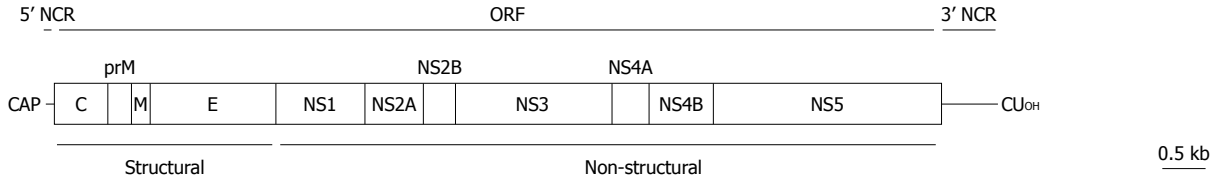

Figure 2 Schematic view of West Nile virus genome organization. The single ORF (boxes) that encodes both structural and non-structural proteins is flanked by two non-coding regions (NCRs). See text for details.

polyprotein in a single open reading frame that is flanked by two non-coding regions (NCRs) located at the 5' and 3' ends of the genome (about 96 and 635 nucleotides in length, respectively)[2,3]. The polyprotein is proteolytically processed by viral and cellular proteases rendering ten major viral proteins: three structural (C, prM and E) and seven non-structural, NS (NS1, 2A, 2B, 3, 4A, 4B y 5)[3]. WNV genome has a cap structure at 5' end, but it lacks a 3' poly(A) tract and ends with CUOH[3]. Proper methylation of the cap structure at the guanine N-7 and the ribose 2'-OH positions of the first transcribed adenine is 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*[4,5].

Virus cell host interactions

Early steps: Attachment, entry and fusion: Infection by WNV is initiated by binding of virions to its cellular receptor (Figure 3). Glycosaminoglycans, c-type lectins, and, although still controversial, integrin $\alpha_v\beta_3$ have been proposed as the cellular receptors for WNV[6-9]. Viral particles are internalized into host cells *via* a clathrin dependent mechanism and are transported to endosomal compartments with the involvement of cellular actin and microtubules[10,11]. Entry of WNV has been suggested to be dependent on cellular protein Cas-Br-M (murine) ecotropic retroviral transforming sequence-like 1[12], although a role of this protein on viral replication rather than in WNV entry has been recently proposed[13]. Transport of WNV particles to early endosomes is dependent on the activity of small GTPase Rab5[14]. Inside endosome, acid pH triggers rapid conformational changes on the envelope protein that result in its fusion with endosomal membrane, thus allowing nucleocapsid release to the cytoplasm for genome uncoating. 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[15,16]. In this way, exposure of viral particles to acid pH in absence of target membranes induces conformational rearrangements that resemble those induced inside endosomes and causes loss of fusion capacity, thus reducing its infectivity[15]. Freezing virus particles in a pre-fusion intermediate by antibodies that neutralize WNV infection after attachment, likely by impairing acid-induced fusion within the endosome, have been described[17,18].

Replication complex assembly: Once viral RNA reaches the host cell cytoplasm it is translated to raise structural and non-structural proteins involved in viral replication and virion assembly (Figure 3). The genome is translated into a single RNA polyprotein that is processed to render mature viral proteins. RNA replication requires the synthesis of a minus-strand RNA which acts as template[3].

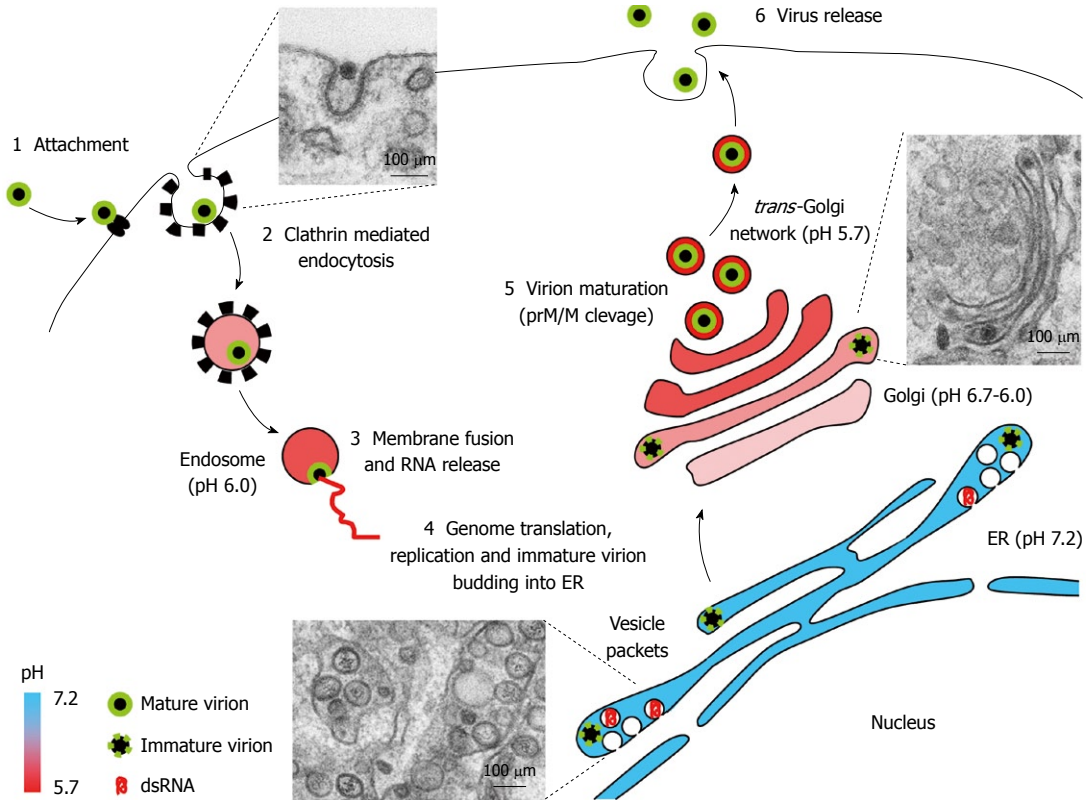

Figure 3 West Nile virus replication cycle. Schematic view of West Nile virus replication cycle in an infected cell. Electron micrographs of West Nile virus-infected Vero cells illustrate distinct snapshots from infectious cycle: entry of virions, vesicle packets formation, and virion trafficking through Golgi complex. See text for details. ER: Endoplasmic reticulum; prM/M: Premembrane/membrane protein.

