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West Nile virus population genetics and evolution

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Abstract

West Nile virus (WNV) (*Flaviviridae: Flavivirus*) is transmitted from mosquitoes to birds, but can cause fatal encephalitis in infected humans. Since its introduction into North America in New York in 1999, it has spread throughout the western hemisphere. Multiple outbreaks have also occurred in Europe over the last 20 years. This review highlights recent efforts to understand how host pressures impact viral population genetics, genotypic and phenotypic changes which have occurred in the WNV genome as it adapts to this novel environment, and molecular epidemiology of WNV worldwide. Future research directions are also discussed.

Keywords

West Nile virus; Molecular epidemiology; Population genetics; Pathogenesis Fitness

1. Introduction

West Nile virus (WNV, *Flaviviridae: Flavivirus*) has emerged in recent decades as a significant burden to public health in Europe and the Americas. This emergence, in particular the recent invasion of WNV into North America in 1999 and its subsequent spread throughout the new world, has stimulated intense interest in its population genetics and evolution. The dynamics of the WNV epizootic/epidemic in N. America have been of special interest because they provide insight into a longstanding question in evolutionary and invasion biology: what happens when an exotic pathogen is introduced into a naïve environment? Both observational and laboratory studies have therefore been undertaken to determine the modes and direction of virus evolution and examine the evolutionary implications of the host–virus interactions. In this review, we highlight recent advances in research into the population and evolutionary dynamics of WNV and identify key areas for further research.

1.1. Molecular biology and replication

WNV is a member of the Japanese Encephalitis virus (JEV) serological complex of the flaviviruses (Calisher et al., 1989). The virion is enveloped, spherical (~40–60 nm in diameter) and contains a single copy of the positive-sense RNA genome (Mukhopadhyay et al., 2003; Brinton, 2009). The WNV genome is approximately 11,000 nt in length and the

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translated polyprotein is co- and post-translationally cleaved by viral and host-cell proteases into three structural (capsid C, premembrane prM/M, and envelope E) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). C, M and E are incorporated into the mature virion, while the nonstructural proteins assemble on host cell membranes where they participate in RNA replication and suppression of the host antiviral response (Brinton, 2009, 2001; Westaway et al., 2002; Evans et al., 2011; Avirutnan et al., 2011; Ambrose and Mackenzie, 2011). Overall, the genome organization of WNV, and its protein coding strategy are similar to other flaviviruses.

WNV is believed to enter host cells by receptor-mediated endocytosis that is dependent on an Ig-like fold present in domain III of the E glycoprotein. Virus-containing vesicles enter the endocytic pathways, where acidification leads to a major reorganization of E homodimers into trimers, exposing a hydrophobic peptide (termed the cd loop) contained in the distal portion of domain II of E. Ultimately this reorganization results in fusion of the viral and host cell membranes. Identifying specific host receptors for all flaviviruses has proved difficult and the literature is currently ambiguous on which host-cell molecules are so-called attachment receptors and which, if any, are absolutely required for virus entry. Candidate receptors that have been proposed for WNV include DC-SIGN, DC-SIGNR and $\alpha_v\beta_3$ integrin (Davis et al., 2006; Chu and Ng, 2004a, b). In mosquito cells, a c-type lectin is secreted from infected cells and binds virions to enhance uptake involving a phosphatase homolog of human CD45, mosPTP-1 (Cheng et al., 2010). Once viral RNA is released into host cells, it is immediately translated by host machinery. The resulting viral nonstructural proteins assemble on host membranes and replicate the viral genome. Notably, several viral nonstructural proteins are multifunctional and the function of others are poorly defined. Excellent reviews on their roles in flavivirus replication and host cell function have been published recently (Bollati et al., 2009). Mature virions exit cells through the trans-Golgi network and are released into the extracellular milieu by exocytosis and/or budding at the plasma membrane. Thus, the life cycle of WNV within cells is similar to other RNA viruses that replicate cytoplasmically. However, WNV and other arboviruses have evolved the ability to replicate in cells of hosts that are widely taxonomically divergent (i.e. arthropods and vertebrates). This requirement for replication in different host types exerts unique evolutionary and selective pressures on the virus, which are discussed below.