Cells infected by WNV undergo notable intracellular membrane remodelling. Major membrane reorganizations leading to different well defined structures aimed to establish the viral replication complex have been described. The primary membrane source for these structures is provided by the endoplasmic reticulum (ER)[19]. The presence of markers from organelles involved in the endocytic pathway (endosomes/lysosomes) or from the Golgi complex have been also suggested[20]. However, recent data did not support this hypothesis[19,21]. These structures are termed convoluted membranes (CM), paracrystalline arrays (PC) and vesicle packets (VP)[22,23]. Viral replication takes place at VPs, which are generated as invaginations of the membrane of ER and contact by pores with the cell cytoplasm[19]. VPs contain dsRNA replication intermediates, and assembled virions bud into the ER[19] (Figure 4). A specific role of cholesterol and fatty acids in WNV-induced membrane structures has been proposed[21,24,25], and proteasome activity seems to be also important for viral replication[13,26]. 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[24,27]. Replication of WNV and accumulation of non-structural proteins at the ER induces ER stress and activates the unfolded protein response[28,29]. In addition, replication of WNV also induces apoptosis of infected cells[30].

Particle maturation and viral egress: To render infectious virions, immature viral particles assembled at the ERs traffic through Golgi complex for maturation (Figure 3). This maturation process requires the cleavage of Premembrane/membrane protein (prM/M) by a furin-like protease located at the acidic environment of the *trans*-Golgi network[3]. After maturation, viral particles are released from infect cells following the secretory pathway.

Non-coding genomic regions

The NCRs located at 5' and 3' ends of viral genome contain conserved secondary RNA structures that play important roles in genome replication, may function as protein translation enhancers and, in the case of 3'NCR, display translational regulatory properties similar to those of a poly(A) tail[3]. Different cellular proteins that interact with both 5' and 3' NCRs have been involved in viral replication, including elongation factor 1α which binds 5' NCR, T cell-restricted intracellular antigen (TIA)-related protein (TIAR), and a closely related protein TIA-1 which bind to 3' NCR in minus-sense strand[3,31-33]. Several short conserved sequences located at both NCR are important for establishment of two pairs of long-distance RNA interactions that mediate genome cyclization and replication[34]. Functional analyses have confirmed that mutations that abolish these interactions affect viral replication, but not RNA translation[35]. As the RNA dependent RNA poly-

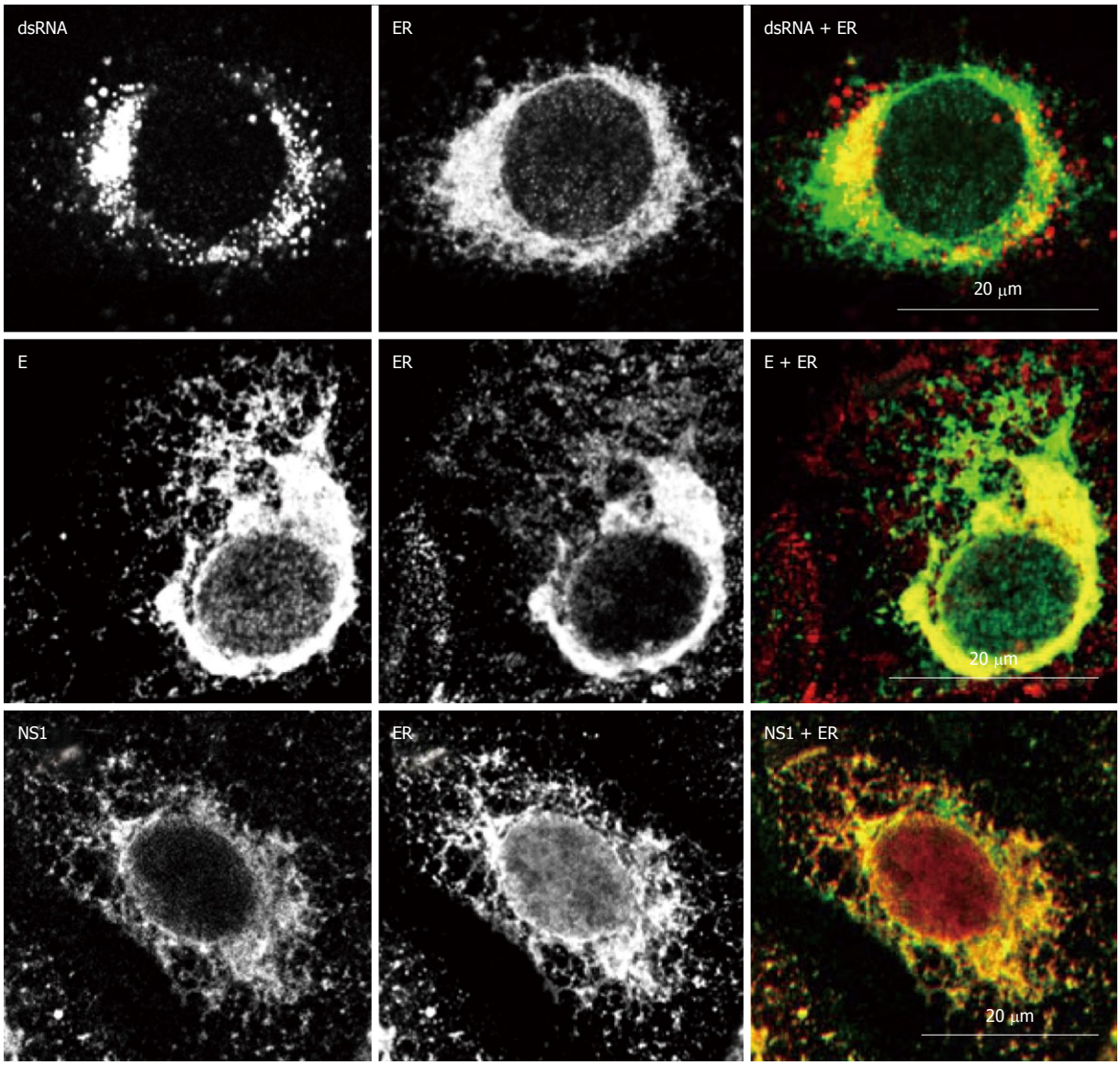

Figure 4 Localization of West Nile virus dsRNA, structural and non-structural proteins at the endoplasmic reticulum of infected cells. Double immunofluorescence labeling of Vero cells 24h post-infection with West Nile virus (MOI of 1 PFU/cell) and stained for dsRNA, using monoclonal antibody J2 (English and Scientific Consulting Bt., Hungary), E glycoprotein or NS1, using monoclonal antibody 3.67G and 3.1112G (Millipore, Temecula, CA), respectively, in combination with rabbit polyclonal antibody against calnexin (Abcam, Cambridge UK) as an specific marker for endoplasmic reticulum. Procedures for immunostaining have been previously described[21].

merase binds to a stem loop within the 5' NCR, genome cyclization should facilitate binding of the polymerase to the 3' end of the genome and, thus, to the initiation of minus strand synthesis[34,36]. The balance between circular and lineal forms of flavivirus genomes are essential for efficient RNA replication and control of translation initiation, as well as for switch between translation and RNA synthesis during the viral life cycle[37].

Apart from the involvement in genome cyclization, 5' NCR acts as a template for recognition of the enzyme responsible for methylation reactions required for cap assembly[36]. Mutational analysis of six terminal nucleotides of the 3' NCR have revealed that it displays a conserved RNA structure, which may function as contact sites for specific assembly of the replication complex or for efficient initiation of minus-sense RNA synthesis[38].