1.2. Ecology

Viruses adapt to available ecological niches or they become extinct. A thorough understanding of what constitutes the “niche” for WNV is therefore critical to formulating hypotheses regarding how the virus might evolve in order to maximize its potential to perpetuate. WNV is maintained in nature in an enzootic cycle involving birds and mosquitoes. Although the specific birds and mosquitoes most important for virus perpetuation in any given focus vary locally, they tend to include birds of the order *Passeriformes* and mosquitoes of the genus *Culex*. However, nearly 60 mosquito and 300 bird species have been found infected, and the species of *Culex* mosquito that is most important in a given locality is highly variable. For example, in the Northeastern US, *Cx. pipiens pipiens* is a major vector and appears to be responsible for the vast majority of virus transmission (Bernard et al., 2001). In the central and western US, however, *Cx. tarsalis* is

the principal vector, while in southern regions of the US, *Cx. p. quinquefasciatus* is most important (Bell et al., 2006; Molaei et al., 2010; Goldberg et al., 2010; Venkatesan and Rasgon, 2010). In Florida, *Cx. nigripalpus* is the dominant vector (Vitek et al., 2008; Kramer et al., 2008). This pattern is repeated at a global scale, with the dominant *Culex* mosquitoes in a given locality driving local WNV transmission (Kramer et al., 2008). *Culex* species tend to feed mainly on birds in the spring and summer, switching focus to take more mammalian bloodmeals in the fall, when outbreaks of WNV are most likely to occur among humans (Kilpatrick et al., 2006). In addition, several mosquito species not thought to be extremely important in WNV perpetuation, but potentially significant as “bridge” vectors (i.e. species that feed indiscriminantly) have been found infected, including *Ae. albopictus* and *Ae. vexans* (Turell et al., 2002). Several laboratory studies have established the competence of these vectors to transmit WNV (Turell et al., 2005), and field studies have detected both avian and mammalian blood in *Ae. Vexans*, although their relative importance in infecting humans and other hosts is currently unclear (Kilpatrick et al., 2005; Molaei and Andreadis, 2006). WNV has also been detected in *Culex pipiens* mosquitoes that have fed on human blood, indicating this mosquito may be the major bridge vector for infecting humans (Hamer et al., 2008). Although WNV may infect taxonomically diverse mosquito species throughout its range, certain *Culex* species appear to be critically important in WNV perpetuation in each geographic region where it persists.

Similarly, several bird species appear to be capable of generating sufficiently high viremias to infect mosquitoes and contribute to virus perpetuation. American Crow (*Corvus brachyrhynchos*) deaths near the Bronx Zoo in 1999 heralded the arrival of WNV, and these birds have served as useful sentinels since then (Eidson et al., 2001; Kramer and Bernard, 2001). Viremia in Crows reaches extremely high levels ($>10^{10}$ PFU/mL of blood) and mortality is nearly uniform (McLean et al., 2001; Komar et al., 2003). Recently it has become clear that other massively roosting birds, mainly American Robins (*Turdus migratorius*) are important both in enzootic maintenance of WNV in highly active transmission foci, and in driving a feeding shift in *Culex* mosquitoes that increases human risk (Kilpatrick et al., 2006). Birds also have been implicated in spreading WNV throughout its distribution. Most importantly, migrating birds have been implicated in transportation of WNV from Africa throughout the Middle East and into Eurasia and within the Americas (Rappole et al., 2006; May et al., 2011; Zehender et al., 2011; Dusek et al., 2009). Clearly a wide variety of birds have been found infected by WNV, but the species most important to virus perpetuation may vary locally.

WNV is capable of being transmitted between a surprisingly large variety of hosts. In contrast, the related Dengue virus (DENV, Flaviviridae, Flavivirus) maintenance is mainly driven by single mosquito and host species (i.e. *Aedes aegypti* and human beings). By comparison, the ability of WNV to act as an ecological generalist is quite clear, and may account, in part, for its dispersal throughout much of the tropical and temperate world. The molecular and/or population mechanisms that form the basis for the relative lack of host-specificity exhibited by WNV are not fully understood, representing a critical area for future research.

2. Historical perspective

The evolutionary dynamics of WNV are of particular interest because of the emergence of the virus as a significant health burden in the last 20 years. Originally isolated in 1937 from the blood of a patient with fever in the West Nile district of Uganda (Smithburn et al., 1940), the first outbreaks of WNV disease were associated with relatively few cases, mild disease and rural settings (Hayes, 2001). Strikingly, an outbreak in Romania that occurred in 1996 and 1997 involved over 500 reported cases, with a case-fatality rate of approximately 10% (Tsai et al., 1989, 1998). This outbreak was also striking in that it occurred in a temperate urban region. Shortly thereafter, epidemics were reported in the south of Romania and in the Volga delta region of Russia. Additional recent epidemics have been reported in Russia, Israel, Greece, France, Hungary, Italy and others (Platonov, 2001; Bin et al., 2001; Papa et al., 2010; Balenghien et al., 2006; Depoortere et al., 2004; Kutasi et al., 2011; Bakonyi et al., 2006; Monaco et al., 2011). Generally, these outbreaks occurred in delta regions of major rivers including the Volga, Rhone and Danube. Comprehensive reviews of WNV in Europe have been published recently (Hubalek and Halouzka, 1999; Zeller and Schuffenecker, 2004).

In 1999, WNV was introduced into North America in the New York City area, resulting in an equine and avian epizootic, and associated human infection, morbidity and mortality (CDC, 1999). The virus rapidly spread throughout the mainland US and into Canada, Mexico, and as far south as Argentina. As has been amply noted, the introduction of WNV at a precisely defined time and place provided a relatively unique opportunity to prospectively observe the adaptation of an exotic RNA virus to an essentially naïve ecosystem. Accordingly, several studies have been conducted to examine the evolution of the virus since its introduction (Anderson et al., 2001; Ebel et al., 2001; Ebel et al., 2004; Beasley et al., 2003; Davis et al., 2005; Bertolotti et al., 2007; McMullen et al., 2011; Armstrong et al., 2011). Several molecular epidemiologic studies have examined nucleotide sequence data from WNV strains found in birds, mosquitoes and human beings. The most recent of these are discussed in detail below and others are reviewed elsewhere (Ebel and Kramer, 2009). The ability of WNV to act as an ecological generalist, in combination with recent increases in intercontinental travel and trade, appear to have facilitated its emergence on a global scale.

3. Taxonomy and classification

WNV is classified as a member of the Japanese Encephalitis complex of the Flaviviruses on the basis of serological cross-reactivity (Calisher et al., 1989). Within WNV, two major lineages (Lineage I and II) are currently accepted, with several additional lineages that differ from one another by 5–25% recently proposed (Vazquez et al., 2010; Bondre et al., 2007). Lineage I is distributed throughout much of the world, and is further subdivided into several clades, one of which includes NY-99 (clade Ia), the genotype introduced to the US in 1999, another includes Kunjin virus (clade Ib), a variant of West Nile virus endemic to Australia (Ebel and Kramer, 2009; Lanciotti et al., 1999; May et al., 2011). Lineage II was thought to be restricted to sub-Saharan Africa until recently. Since 2004, lineage II has been associated with outbreaks of West Nile virus in Western and Eastern Europe, and appears to have

established endemic cycles in Spain and Greece (Papa et al., 2010; 2011; Bakonyi et al., 2006; Vazquez et al., 2010). Lineage III, also known as “Rabensburg virus”, is represented by several isolates made from the same region of the Czech Republic in 1997 and 1999 from *Cx. pipiens* mosquitoes, and 2006 from a pool of *Ae. rossicus* (Bakonyi et al., 2005; Hubalek et al., 2010). Lineage IV encompasses numerous isolates made in Russia, first detected in 1988 from a *Dermacentor* tick, and since isolated from mosquitoes and frogs in 2002 and 2005 in Russia (May et al., 2011). Lineage V comprises 13 isolates from India, collected from humans and *Culex* mosquitoes from the 1950s through 1980, which differ from other West Nile lineages by 20–25% at the nucleotide level (Bondre et al., 2007). Recent publications show these strains as basal to lineage I, comprising an independent cluster, lineage Ic (May et al., 2011). An additional, putative sixth lineage has been isolated in Spain from a pool of *Cx. pipiens* mosquitoes, and appears to be most closely related to lineage IV WNV (Vazquez et al., 2010). Additionally, Koutango virus (KOU), a Flavivirus isolated in Senegal, may represent a seventh lineage, as it is ~25% divergent from other WNV isolates, although it is currently categorized as a separate species (King et al., 2011). Human infection by KOU has not been reported, and its serological relationships to established WNV strains and transmission cycle are unclear, although partial cross neutralization with WNV and KUN has been shown (Charrel et al., 2003; Calisher et al., 1989). The distribution of all described lineages of WNV is shown in Fig. 1, by country where isolations have been made. The diversity of proposed WNV lineages worldwide reflects the diversity of the vectors involved in virus perpetuation and suggests that WNV or closely related agents have been introduced, and adapted to local transmission cycles on several occasions.

Taxonomic relationships are not entirely clear, and require reevaluation, especially with the recent proposal of so many new WNV lineages. In terms of nucleotide identity, they may be too disparately related to qualify as part of the same virus species, as the cutoffs proposed by researchers are >84% pairwise sequence identity (Kuno et al., 1998) or >79% for inclusion within a species (Ebel and Kramer, 2009; Charrel et al., 2003), although identity limits for inclusion within a lineage or species are generally arbitrary. According to the first estimate, lineage II WNV would have to be separated into its own species, as it has between 17% and 20% pairwise distance from lineage I, while the second pairwise distance might prevent proposed lineages III–VI from inclusion in WNV, as most show greater than 21% pairwise distance from the first two lineages at whole and partial genome levels (Vazquez et al., 2010; Bondre et al., 2007). These lineages appear to show some cross reactivity (Bondre et al., 2007; Bakonyi et al., 2005), may persist in similar transmission cycles, as most have been isolated from mosquitoes and birds, and appear to form a monophyletic clade when examined alongside Japanese encephalitis virus and Usutu virus (from the same serogroup) but a more systematic examination of relationships between the different lineages is warranted, as they have not been universally accepted (Ebel and Kramer, 2009; Vazquez et al., 2010). The relationships between these different lineages are further elucidated by Fig. 2, a phylogram based on the complete coding sequences for WNV lineages available in Genbank.