Structural proteins

Capsid (C): The capsid, or core, (C) protein contains a large number of scattered charged amino acids[39] and is implicated in viral assembly and replication[40]. 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. The N and C-terminal 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[41]. X-ray crystallography analysis of the central part of the C protein structure showed the presence of four α-helices[39]. 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[42]. Nuclear location of the

C protein is mediated by a bipartite nuclear location signal and requires specific interaction with cellular importins[43]. The capsid protein also interacts with other cellular factors, as the inhibitor of the serine/threonine phosphatase PP2A, I(2) (PP2A), Hsp70 and Jab1[44-46]. The phosphorilation status of the protein and Jab1 can regulate nuclear location and RNA binding activity[46-48]. The C protein has been also implicated in degradation of claudin proteins and disruption of epithelia barrier, thus helping to virus dissemination[49].

prM/M: The prM/M is a short transmembrane glycosylated protein associated to the lipid bilayer of the virion. The cleavage of this protein by a furin-like protease occurs within the *trans*-Golgi network and is necessary for particle maturation[3]. This protein protects virions from fusion inside acidic vesicles of the Golgi complex[50]. The furin-like protease cleaves the prM/M membrane protein, enabling a conformational rearrangement in the viral particle from immature particles[51] to mature ones[1]. Modulation of the proportion of prM/M cleavage can also modulate the sensitivity of antibody-mediated neutralization[52].

Envelope: 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[53]. Glycosylation is important for efficient transmission in mosquito and birds[54,55] and may be related to neuroinvasiveness[56]. The atomic structure of the E glycoprotein as a soluble ectodomain has been solved by X-ray crystallography[57,58], showing that it presents the typical folding of the flavivirus E glycoproteins and is organized in three domains (Figure 1): DⅠ, DⅡ, that contains a hydrophobic peptide responsible for virus fusion termed fusion loop, and DⅢ, an immunoglobulin-like domain. DⅡ mediates the homodimerization of the protein on the surface of the virion (Figure 1). DⅢ is involved in receptor binding and 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[59] that should act as a critical pH sensor. However, this hypothesis has not been validated for WNV[60], although point mutations can modulate the fusion threshold[15].

Non-structural proteins

NS1: This viral glycoprotein, which can be secreted from infected cells[61], may vary its oligomeric state between monomers, dimers (the primary form) and hexamers, and this seems to be related to its cellular retention or secretion stage[3,53]. Intracellular NS1 functions as an essential cofactor for viral replication, and it localizes to WNV replication sites[62], whilst cell surface and secreted NS1 act as immunomodulators. NS1 antagonizes complement activation[63] and inhibits Toll-like receptor 3 (TLR3) signalling[64]. 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[65].

NS2A: This is a small hydrophobic transmembrane protein involved in the production of intracellular virus-induced membranous structures and virion assembly[66,67]. In fact, NS2A has been detected by immunogold labelling primarily within VP, associated with labelled dsRNA[62]. In addition, NS2A seems to have an immunomodulatory role because it inhibits α/β interferon production[68], and mutations in this protein result in viral attenuation *in vivo*[68,69].

NS2B: It is also a small hydrophobic protein that acts as a cofactor of NS3 protease and may function as a membrane anchor for viral protease[3,53,70]. Alanine scanning approaches of NS2B has revealed two sites critical for regulation of the proteolytic activity of NS2B-NS3 complex[71]. The interaction between NS2B and NS3 may also confer specificity for RNA unwinding of NS3 discriminating from DNA[72].

NS3: This is a highly conserved protein, which N-terminal encodes the viral trypsin-like serine protease. However, it is not active unless tethered to its cofactor, NS2B[70]. This protease cleaves the viral polyprotein to release structural and non-structural proteins and, thus, disruption of its activity is lethal for virus replication. The NS3 protein also encodes other enzymatic activities within its sequence (helicase, nucleoside triphosphatase, RNA triphosphatase) important for viral replication[3,53,70]. NS3 (and also its cofactor NS2B) has been localized within PC or CM, suggesting that these membranes are the sites of proteolytic cleavage[62]. The structure of the helicase domain has been solved by X-ray crystallography[73] and a single substitution in this domain has been related to increased virogenesis in American crows[74]. All these properties of NS3 made of this protein and its active form, NS2B-NS3, a promising antiviral target.

NS4A: It is also 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)[62]. It may be responsible of membrane rearrangements in infected cells upon cleavage of its C-terminal region (designated 2K fragment) that, in dengue virus, acts as a signal sequence for translocation into the ER of the adjacent NS4B protein[75]. NS4A has been also related, together with NS2A and NS4B, to the inhibition of interferon signalling in flavivirus infected cells[76]. 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[29]. Mu-

tations in the 2K fragment have been related to resistance against the antiviral action of the interferon-inducible 2', 5'-oligoadenylate synthetase 1b protein[77], and also to resistance against the flavivirus inhibitor lycorine thanks to the enhancement of RNA replication[78]. NS4A has been proposed to also act as a cofactor regulating ATPase activity of the NS3 helicase[79].

NS4B: This small hydrophobic protein plays a major role on inhibition of WNV interferon signalling[80,81]. Mutations in NS4B can result in attenuation of WNV *in vivo*[82,83]. NS4B has been found in perinuclear membranes and in the nucleus of WNV infected cells[84] and it may be involved in the formation of viral replication complex.

NS5: It is the largest protein encoded by the virus. NS5 localizes to virus induced membranes in infected cells and colocalizes with dsRNA at viral replication complexes[85]. It has two different enzymatic activities: the N-terminal encodes the methyltransferase[86], while the C-terminal encodes the viral RNA-dependent RNA polymerase for replication of viral genome[87]. 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. The methyltransferase activity is necessary for capping the 5' end of the viral RNA, which is performed by sequential methylation reactions[88]. NS5 is also a potent antagonist of interferon signalling[89]. The methyltransferase activities together with the polymerase activities made of NS5 also a promising antiviral target.

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[53,90]. Recent advances on molecular phylogeny support this antigenic classification and reveal the existence of up to five distinct genetic lineages of WNV[91-94].

Lineage 1 is subdivided into three clades (Figure 5). Clade 1a includes African, European and American isolates; clade 1b groups the Australian Kunjin virus (KUNV), which has been shown to be a subtype of WNV[95]; and clade 1c clusters isolates from India[53,92]. Interestingly, clade 1a displays close genetic relationships between geographically distant areas which are supposed to be the result of WNV introductions *via* migratory birds (Figure 5). WNV inside clade 1a can be further grouped into different clusters[96]. 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 case in the American continent, where WNV was introduced in 1999 in the East Cost of the US[2,96]. 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[2]. 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[96].