Lineage I has been subject to the most intensive study. It is now distributed worldwide, and includes the genotype introduced to the US in 1999 (NY99). Some genotypes appear to be

more pathogenic than others, for example NY99 shows enhanced pathogenesis in birds (see below), whereas Kunjin virus (clade Ib) is associated with attenuated infection and decreased neuroinvasion (Brault et al., 2007; Daffis et al., 2011). Lineage II is mainly associated with less severe disease, and less frequent neuroinvasion. However, recent reports describe encephalitis produced by infection with lineage II strains in both humans and horses in South Africa (Venter and Swanepoel, 2010; Venter et al., 2009). Lineage III has only been isolated from mosquitoes, and did not produce mortality in adult mice infected subcutaneously, intraperitoneally, or intracranially (Hubalek et al., 2010). Lineage V viruses from India are also associated with lower virulence (Davis et al., 2005; Bondre et al., 2007). WNV has thus clearly adapted to a wide array of transmission cycles and environments worldwide. This process of migration and adaptation to these environments has produced the currently observed lineages. Additional studies are required in order to define differences in virulence, neuroinvasiveness, natural hosts and vectors, and basic ecology for each putative lineage.

4. Molecular epidemiology

Upon its introduction to the United States, WNV was initially recognized by sequence comparisons and phylogenetic analysis (Lanciotti et al., 1999). The genotype introduced to the New World, dubbed NY99 for its initial isolation in New York in 1999, is most closely related to isolates made in Israel in 1998 and Hungary in 2003 (Zehender et al., 2011; Lanciotti et al., 1999; Jia et al., 1999). Initial sequence analysis of the WNV strains isolated during the first two years in New England showed a remarkable amount of genetic conservation, indicating a single point of introduction and very little diversification in WNV populations during this time period (Anderson et al., 2001; Ebel et al., 2001; Lanciotti et al., 1999; reviewed in Kramer et al., 2008; Ebel and Kramer, 2009). Subsequently, an additional subtype of WNV, WN02, with an amino acid substitution in the envelope protein, A159V, was detected in samples isolated in Texas (Beasley et al., 2003). From 2001 to 2003, WN02 rapidly displaced NY99, becoming the dominant genotype in North America (Ebel et al., 2004; Davis et al., 2005). WN02 strains require a shorter extrinsic incubation period in mosquitoes, which appears to be the mechanism for its increased fitness relative to NY99 (Ebel et al., 2004; Moudy et al., 2007). Thus, shortly after WNV was introduced into North America, the process of evolution led to increases in the basic reproductive rate of this pathogen.

As WNV became established throughout North America, the genetic diversity present in different types of data sets has led to insights into its emergence and expansion. Studies have found increased genetic diversity in mosquitoes relative to birds (Bertolotti et al., 2007; Amore et al., 2010), perhaps due to different selective pressure from the immune pathways used by these different hosts, which will be discussed in the section on genetic diversity below (Brackney et al., 2009). Genetic diversity and therefore estimated virus population size appeared to initially increase yearly after introduction to the US, although studies suggest this may be leveling off as WNV becomes established endemically (Bertolotti et al., 2007, 2008; Amore et al., 2010; Snapinn et al., 2007).

Several studies (Armstrong et al., 2011) showed a lack of geographical partitioning among sequences, especially those that examined sequence data from isolates sampled immediately after the introduction of WNV to novel environments and relied primarily on envelope sequences (Bertolotti et al., 2007, 2008; Davis et al., 2007). Recent studies, relying on full genome sequences and encompassing samples taken over a number of years after introduction of WNV uncovered more evidence for geographical structure to samples (McMullen et al., 2011; Armstrong et al., 2011; Herring et al., 2007; Grinev et al., 2008). A recent analysis indicates sequences from the envelope coding region may not be the most phylogenetically informative, and suggests NS3 or NS5 may be better partial sequences for reconstructing the phylogenetic relationships between different isolates, and can provide reconstructions that more closely resemble those resulting from whole genome sequences (Gray et al., 2010). Several distinct genetic variants of WNV have arisen in certain geographical areas, such as Texas (McMullen et al., 2011; Davis et al., 2004). One attenuated genetic lineage seems to have become extinct after being detected over the course of two years (Davis et al., 2005, 2004; Ebel and Kramer, 2009; Ebel, 2010). Another distinctive genotype, SW/WN03, contains several amino acid changes relative to other WN02 and is recently reported to be spreading through numerous states, although the phenotype associated with this new genotype has not been characterized (McMullen et al., 2011). It may be that more genetic changes accumulated in these WNV populations as they adapted to local transmission cycles.

Recent studies using full genome sequences of WNV from isolates made globally have uncovered phylogeographical influences on clade 1a distribution (May et al., 2011; Zehender et al., 2011). This clade seems to have a common ancestor that existed in sub-Saharan Africa in the early 20th century, which had multiple migrations to both Western and Eastern European countries in the 1970s and 1980s, and single introductions to India and Australia around the same time (May et al., 2011; Zehender et al., 2011). The patterns of distribution from Africa to Europe seem to follow white stork migration routes, indicating a possibly important role for this bird species in the spread of WNV into that continent (Zehender et al., 2011). Other mosquito borne Flaviviruses have also apparently originated in Africa, including yellow fever and dengue virus (Bryant et al., 2007; Gaunt et al., 2001; Holmes and Twiddy, 2003).

Arboviruses are unique in that they require replication in taxonomically divergent hosts – vertebrates and invertebrates (Weaver, 2006). This requirement is thought to restrict the amount of mutation that can occur in arboviruses, relative to single host viruses (Jenkins et al., 2002). Experimental studies have shown lower mutation rates in viruses serially passaged in alternating hosts, relative to those passaged in a single host type (Jerzak et al., 2007; Coffey et al., 2008; Coffey and Vignuzzi, 2011). To date, numerous studies have shown purifying or negative selection is dominant in arbovirus populations, including West Nile virus (Bertolotti et al., 2007, 2008; McMullen et al., 2011; Armstrong et al., 2011; Amore et al., 2010; Jerzak et al., 2005). In WNV phylogenetic analyses, only a few genetic changes have been identified that appear to be the subject of positive selective pressure. These include the amino acid residue associated with increased pathogenesis among North American birds, NS3 T249P and a mutation to NS4A, or the 2 K protein (V135M or V9M) (Armstrong et al., 2011; Brault et al., 2007). The valine to methionine mutation in NS4A/2K

is associated with OAS1b resistance, resistance to the flavivirus specific antiviral lycorine, and ability to overcome super-infection exclusion in replicon containing cell lines, which appears to be related to enhanced viral RNA synthesis (Mertens et al., 2010; Zou et al., 2009a,b). Adaptive evolution has also been detected at amino acid sites: E-V431I, NS2A-A224V/T, NS4A-A85T, NS5-K314R, and NS5-R422K although the functional significances of these sites are unclear (May et al., 2011; McMullen et al., 2011). Thus, several of the encoded WNV proteins are subject to positive selection that may lead to increased transmission efficiency and the likelihood for perpetuation in different transmission cycles.

Synonymous changes to the WNV genome could also impact its pathogenesis and evolution. Numerous synonymous changes were associated with the new genotype, WN02, and although some of these are assumed to have become fixed by association with other mutations that might confer a selective advantage, they could also exert an effect through codon bias or changes to the RNA genomic structure. The 5' and 3' untranslated regions are well conserved and have essential roles during viral replication (Khromykh et al., 2001; Zhang et al., 2008). Additional studies have shown that other RNA genome structures present in the capsid coding region can operate to upregulate flaviviral replication (Tuplin et al., 2011; Clyde and Harris, 2006). Additionally, codon bias in flaviviruses reflect the host usage (vertebrate only and alternating seem to display vertebrate codon biases, invertebrate only have more invertebrate bias), so examination of codon bias for a given virus can provide insight into its evolutionary history (Schubert and Putonti, 2010). Additional studies are required in order to determine the extent to which nonsynonymous variation impact RNA genomic structure in a way that influences WNV phenotype.

5. Within-host population dynamics

Molecular epidemiologic studies such as those discussed in the preceding section have provided insights into the selective forces that act on WNV and shown clearly that the virus is a dynamic, evolving entity with the capacity to adapt to a wide range of hosts and environments. These findings have stimulated studies aimed at understanding the viral population genetic mechanisms that account for this, and to assess whether the two very different kinds of host required for WNV perpetuation (mosquitoes and birds) influence the WNV population in different ways. Early studies suggested that within hosts, WNV forms a genetically complex distribution of mutants that vary in their degree of nucleotide divergence from the population consensus sequence. Further, Jerzak et al. (2005) showed that whereas WNV populations in naturally infected birds are relatively genetically homogeneous and purifying selection is strong, in field collected WNV infected mosquitoes they are very diverse, and purifying selection seems to be relaxed. The observations were supported by a series of laboratory studies that passed WNV in colonized mosquitoes and chickens (Jerzak et al., 2007), and cultured cells (Ciota et al., 2007). Importantly, the mosquito passed virus was inoculated intrathoracically and whole mosquitoes were triturated to obtain passed WNV, bypassing putative transmission barriers in the midgut and salivary glands (Hardy et al., 1983; Ciota et al., 2008). A highly similar study conducted using virus obtained from mosquito saliva failed to confirm these results raising the possibility that infection of, or escape from salivary glands might constitute a population bottleneck in the WNV system (Ciota et al., 2008; Ciota and Kramer, 2010). Nonetheless,

several studies have clearly established that mosquitoes and birds exert different evolutionary pressures on WNV.