Lineage 2 initially included WNV strains only detected in Africa and Madagascar, which have been speculated to be less neurovirulent than those included in lineage 1[92]. However, recent outbreaks in Europe (Austria, Hungary and Greece) have been associated to lineage 2 strains[97]. Other lineages of WNV of unknown human pathogenicity are lineage 3 (Rabensburg isolate 97-103), isolated from *Culex pipiens* mosquitoes in the Czech Republic in 1997[93], and Lineage 4 (LEIVKrnd88-190), isolated from *Dermacentor marginatus* ticks in 1998 in Russia[94]. It has been proposed that WNV Indian isolates that were grouped as linage 1c make a new cluster termed lineage 5[91]. A putative new lineage of WNV (strain HU2925/06) that forms a common evolutionary branch with lineage 4 has been recently reported in Spain[98]. In addition to these minor lineages, an unusual KUNV isolate from Malaysia (KUN MP502-66) and the African virus Koutango (KOUV) could also constitute additional lineages of WNV[95].

TRANSMISSION CYCLE

Mosquitoes and other arthropods

WNV is maintained in nature in an enzootic transmission cycle between avian hosts and ornithophilic mosquito vectors. At least 60 species of mosquitoes from 11 different genera have been described as competent vectors in North America, being those of the *Culex* species the most efficient ones (*Cx. pippiens*, *Cx. quinquefasciatus*, *Cx. restuans*, *Cx. salinarus*, *Cx. tarsalis*, and *Cx. nigripalpus*), although other species such as *Aedes albopictus*, *Aedes vexans*, *Ochlerotatus japonicus* and *Ochlerotatus triseriatus* may also play a role on viral transmission as bridging vectors that can transmit the virus to mammals[99]. In Europe, the virus has been isolated from more than 40 different species, being again those of the *Culex* species the main vectors[100]. Several other species have been also described as competent vectors in other geographical areas, *Cx. univittatus* in Africa, *Cx. annulirostris* in Australia, and *Cx. vishnui* and *Cx. tritaeniorhynchus* in Asia[99,101,102].

Laboratory analyses have shown that *Cx. 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[103]. 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[104].

Vertical transmission, overwintering, and survival of adult mosquitoes with some low activity during winter

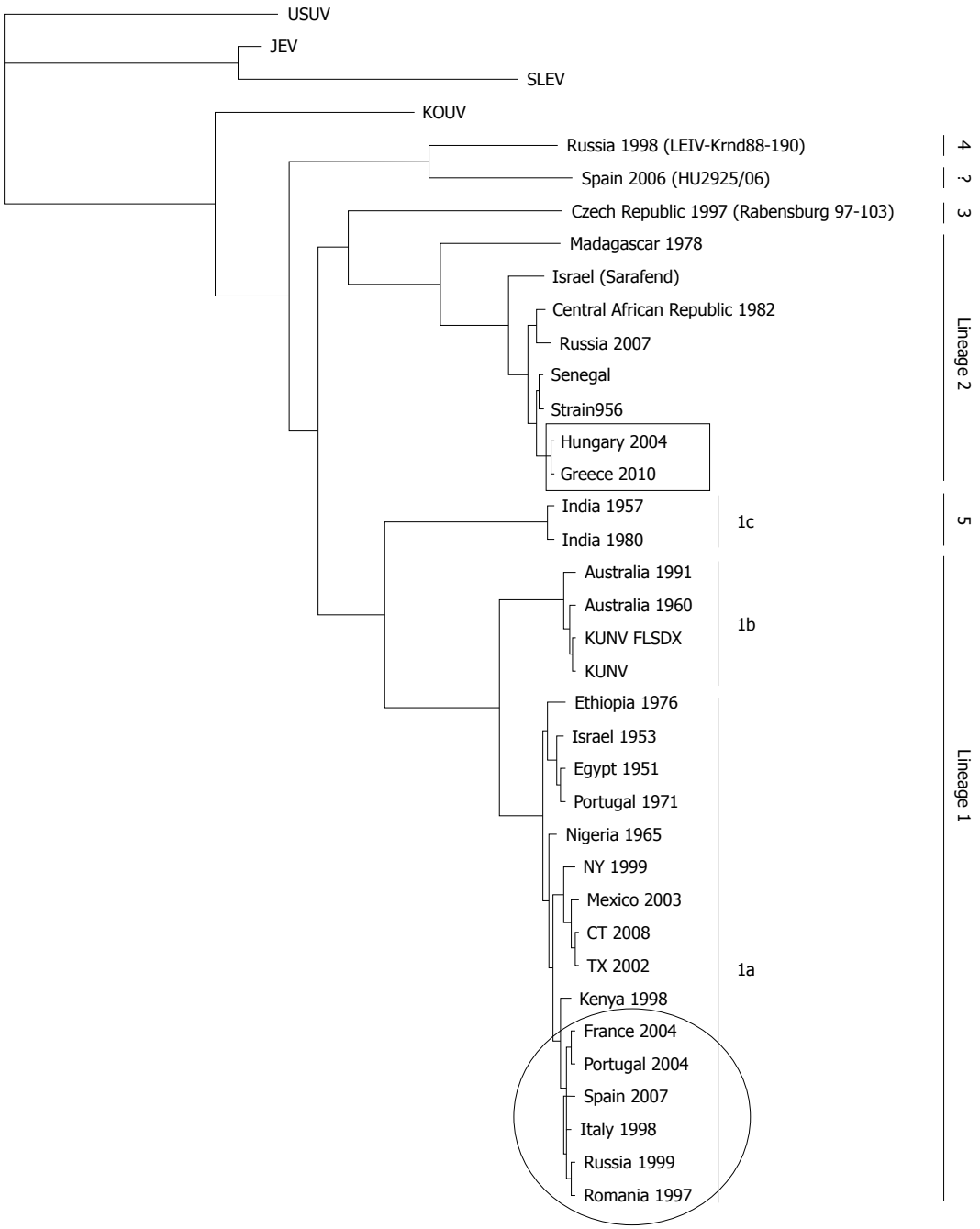

Figure 5 Representative phylogram showing the relationships between West Nile virus strains. The country of origin and year of isolation is displayed when available. The scale indicates 0.1 substitutions/site. The tree was based on complete NS5 nucleotide sequence (except for HU2925/06), built from a multiple alignment using ClustalW and Phylogeny.fr[231] and visualized with TreeView. Bootstrap values correspond to 500 replications. Squared and circled isolates correspond to lineage 1 and 2 recently circulating in Europe. GeneBank Accesions: Usutu virus (USUV) (AY453412), Japanese encephalitis virus (JEV) (NC_001437), St. Louis encephalitis virus (SLEV) (AY167443), KOUV (EU082200), Russia 1998 (AY277251), Spain 2006 (GU047875), Czech Republic 1997 (AY765264), Madagascar 1978 (ABB01532), Sarafend (AY688948), Central African Republic 1982 (DQ318020), Russia 2007 (FJ425721), Senegal (DQ318019), Strain 956 (NC_001563), Hungary 2004 (DQ116961.1), Greece 2010 (HQ537483), India 1957 (GQ851605), India 1980 (ABC40712), Australia 1991 (GQ851603), Australia 1960 (GQ851602), KUNV_FLSDX (AY274504.1), KUNV (D00246.1), Ethiopia 1976 (AY603654), Israel 1953 (HM051416), Egypt 1951 (EU081844), Portugal 1971 (AM404308), Nigeria 1965 (GQ851607), NY 1999 (AF196835), Mexico 2003 (AY660002), CT 2008 (AEF33448), TX 2002 (DQ176637), Kenya 1998 (AAP20887), France 2004 (DQ786573), Portugal 2004 (AJ965626), Spain 2007 (FJ766332), Italy 1998 (AF404757), Russia 1999 (AF317203), Romania 1997 (AF260969), Italy 2008 (HM641229), and Italy 2009 (HM641225).

time have been observed in temperate regions[105,106]. It should be also noted that the minimal infection rate (MIR), expressed as the number of infected mosquito pools per 1000 mosquitoes tested, is a good indicator of the intensity of viral transmission in a given area, and is often related to the human risk of disease, so that, a MIR $\geqslant$ 1.0 seems to be needed for a mosquito species to represent a high risk for human transmission[107].

Beside from mosquitoes, WNV has been sporadically isolated in other arthropods such as soft and hard ticks,

mites and hippoboscid flies[108-110] but, although their implications on WNV transmission still need to be analyzed in detail, it seems unlikely that they play a substantial role on the viral transmission cycle in nature.

Birds

Birds are the natural reservoir of WNV. Hundreds of avian species representing over 20 birds families from North America have been described as susceptible to WNV infection after its first introduction in 1999. However, the most affected ones have been the Passeriformes, mostly those of the *Corvidae* family, in many of which the virus replicates to high titers before the birds become moribund and die a few days after being infected[53]. Up to 100% mortality has been described in experimentally infected crows[111,112], and an estimated 45% decrease in crow population has been reported since the introduction of the virus in US[113]; however, recent data indicate that this mortality rate is decreasing[114]. A study carried out with several different avian orders from America reported that blue jay, common grackle, house finch, American crow, and specially house sparrow are among the most important WNV amplifying avian species, while many other did not show any evident sign of WN disease (WND)[112]. For instance, no significant mortality has been reported in chickens, beside they showed titers in blood of 10^5 PFU/mL and virus can be isolated from several organs after experimental infection[115].

Many avian species shed large quantities of virus in their feces or oral secretions when infected[112], allowing direct transmission form bird-to-bird and even from bird-to-human. Experimental oral infection of birds has been demonstrated[116] and prey-to-predator infection has been suggested[117].

Although high bird mortality has been a common feature of WNV activity in US and Israel[118], no such trait has been observed in other regions of the world[100,101,118-120].

Humans, horses and other animals

WNV infection in humans, horses and other mammals are generally considered as "dead end" infections because viral replication does not yield significant viremia to allow transmission from vertebrate hosts to feeding mosquitoes, which is very dependent on the level of viremia in the host. For instance, in experimentally infected horses viremia levels are around 10^3 PFU/mL[121], thus being usually insufficient to sustain infectivity cycles.

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, sheep, pigs, cows, rabbits, llamas, alpacas, deers, reindeers, raccoons, bears, wolfs, squirrels, chipmunks, and bats, among others[53,122]. As it has been described for humans and horses, in most cases the viremia raised in these animals is low and probably not enough to initiate a new transmission cycle.

Apart from mammals, several reptiles and amphibians, such as snakes, crocodiles, alligators, iguanas and frogs[123-126] have been also described as susceptible to WNV infection and some of them raise high viremia, but the real contribution of animals other than birds and mosquitoes in maintaining WNV cycle in nature is still uncertain.

Non-vector

WNV transmission to vertebrates usually involves mosquitoes; however sporadic reports of non-vector-borne transmission have been documented in humans throughout liver and kidney transplantation, dialysis, needle-stick injury, breast-feeding, by transplacental route, and by blood transfusion, which drove to the consequent screening of blood donors[127-130].

These transmission routes seem to be mainly anecdotic and have minor effects on disease burden. For instance, no fatalities were recorded among 71 WNV infected pregnant women included in a retrospective study, most of whom gave birth healthy children with only a few cases of newborns presenting malformations, though no conclusive association to WNV infection could be established in any of them[130]. In any case, assessment of the fetus or child is recommended when mothers are infected by WNV and clinicians are encouraged to report known or suspected cases of WNV infection during pregnancy.

WNV experimental transmission by oral, intrauterine and lactation routes has been described in mice without evidence of mice-to-mice infection[131,132] and, contrary to what has been observed in humans, it has been reported that WNV infection increases mortality rates in pregnant mice[131]. Direct or experimental oral exposure to the virus resulted in infection of cats, dogs, hamsters, mice, fox squirrels, alligators, crocodiles, and snakes[53,102,122]. Transmission from experimentally infected alligators to uninfected[124], and from infected hamster to uninfected cage-mates has been also reported[133,134].

ECOLOGY

A primary role of migratory birds in WNV introduction, reintroduction, and spread across Europe[135-139] and the Americas[140,141] is well documented. However, comparison of the levels of neutralizing antibodies in migratory and resident birds in the south of Spain[135,136], the seroconversion observed in some recaptured resident birds of the same area[137], and the significant number of positive resident birds found in Great Britain[142] and Italy[143] support that there is also local circulation of the virus. Contribution of resident birds to the initiation of annual infection cycles in the USA has also been suggested[144].

Since viral cycle may persist in a given geographical region from year to year without reports of human or horses cases, epidemics seem to be more related to the concomitant profusion of birds and mosquitoes populations in the same area, generally wastelands and mashes where migratory birds are abundant, than to other factors. Thus, in addition to the viral load present on the

blood of the hosts[103], the efficiency with which mosquitoes transmit WNV seems to be clearly dependent upon various environmental and climate factors, particularly temperature, humidity and rainfall[122,145].

RNA viruses have the capability to replicate at elevate temperature, particularly arboviruses present a wide range of temperatures at which they replicate in order to be propagated within different hosts, vertebrates and invertebrates. *In vitro* experiments have demonstrated that selection of viruses with increased capability to replicate in vertebrates resulted in a lower capacity to replicate in mosquito[146]. WNV replication in mosquitoes is constrained below 14 ℃[147] and strains isolated in 2002 in the USA disseminate more rapidly and efficiently at elevated temperatures than the original strain isolated in New York in 1999[148]. All these data strongly suggest that temperature is a key factor on WNV evolution and dissemination.

Climate changes have a direct effect on WNV amplification and transmission. Lately, mosquitoes expanded to more extreme latitudes and elevations, thus reaching new host populations to feed on and sharing new ecological niches with different vector species. Heavy rainfall as well as warm and dry temperatures benefit the increase of mosquitoes population and positively correlate with WNV transmission[149,150].