The mechanistic basis for this difference has been addressed from a variety of perspectives. First, vertebrates and invertebrates respond to virus infections differently. In vertebrates, the earliest responses to infection by RNA viruses are dominated by type I interferon (IFN α/β). This response is triggered when RIG-I senses dsRNA in host cell cytosol, initiating signaling cascades that ultimately result in an antiviral state in the cell (reviewed in (Daffis et al., 2009)). Therefore, in vertebrates, WNV may be required to essentially “outrun” the antiviral state in infected individuals. This would result in strong purifying selection that has been observed after virus replication in these hosts (Ding, 2010; Jerzak et al., 2007), where presumably all or nearly all nonsynonymous mutation results in genomes of diminished fitness.

In contrast, insects respond to virus infection mainly through RNA interference (RNAi), which is also triggered by dsRNA within cells (reviewed in (Ding, 2010)). Ultimately, virus-derived small-interfering RNAs (viRNAs) are loaded into the RNA induced silencing complex (RISC) to degrade target viral RNA in a sequence-specific manner. Therefore, the antiviral state in mosquito cells seems to drive WNV diversification through a mechanism akin to negative, frequency-dependant selection, wherein rare genotypes (i.e. those that do not match common guide sequences loaded into the RISC) are favored because they are less efficiently degraded (Brackney et al., 2009). The precise relationship between this mechanism and the observed lack of purifying selection in mosquitoes has not been resolved or adequately addressed, and may represent two sides of the same coin. Overall, WNV population biology seems to be dominated by largely opposing forces that exist within its natural transmission cycle. Specifically, WNV undergoes alternating cycles of genetic expansion in mosquitoes that generates novel genotypes, and purification in birds that ensures that high fitness is maintained.

Other forces that influence WNV genetic diversity also have been examined recently. Population bottlenecks can stochastically reduce population diversity and lead to fitness declines through the action of Muller’s ratchet (Duarte et al., 1992). Convention holds that in natural transmission cycles, arboviruses undergo population bottlenecks as they pass through mosquitoes, where they seem to sequentially infect the epithelium of the mosquito midgut, peripheral tissues and ultimately the salivary glands, from which they are released into salivary secretions that are inoculated during mosquito feeding (Hardy et al., 1983). Such population bottlenecks have been described for alphaviruses and flaviviruses. Studies examining early mosquito infection by Venezuelan equine encephalitis virus (VEEV; *Togaviridae*, *Alphavirus*) and WNV demonstrated that only a few midgut cells are susceptible to infection, suggesting that anatomical bottlenecks may reduce genetic variability (Smith et al., 2008). Conversely, identical non-consensus WNV genomes have been detected in intrahost populations infecting birds in a single transmission focus, suggesting that population bottlenecks may not be as restrictive as had been assumed (Jerzak et al., 2005), and defective DENV genomes appear to perpetuate in transmission cycles through complementation (Aaskov et al., 2006). Supporting this, Brackney et al. recently failed to document significant population bottlenecks during infection of *Cx*.

quinquefasciatus mosquitoes by WNV (Brackney et al., 2011). It may be that the importance of bottlenecks during arbovirus transmission is a function of the specific virus–host system under study, and not consistent across systems.

6. Genetic correlates of pathogenesis and fitness

Molecular genetic and phenotypic studies of WNV mutants and engineered clones have revealed multiple genetic variations correlated with increased or decreased pathogenicity. WNV was long thought to be a less pathogenic flavivirus, with sporadic epidemics producing little or no mortality in human populations up until the early 1990s (Hayes, 2001). The recent introduction of WNV to the United States was marked by large die offs in bird populations, and a wave of epidemic cases among humans (Murray et al., 2010a). Studies have identified a single amino acid substitution in the NS3 helicase coding region, T249P, that increased morbidity and viral load in American crows, and appeared to be under selective pressure in areas with multiple genotypes present (Brault et al., 2007, 2004). After establishment of this initial pathogenic strain of WNV across the US, phylogenetic analysis of WNV sequences detected a new genotype, WN02, which displaced the initial strain NY99 in less than 4 years (Ebel et al., 2004; Davis et al., 2005). This new genotype had a single amino acid substitution in the envelope coding protein, V159A, that significantly decreased the extrinsic incubation time from virus infection until transmission by *Cx. pipiens* mosquitoes important vectors in the northeastern United States (Moudy et al., 2007; Kilpatrick et al., 2008). This mutation occurs nearby the envelope glycosylation motif for WNV, which is at nucleotide positions 154–156 in the envelope coding sequence.