Although the way in which the virus causes sporadic outbreaks in humans and horses is still unclear, it is quite reasonable to think that overabundance of birds that share urban and rural areas with ubiquitous fastidious mosquitoes that feed in birds and mammals plays an important role on it. In fact, in two of the major urban outbreaks reported, the ones that took place in Bucharest (Romania)[151] and Volgograd and Volzhskiy (Russia)[152], the regions were heavily infected by potentially mosquito vectors. Hence, new human and animal behaviors and climate changes are facilitating contact between vectors and hosts.

EPIDEMIOLOGY

WNV was first detected in the blood of a febrile woman in the West Nile district of Uganda, currently the Arua district, in 1937[153]. Further studies showed that the virus was widely distributed across Africa, Europe, Asia and Australia[100-102,108] but, since only sporadic outbreaks with low clinical incidence were reported until recently, WNV infection was not considered a serious animal or human health treat.

Europe and the Mediterranean basin

The first recorded human WNV outbreak took place in Israel in 1950s[154,155]. Since then, there were some sporadic reports of WNV circulation in Albania, Bulgaria, Belarus, Ukraine, and Moldavia[108], but viral activity was low until the 1990s, when the virus re-emerged with a worrisome increase in the number, frequency and severity of the outbreaks reported in human and horses. In fact, evidence of WNV activity, with or without recorded human or horse clinical cases, have been lately reported in Algeria, Morocco, Tunisia, Egypt, Israel, Romania, Russia, Poland, Czech Republic, Hungary, Croatia, Serbia, France, Portugal, Spain, and Italy, which, overall, have accounted for hundreds of cases and dozens of deaths[100,156].

Early serological studies in Greece had detected anti-WNV antibodies in mammals and humans, but later intensive serosurveillance studies in blood donor in 2006-2007 failed to do so[157]. Along 2010 (Figure 6) WNV outbreaks were reported in Greece and neighboring countries with more than 250 laboratory confirmed cases and 27 deaths[158]. Analysis of the circulating virus has shown that it was genetically closely related to the virus that emerged in Hungary in 2004[158]. An increasing activity of WNV in horses has also been observed lately in Europe[156,159,160], including a large outbreak that took place in northeast Italy in 2008 involving 251 stables with 794 cases and 5 deaths[156,161]. At the same time, nine human cases of West Nile fever (WNF) were reported in the area[161]. In southern Spain, during summer 2010, the first outbreaks of severe WND in horses were reported with 41 diagnosed cases and 10 deaths, and 2 laboratory confirmed human cases (http://www.oie.int/wahis/). Similar figures have been reported during 2011. The strains responsible for the Italian and Spanish outbreaks are closely similar to those previously circulating in the western-Mediterranean region (Figure 5) and differ from the one that circulate in Greece[162]. All these data suggest that new epidemiological scenarios are being developed in Europe and, thus, that assessment of WNV activity and implementation of coordinated surveillance programs are necessary across the continent.

Africa, Asia and Oceania

Since the first detection of WNV in Uganda[153], little information has been available about its activity there. Studies conducted in several sub-Saharan countries have reported a quite variable seroprevalence in humans[120,163-165], with few documented clinical cases, but these data should be taken carefully since most studies have not addressed possible cross-reactivity with other circulating flaviviruses, and clinical cases may have been attributed to other diseases that present similar symptoms. Serological evidence of WNV infection has also been described in horses in the continent[165].

WNV is frequently detected in southern Asia, where some human cases have been reported, and sporadically in South-East Asia[166]. In Australia, the lineage 1 circulating WNV strain is known as KUNV[101] and, although may cause febrile illness and even encephalitis, its clinical effects are usually mild[101,166].

The Americas

WNV was first detected in North America in the New York metropolitan area in 1999[2], but the way the virus was introduced is still unknown, and several options have been proposed: transport of infected mosquitoes by air-

Figure 6 West Nile activity in Europe and the Mediterranean basin in 2010. Areas with West Nile virus confirmed cases in horses (gray) and humans (black). Data collected from http://ecdc.europa.eu.

plane or other means, arrival of an infected person, or, what it seems more probable, carried by infected migratory or imported birds.

During the first outbreak in New York the virus caused 62 human cases with seven deaths, 25 equine cases with nine deaths, and an enormous mortality of birds, particularly among corvids. Since then, the virus has spread quickly across the country, being, so far, responsible for over 1100 fatalities, over 12 000 cases of meningitis/encephalitis, and more than 30 000 diagnosed human infections (http://www.cdc.gov). In addition, more than 25 000 accumulated cases were reported in horses until the introduction of veterinary vaccines that have greatly contributed to reduce equine mortality and morbidity in the country[167].

Several studies conducted on experimental animal models and birds have demonstrated that the strain introduced, belonging to lineage 1, was highly virulent[111,168]. Further analyses have identified possible determinants in the structural and non-structural proteins of the virus that may account for its neurovirulence[169,170]. Then, the combination of the introduction of highly virulent strains acting over a naïve population in the presence of competent amplifying vectors may have been responsible for the devastating consequences that WNV had in the US early after its introduction.

The virus has also expanded to neighboring regions, and serological evidence of 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[53,122]. Sequencing analysis of the Mexican strains suggests that the virus was introduced into Mexico in two different events, once in the northern states and another in the Yucatan region, more probably by migratory birds. However, WNV pathogenicity in these regions seems to be lower than in the US, as very few human or horse cases have been reported[122]. In fact, only 5 and 7 human cases have been reported in the Caribbean and in Mexican states bordered to the US, none of them fatal. These differences are intriguing and could be due to a combination of several factors, like to the wide distribution of other related and potentially cross-protective flaviviruses in the region (because individuals that had previously been in contact with them may have natural acquired immunity against WNV), to the species composition, abundance and/or susceptibility of hosts and vectors in the regions, or to a different virulence of the circulating WNV strains that could also contribute to the apparent paucity of the disease there[171,172]. In fact, although slightly divergent in their genomes, strains with attenuated phenotypes in cell culture and/or lower virulence have been isolated after the first introduction in America[173,174]. Even more, although stasis of WNV evolution was initially described in the US[175], recent data suggest that the virus is continuously evolving[176].

CLINICAL MANIFESTATIONS AND PATHOGENESIS

Humans

Most of WNV infections in humans are asymptomatic, but approximately 20% of the infected people develop clinical symptoms, although severe neurological diseases

are observed in less than 1% of them. WNF is characterized by several non-specific flu-like symptoms, such as fatigue, fever, headache, muscle pain, weakness, rash, and neck-pain. Symptoms usually develop between 2 to 15 d after infection and last for 2 to 5 d[177]; however, in some instances, they can result in hospitalization and many patients with WNV encephalitis have movement disorders, including severe tremors and parkinsonism[178,179]. Severe WND is associated with neurological involvement that varies from meningitis and/or encephalitis to poliomyelitis-like condition with acute flaccid paralysis that can result in respiratory failure, with an estimated 10% of the neuroinvasive cases resulting fatal[180]. Analysis of the long-term outcomes of WND has estimated that more than 50% of survivors that presented severe symptoms reported physical and cognitive deficits up to 2 years after being diagnosed[180,181].

Older age use to be associated with a worst prognosis, but neuroinvasive diseases have been also reported in young people and children[177,182]. Hypertension and diabetes have been suggested to be a potential risk factor for severe WND, but the most important factor, beside old age, seem to be immunosuppression, such as that of transplanted people or human immunodeficiency virus infected patients[177]. In fact, a fully functional immune (innate and adaptive) response (humoral and cellular) has been shown to be essential to fight WNV infection in animal models[183-186]. It is known that, overall, humoral immune response is capable of control viral load and dissemination; whereas T-cell mediated response is required for clearance of the virus from the central nervous system (CNS). A TLR3 mediated inflammatory response has been implicated in a higher neuroinvasiveness of the virus in the mouse model[187], but it has not yet been confirmed in humans. Nevertheless, WNV is capable to directly infect neurons, brain stem and spinal cord[188].

The pathogenesis of WNV infection is similar to that of other Flaviviruses. After primary inoculation, WNV is believed to replicate in resident skin Langerhans dendritic cells before it traffics to the lymph nodes and blood stream from where it reaches the spleen and kidneys and, finally penetrates the CNS resulting in inflammation of the medulla, brain stem and spinal cord[184]. In any case, the mechanisms by which the virus enters the CNS are still to be fully elucidated. The proposed routes include: *via* leukocytes, direct entry across the brain barrier, and by retrograde axonal transport *via* the peripheral nervous system[189,190].

As many viruses, WNV has developed different strategies to block the action of type I interferon (IFN) and, thus, to evade the host antiviral activity of IFN-stimulating genes, ISG[184,186]. Different reports indicate that non-structural proteins NS1, NS2A, NS4B and NS5 contribute to control IFN α/β signaling by different ways[186,190]. On the other hand, genetic polymorphism of the IFN-inducible 2'5-Oligodenylate synthetases has been implicated in the host innate resistance to WNV infection in horses[191], humans[192], and mice[193].

Horses

As in humans, the majority of WNV infections in horses are usually not accompanied by presentation of clinical signs, which are only observed in around 10% of the animals[121] and are characterized by fever and, sometimes, by ataxia and muscular weakness; however, some others signs like dysmetria, somnolence, or hyperexcitation have also been observed[159]. Illness usually last for 3 wk[194]. Even though outbreaks resulted in 25% to 45% of mortality rates among affected horses[194,195], implementation of vaccination campaigns has strongly reduced these mortality rates[167], and treatment guidelines for horses with WND have been published. Lesions by WNV infection in horses are mainly limited to the CNS such as polioencephalomielitis and, in the most severe cases, neuronal degeneration[159]. Even though the underlying mechanism is uncertain, it has been suggested that there is an immunopathological component involved, because inflammatory changes were present in the absence of abundant viral antigens. Contrary to other Flavivirus infections, clinical cases of WND in horses do not precede that in humans, discarding their use as sentinels[196].

Birds

Typical clinical signs in birds are neurological, such as ataxia and paralysis, as well as non-neurological, such as depression, lethargy, ruffled feathers, weight loss and myocarditis[112]. When birds die from WND, they use to do it in the first 24 h after the onset of clinical signs. WNV infection causes damage in multiple bird organs, as brain, kidney, heart, lungs, liver, gonads, spleen, intestines, esophagus, and skin. As mentioned early, crows and blue jays are highly susceptible to WNV infection[112] and, thus, monitoring of crow mortality has been successfully adopted as an epidemiological indicator for tracking WNV activity in the USA, predicting an increase of the risk for human infections[197,198].

Others animals

Beside birds, horses and humans, WND is unusual in other vertebrates, but neurological manifestations have been reported in squirrels[199], dogs and cats[200], and alligators[124]. Hamsters[134,201] and mice[131,132,168] are frequently used as experimental models, as they present some disease signs that parallel those exhibited by humans with severe neuroinvasive disease, such as confusion, tremor of extremities and paralysis.

Persistent infections

Persistent infection was early described in rhesus monkeys in which virus could be recovered post-mortem from different tissues of sacrificed animals that did not show any sign of WND and presented specific antibodies in serum[202]. Later on, persistent infection of brains and kidneys has been reported in the hamster model with viral shedding in urine for up to 8 mo[133,134]. Chronic infection has also been recently described in immunocompetent mice without any clinical signs of disease[203].

All these animal experimental data suggest that persistent infections may occur in humans and should be carefully monitored. In fact, a recent study has detected WNV-RNA in the urine of 20% of a study cohort 7 years after being infected, even though no infectious virus could be recovered[181]. Similarly, WNV-RNA and antigens were also detected in the CNS of an encephalitis patient with B cell lymphoma 99 d after symptoms onset[204].

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.

Antibody

Cross-reactivity between Flavivirus antigens is the greatest drawback for proper serological diagnosis and epidemiological studies and, thus, sera have to be tested against different related viruses and results have to be subsequently confirmed by different assays, namely hemagglutination inhibition, immunoflourescence or plaque reduction neutralization test (PRNT), considered as the gold-standard[205]. A 4-fold increase in PRNT titers between 2 sequential serum samples collected 2-3 wk apart usually confirms an acute WNV infection, and WNV neutralizing titers 4-fold higher than titers to other related-flavivirus is usually taken as a probe of the specificity of the infection.

Initially, serological testing was based on IgM antibody capture assays (MAC-ELISA) and in indirect IgG ELISAs, followed by retesting of positive samples by PRNT. Later on, ELISAs using monoclonal antibody blocking assays were set up. Presence of IgM in the cerebrospinal fluid is indicative of infection of the CNS, because IgM does not cross the blood-brain barrier; however, data should be taken with caution since IgM may persist for extended period of time[206]. All these assays have been extensively used for detection of anti-WNV antibodies in human and animal samples[53].

ELISAs, both commercial and *in-house*, were mainly based in the use of inactivated whole virus as antigen, either produced in mammalian cells or suckling mice; however, its production implies risks for laboratory personnel and needs highly sophisticated biosafety level 3 (BSL-3) containment facilities to grow the virus. For these reasons, several ELISAs have been developed using recombinant viral proteins, mainly the envelope E protein or parts of it, because this protein is highly exposed to the host immune system and bears most of the neutralizing epitopes described[206]. These recombinant antigens have been expressed in a variety of systems, including bacteria[207], mammalian cells[208,209], insect cells[209-211], and larvae[211]. Other formats, such as microsphere particles in conjunction with fluorescent labelled antibodies and lateral-flow assays have also been recently assayed[53].

Virus isolation in susceptible cell culture is the gold standard for virus detection, but it is usually hampered by the typical short duration and low levels of viremia and by the need of BSL-3 facilities, which has lead to the development of alternative methods. Detection of viral antigens is based on antigen-capture ELISAs, dipstick assays, or immunohistochemical methods[212,213].