Envelope protein glycosylation sites are conserved throughout the genus *Flavivirus*, although natural variation in glycosylation is present in populations of WNV (Adams et al., 1995; Berthet et al., 1997; Shirato et al., 2004; Hanna et al., 2005). An N linked glycosylation site at position 154 in the envelope protein has been associated with increased neuroinvasiveness for WNV in mice, and increased virulence and viremia in young chicks (Shirato et al., 2004; Beasley et al., 2005; Murata et al., 2010). Envelope protein glycosylation is also necessary for efficient transmission by *Cx. pipiens*, *Cx. tarsalis*, and *Cx. quinquefasciatus*, but not *Cx. pipiens pallens*, thus it influences vector competence in a species specific way (Murata et al., 2010; Moudy et al., 2009). Glycosylation patterns from virus propagated in insect versus vertebrate cells also seem to influence the ability of envelope protein to modulate innate immune response, and leads to different patterns of infectivity and propagation in different cell types, thus the role of glycosylation is also host specific (Hanna et al., 2005; Arjona et al., 2007).

The mechanism behind envelope protein glycosylation and modulation of WNV activity could be related to a number of different phenomena. The ability of WNV envelope to suppress dsRNA activated innate immune response is dependent on glycosylation status, which leads to increased inflammatory cytokine production for cells infected with virus lacking glycosylation (Arjona et al., 2007). Envelope glycosylation status influences ability to survive in lower pH environments (Beasley et al., 2005; Langevin et al., 2011). Genomes lacking the envelope glycosylation site have decreased replication, which may be related to budding of mature virions from the lumen of the endoplasmic reticulum rather than the

plasma membrane (Berthet et al., 1997; Shirato et al., 2004; Li et al., 2006). Abolishing the N linked glycosylation site on WNV envelope also influences receptor interactions, as it decreases greatly the ability of WNV to bind DC-SIGNR (Davis et al., 2006). One or a combination of these mechanisms, or perhaps a mechanism yet to be uncovered may explain the increased virulence of WNV with envelope N-linked glycosylation.

Strains associated with greater neuroinvasiveness and pathogenesis in mice and humans tend to be better controllers of interferon mediated responses (Daffis et al., 2011, 2009).

Numerous WNV proteins may modulate the interferon signaling cascade in vertebrate hosts, including all nonstructural coding proteins (NS1: Wilson et al., 2008, NS2A/B: Liu et al., 2006, NS3: Liu et al., 2005, NS4A/B: Muñoz-Jordán et al., 2003 NS5: Laurent-Rolle et al., 2010; reviewed, Diamond et al., 2009; Samuel and Diamond, 2006). With the advent of reverse genetics, recent studies have determined distinct amino acid changes in certain residues that are correlated with changed ability to control host immune responses. For example, a single residue at position 653 in NS5 appears to be responsible for the enhanced ability of NY99 like viruses to suppress interferon response. In North American genotype 1a viruses, position 653 of NS5 is a phenylalanine, whereas in the less pathogenic Kunjin virus, this position is a serine, and if these residues are switched through reverse genetics, suppression is enhanced for Kunjin and depleted for NY99 (Laurent-Rolle et al., 2010). Another example comes from studies of the host factor OAS1b which appears to confer natural resistance to WNV (Samuel and Diamond, 2006; Lucas et al., 2003). Virus cultivated in the presence of OAS1b can circumvent this factor by mutating at several residues, including NS3-S365G, which seems to lower the requirement of ATP for the ATPase dependent cleavage activity of this protease, and 2K-V9M, which generally enhances viral RNA synthesis (Mertens et al., 2010). Virus strains with higher rates of replication may be positively selected (Armstrong et al., 2011). Studies like this that correlate genotype with phenotype, and determine the underlying mechanisms, should expand our understanding of virus pathogenesis and the forces shaping the emergence of pathogenic phenotypes.

Attenuated genotypes of WNV emerged during the course of its spread across the United States. In Texas, a number of small plaque variants of WNV that displayed reduced neuroinvasiveness in mice were detected in 2003 (Davis et al., 2004). Comparison to NY99 strain followed by introduction of similar mutations into an infectious clone identified a combination of mutations to NS4, NS5, and the 3' UTR as being necessary for the attenuated phenotype found in one bird sample (Davis et al., 2007, 2004). Further analysis of other samples from the same region found that different amino acid substitutions in these strains appear to confer attenuation, indicating multiple pathways towards attenuated phenotypes (May et al., 2010). A single amino acid substitution in the central portion of NS4B, C102S, was enough to attenuate neurovirulence in mice (Wicker et al., 2006). Lineage II WNV has been associated with encephalitis and more severe disease only rarely, but comparison of the strains isolated from patients with more severe disease to less virulent strains indicates an enhanced role for NS proteins in determining virulence, relative to structural proteins, similar to findings for lineage I (Botha et al., 2008).