Nucleic acid

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[214]. 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.

PROPHYLAXIS

Although great effort on the understanding of the molecular biology of WNV has been paid recently, to date no vaccine or specific therapy has been approved for humans, and clinical treatment is only supportive[186]. However, promising approaches are developing, which can be summarized as the search for antivirals, therapeutic antibodies and the design of vaccines.

Antiviral compounds

The search for antiviral compounds active against WNV infection includes the identification of those targeting distinct aspects of the viral replication cycle. The candidate may be targeted against the viral particle itself, impairing its entry and/or infection, or it may block multiplication of WNV within infected cells. This search has lead to the identification of multiple promising compounds interfering with these processes. However, long way remains to be completed before these novel compounds could to be administered to humans or animals, as they should be first tested for antiviral activity in animal models and adverse effects should be ruled out. The availability of multiple approaches based on sub-genomic replicons and viruses encoding reporter genes has contributed to the initial identification of novel putative antiviral compounds, including those that interfere with viral entry and membrane fusion[215], compounds directed against NS5 enzymatic activities as polymerase or methyltransferase inhibitors[5,216], and inhibitors of the NS2B/NS3 protease complex[70]. Candidates for antiviral molecules include a wide panel of options that range from small molecules (either developed by rational design, identified by structure-based approaches, or by high throughput screening of libraries of chemical substances), peptide inhibitors, antisense oligonucleotides, small interference RNAs, or imino sugars that inhibit glycosylation of WNV proteins[186].

The search for antiviral compounds against WNV not

only includes development of novel molecules, but also repositioning of drugs approved for other purposes that have also shown antiviral activities against WNV. Ribavirin, a widely used antiviral drug, has been tested against WNV and, albeit promising anti-viral effect was derived from *in vitro* assays and also from animal models, treatment of infected patients has not rendered satisfactory results[217]. Mycophenolic acid, an immunosupresant used to prevent rejection of transplanted organs, also inhibits WNV replication *in vitro*, however antiviral properties *in vivo* have not been observed[186]. Sodium valproate, a drug that is currently used in humans for treatment of epilepsy and bipolar disorder, has antiviral effect against WNV in cultured cells[218], but no significant anti-WNV activity in experimental animal models has been observed (Saiz JC, Martín-Acebes MA, Vázquez A, and Sobrino F, unpublished results).

Other interesting antiviral tools for the treatment of WNV are drugs that help the immune system to fight the infection. These immunomodulators comprise interferon derivatives or compounds that induce interferon expression[53]. In fact, infection by WNV is highly sensitive to interferon and, when administered to a limited number of WNV-infected patients, help to reduce disease complications[186]. Progress in WNV therapies should thus pass through a combination of drug strategies that targets viral replication, boosts protective immune responses, minimizes neuronal injury, and limits the development of resistant variants.

Therapeutic antibodies

Therapeutic antibodies that either specifically target WNV particles (mainly directed against E glycoprotein and a minor proportion against prM/M) and neutralize infection or recognize NS1 protein and contribute to protection have also been assayed[219]. Passive administration of anti-WNV antibodies is both protective and therapeutic, and does not cause adverse effects related to the immune enhancement observed for other flaviviruses[186]. Therapeutic antibody administration should be useful for elderly and immunocompromised people, two sectors of the population with major risks of WNV neuroinvasive disease. Treatment with intravenous immunoglobulins obtained from pooled human sera help recovery of infected patients[186,220,221]. These antibodies, which are naturally raised by infected patients, can also be produced in the laboratories, thus reducing the problems associated with their purification from human plasma and the presence of other pathogens, and increasing their availability and reproducible antiviral efficacy. Currently, monoclonal antibodies, either from human origin or humanized, are being tested and it is expected that they should be available for human therapy within a reasonable period of time[222].

Vaccines

Even though notable progress for WNV vaccine development has been made, no approved vaccines exist for human use[205,220,223], and their cost-effectiveness for human treatment is still uncertain. On the other hand, there are effective, licensed vaccines for horses that had greatly contributed to the decrease incidence of equine cases in the US, whilst the number of human cases still remains growing[205,224].

Several experimental vaccines strategies have been tested in preclinical models and some have undergone clinical trials[223,225,226], including classical development of vaccines based on the use of live attenuated or chemically inactivated virus obtained from infected cell cultures or from inoculated suckling mouse brains. Recombinant DNA technology has been also applied for engineering DNA and recombinant vaccines based on the use of viral proteins (or fragments of them) have been synthesized in diverse systems (from bacteria to insect cells and larvae)[205,220,223]. Vertical transfer of acquired maternal immunity to the offspring has been demonstrated in mice immunized with recombinant proteins[227].

For horses, three vaccines are commercialized in the US: one based on formalin-inactivated WNV (Innovator®, FortDodge, Princeton, NJ, US), a recombinant live canarypox vaccine that express prM and E genes formulated with Carbopol adjuvant (Recombitek®, MerialLtd., Athens, GA, US), and a plasmid DNA vaccine, pCBWN, that encodes WNV structural antigens (prM-E) (Fort Dodge and Center for Disease Control and Prevention). 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[228,229]. The success of veterinary vaccines has encouraged the development of human vaccines that should induce a good response on higher risk groups and achieve an affordable cost/benefit ratio.

Preventive measures

Prevention and control of WND require an integrated approach that includes vaccination, enhanced mosquito control and improved clinical management. Preventive tools other than drugs and vaccines can also help to combat the spread of the infection by avoiding mosquito bites responsible for disease transmission. These simple measures can be summarized under the topic 'fight the bite!' and include the use of insect repellents, the minimization of skin surface exposed to mosquito bites, the elimination of standing water where mosquitoes can lay eggs, the installation of window and door screens, the minimization of outdoor activities coincident with the maximum activity of mosquitoes, reporting dead birds to local authorities and supporting mosquito control programs (http://www.cdc.gov/).

CONCLUSION

The recent emergence and expansion of WNV in America and the increase in the number, frequency and severity of outbreaks in Europe represent one of the major zoonotic treats in recent years, and indicate that

new epidemiological and ecological scenarios are being developed around the world. Although our knowledge about WNV infection has greatly increased during recent years, some aspects of WNV activity still need to be further addressed: the ways WNV colonizes new ecological niches and the role that climate (temperature, humidity, *etc*.) and anthropogenic factors play; the differences in WNV disease manifestations between the US and other regions of the world, mainly Central and South America; a better understanding of WNV immunity, pathogenicity, and the molecular basis of virulence; the long-term manifestations and sequelae of WNV infection and the outcome of persistent infections; the development of national and international surveillance programs to monitor WNV spread and to take appropriate measures to control it; the search for more efficient, rapid, and specific diagnostic assays that can be easily adopted worldwide; and the search for cost-effective human vaccines for high-risk targeted populations and for new antiviral targets for therapeutic usage. Advances on our current knowledge on WNV infection will greatly help to fight not only future expansion of WNV to new niches around the world, but also of other arboviruses.

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