7. Conclusions and future research directions

In recent decades, WNV has emerged as one of the most intensely studied arthropod borne viruses. The spread of WNV throughout North America and periodic outbreaks in Eastern and Western Europe have enhanced worldwide interest in understanding viral, host, and ecological factors that result in outbreaks. Due to the availability of an increasing number of complete genome sequences (as of October 12, 2011, 479 are currently available on GenBank) major advances have occurred in understanding its molecular epidemiology, particularly in North America. Collectively, these studies have clearly demonstrated that WNV is a dynamic virus population that is able to perpetuate in and adapt to a wide range of ecological settings through mutation and selection. In addition, since the virus transmission cycle is relatively tractable in the laboratory using colonized mosquitoes and captive wild or domestic birds, experimental studies have provided insights into how different components of the transmission cycle influence virus population genetics and evolution. These studies have shown that mosquitoes drive WNV diversification through their innate antiviral response and because purifying selection is weak in these hosts, provide a reservoir of genetic diversity that allows the observed adaptation. In contrast, infection of birds ensures that the resulting variants are of high fitness through strong purifying selection. The processes that underpin WNV evolution are thus beginning to be understood in greater detail.

Several important areas for future research, however, remain. First, persistent infection of WNV in humans and vertebrates is emerging as a significant health issue in particular regions of the US (Murray et al., 2010b). The extent to which viral genetic and population determinants influence this has not been adequately addressed. In cell culture, establishment of persistent infection with Flaviviruses can occur with subgenomic replicons or the development of defective interfering particles (DIPs) (Zou et al., 2009b; Yoon et al., 2006). A second pressing matter is a reevaluation of the serological relationships within the Japanese encephalitis serogroup. Molecular genetic studies have proposed numerous lineages of WNV beyond the traditional two lineages recognized previously. The basic biology, transmission cycle, host range and pathogenicity of these putative lineages should also be studied further. Additionally, numerous amino acid residues may be under positive selection, but the roles of these residues are still unclear. Reverse genetic studies should be undertaken to determine the influence of these changes on WNV biology. With the advent of new sequencing technologies, our ability to design and conduct experiments into immune responses of hosts to viral infection and viral population biology has greatly increased. The role of RNAi in generating viral diversity, the potential bottlenecks associated with the WNV transmission cycle, and the interaction of individuals within a viral population may all become better understood with deep sequencing approaches. Finally, more collaboration between people studying ecology, epidemiology, molecular genetics, and pathology of WNV could lead to greater insight into its overall biology.

We have highlighted major advances in WNV biology over the past decade, including understanding of host specific selective pressures on viral populations, genotypic correlates with pathogenic phenotypes, and phylogenetic relationships between different lineages,

strains, and genotypes. Molecular epidemiology studies continue to elucidate the spread and evolutionary change that is ongoing in WNV populations.

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Fig. 1.

Worldwide map with countries where West Nile virus has been isolated colored as follows: Lineage Ia in light blue; lineage Ib in medium blue; lineage Ic in dark blue, lineage II in red, lineage III or “Rabensburg” virus in purple, lineage IV in orange, recent Spanish lineage (Vazquez et al., 2010) in green, and Koutango virus is colored yellow. Hatched coloring indicates more than one lineage has been isolated from that country. Lineage I distribution is adapted from May et al., 2011, other lineage isolates adapted from Charrel et al., 2003; Vazquez et al., 2010. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 2.

Radial phylogram showing relationships between different lineages of WNV. Complete coding sequences were downloaded from Genbank and aligned manually in BioEdit. Strains used and accession numbers are as follows: JEV, NC_001437; NY99, lineage Ia, NC_009942; Kunjin, paKUN, lineage Ib, AY274505.1; Indi804994, Indian lineage Ic, DQ256376.1; 956, lineage II, NC_001563; Rabensburg, lineage III, AY765264.1; RussianLEIV, lineage IV, strain Krnd88-190, AY277251.1; Koutango virus, EU082200.1. Bayesian phylogeny is shown, generated with MrBayes 3.1.2 run with a general time reversible (GTR) model with gamma shaped rate variation and invariable sites (Ronquist and Huelsenbeck, 2003). Two Markov chain Monte Carlo (MCMC) tree searches of 5000,000 generations each were run in parallel with sampling one in every 1000 trees. Radial 50% majority-rule consensus tree is shown based on the last 3750 trees. Posterior probabilities are given as numbers at each node.